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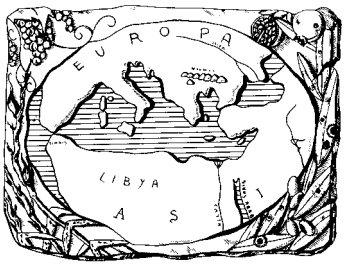

PHYTOPATHOLOGIA MEDITERRANEA

Plant health and food safety

The international journal edited by the Mediterranean Phytopathological Union
founded by A. Ciccarone and G. Goidànich

Phytopathologia Mediterranea is an international journal edited by the Mediterranean Phytopathological Union The journal's mission is the promotion of plant health for Mediterranean crops, climate and regions, safe food production, and the transfer of knowledge on diseases and their sustainable management.

The journal deals with all areas of plant pathology, including epidemiology, disease control, biochemical and physiological aspects, and utilization of molecular technologies. All types of plant pathogens are covered, including fungi, nematodes, protozoa, bacteria, phytoplasmas, viruses, and viroids. Papers on mycotoxins, biological and integrated management of plant diseases, and the use of natural substances in disease and weed control are also strongly encouraged. The journal focuses on pathology of Mediterranean crops grown throughout the world.

The journal includes three issues each year, publishing Reviews, Original research papers, Short notes, New or unusual disease reports, News and opinion, Current topics, Commentaries, and Letters to the Editor.

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Review

Emerging and re-emerging fungus and oomycete soil-borne plant diseases in Italy

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Summary. A disease is recognized as emerging if it is new, it occurs in a new host, there is an unexpected outbreak, its economic importance increases, if it attracts public opinion and the scientific community regardless of economic importance, or if it appears in an area for the first time (referred to as geographic emergence). This review deals with major driving factors of the emergence of plant diseases caused by soil-borne fungi and oomycetes (here indicated as "fungi"), in Italy during recent years. These factors include: accidental introduction of alien pathogens by human activities; effect of climate change; unusually severe weather events; favourable environmental and ecological conditions; pathogen genetic variation; host shifts and expansion of host ranges; introduction or expansion of the geographic range of a susceptible plant species or variety; limited availability of fungicides or development of fungicide-resistance pathogen strains; changes of cropping systems; and/or increased pathogen in soil as a consequence of intensive monoculture of crops. Although in most cases more than a single driving factor contributes to the emergence of an infectious disease, there are examples where a determinant may prevail over others. The case studies reviewed include pathogens belonging to major genera of soil-inhabiting fungi and oomycetes, including *Armillaria*, *Calonectria, Coniella*, *Fusarium sensu lato*, *Ilyonectria*, *Monosporoascus*, *Plectosphaerella*, *Rhizoctonia*, *Rosellinia*, *Sclerotinia*, *Sclerotium, Verticillium*, *Pythium* and *Phytophthora*. The examples encompass natural and forest ecosystems, economically important agricultural crops including citrus, fruit trees, olive, legumes, vegetables, and ornamentals, as well as exotic or expanding minor crops, such as avocado, goji berry, and pomegranate. Whatever the prevailing driving factor(s) these case studies all show that the large-scale emergence of soil-borne fungal diseases of plants is the consequence of human activities.

Keywords. Exotic pathogens, endemic pathogens, agricultural crops, minor crops, forest ecosystems.

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INTRODUCTION

There are different definitions of the term soil-borne when referring to plant pathogens. According to Koike *et al.* (2003), this includes pathogens that infect the plant through the soil while Katan (2017) broadened the con-

cept and defined as soil-borne those pathogens surviving and acting in the soil, at least during part of their lives. Major genera of fungi and oomycetes (Chromista) recognized as typical soil-borne plant pathogens include *Armillaria*, *Fusarium sensu lato* (including *Neocosmospora* spp., formerly the species complex *Fusarium solani*, and other *Fusarium*-like genera recently segregated from *Fusarium sensu lato*), *Gaeumannomyces*, *Macrophomina*, *Cylindrocarpon*-like asexual morphs, *Monosporascus*, *Phytophthora*, *Phytopythium*, *Plectosphaerella, Pyrenochaeta*, *Pythium*, *Rhizoctonia*, *Rosellinia*, *Sclerotinia*, *Sclerotium*, *Thielaviopsis* and *Verticillium*. These pathogens are well-studied because of their widespread occurrence in many important world food and fibre crops. However, other genera of plant pathogens, such as *Colletotrichum* as causal agents of anthracnose of many host plants, include species living in soil and affecting important crops (Gilardi *et al.*, 2014c). Whatever the definition, soil-borne fungal pathogens have the following characteristics: they have adapted to living in terrestrial habitats; they infect plants through belowground organs but are also able to cause infections of aboveground parts of the plants; they are saprobic, hemibiotrophic or parasitic on roots, stems and/or leaves of herbaceous or woody plants; and they cause monocyclic, in some cases polyetic and, more rarely, polycyclic diseases.

Soil-borne pathogens causing polycyclic leaf diseases have been frequently reported in Italy in nurseries of ornamentals, as consequences of conducive environments. They include *Calonectria* (Saracchi *et al.*, 2008; Vitale *et al.*, 2013) and *Phytophthora* species (Pane *et al.*, 2007a; Cacciola *et al.*, 2011b; Ginetti *et al.*, 2014). Microsclerotia of *Calonectria* species are the primary inocula and the survival form of these pathogens in the soil, while aerial dissemination occurs through conidia of their *Cylindrocladium*-like anamorphic stage and ascospores. As far as *Phytophthora* is concerned, few species, like *P. infestans*, have adapted to almost completely aerial lifestyles, while the majority of *Phytophthora* species are typically terricolous. A number of species that produce deciduous sporangia have partially adapted to aerial lifestyles (Jung *et al.*, 2018). *Phytophthora palmivora*, in particular, has developed evolutive transitional dispersal conidium-like propagules (sporocysts). Sporocysts are non-papillate caducous sporangia that can be easily detached and dispersed by rain.

A second aspect to be defined is when an infectious disease can be considered to be emerging. In this review, we recognize diseases as "emerging" when they are new, occurring in an area for the first time (geographic emergence), if they occur in new hosts, if there is an unexpected outbreak, if their economic importance increases, or if for some reason they attract public or scientific attention.

In recent years there has been considerable increase in the number of new *Phytophthora* species discovered. The number of validly described and recognized species has increased from approx. 40 in 1990 101 by 2011 (Kroon *et al.*, 2011) and now exceeds 150 (Ruano-Rosa *et al.*, 2018b, Albuquerque Alves *et al.*, 2019). The increase in the number of newly described *Phytophthora* species probably results from the scientific interest for these Oomycetes, their spread through the nursery plant trade and the rapid progress and evolution of molecular diagnostic techniques and taxonomy (Cooke *et al.*, 2007; Lamour *et al.*, 2007; Blair *et al.*, 2008; Martin *et al.*, 2012; Scibetta *et al.*, 2012; Bilodeau *et al.*, 2014; Jung *et al.*, 2016a; Yang *et al.*, 2017).

Molecular studies have also revealed the complexity of *Fusarium*, which encompasses numerous soil-borne pathogens of economically important crops. The taxonomy of this genus has undergone substantial revision and numerous new cryptic species have been described which differ in subtle morphological details and were previously assigned to the species complexes of *F. fujikuroi*, *F. incarnatum-equiseti*, *F. oxysporum*, *F. sambucinum* and *F.solani*. These have been separated or reallocated in the genus *Neocosmospora* on the basis of multi-locus phylogenetic analyses (O'Donnell *et al.*, 2015; Schroers *et al.*, 2016; Sandoval-Denis and Crous, 2018; Sandoval-Denis *et al.*, 2018; Lombard *et al.*, 2019; Maryani *et al.*, 2019).

Major determinants of the emergence of infectious plant diseases have been reviewed elsewhere (Fisher *et al.*, 2012; Gonthier and Garbelotto, 2011; Santini *et al.*, 2013; Gilardi *et al.*, 2018a,b; Moricca *et al.*, 2018). These determinants include anthropogenic introduction of alien pathogens, climate change, severe weather events, favourable environmental and ecological conditions, pathogen genetic recombination or mutation, host shifts or expansion of host range, introduction or expansion of the geographic ranges of susceptible plant species or varieties, limited availability of fungicides and development of resistance, changes of cropping systems, and increases in soil inoculum consequences of monoculture. These factors may be involved individually or together, instantaneously or continuously, simultaneously or in succession.

There is a body of direct or circumstantial evidence indicating that emergence of infectious plant diseases results from human activities (Andjic *et al.*, 2011; Mammella *et al.*, 2013; Santini *et al.*, 2013; Barnes *et al.*, 2014; Biasi *et al.*, 2016; Engelbrecht *et al.*, 2017), and several driving factors usually concur to cause emergence of a

disease (Desprez-Loustau *et al*., 2010; Stenlid *et al*., 2011; Garbelotto and Pautasso, 2012). The recent study by Serrano *et al*. (2019), of the genetic structure of *P. cinnamomi* isolates of worldwide origin, using four microsatellite markers, showed that identical genotypes of this pathogen were associated with the same hosts on different continents. This indicated long-distance transport by man, while the presence of identical genotypes in agricultural settings and neighbouring wildlands would suggested that specific commodities may have been the common sources of recent infestations caused by new invasive genotypes.

The present review considers emerging soil-borne plant diseases reported in Italy during recent years. These examples exemplify prominent roles of disease emergence drivers.

INTRODUCTION OF ALIEN PATHOGENS

In the modern era, the most devastating plant disease epidemics have been caused by exotic invasive pathogens. The causal agents of several devastating *Phytophthora* epidemics in Europe, Australia, and North America, including those caused by *P.* ×*cambivora, P. cinnamomi, P. lateralis, P. plurivora* and *P. ramorum*, have probably originated in South-east Asia (Jung *et al*., 2016a; Jung *et al*., 2018). Intensification of international trade of plant material and increased efficiency and speed of transport have favoured the introduction of pathogens into new areas. Most alien emerging pathogens have been inadvertently introduced with exotic plants or imported plant materials. Seeds and timber can be vectors of soilborne plant pathogens, but the nursery trade has been increasingly identified as responsible for the movement of this group of pathogens, despite phytosanitary regulations of international trade of plants and commodities (Migliorini *et al*., 2015; Jung *et al*., 2016b; Simamora *et al*., 2017; Jung *et al*., 2019). During visual inspections, soil-borne pathogens often go undetected because they induce obvious symptoms only in advanced stages of infection. The most commonly moved soil-borne pathogens globally are species of *Phytophthora* (Brasier, 2008). Genetic analysis of the variability of large numbers of isolates of the cosmopolitan and polyphagous plant pathogen *P. nicotianae*, from a wide range of geographic origins and hosts including ornamentals and agricultural crops, revealed that isolates from *Citrus* spp. were genetically related. This was regardless of their geographic origin, and these pathogens were characterized by genetic uniformity and high inbreeding coefficients (Mammella *et al*., 2013; Biasi *et al*., 2016). Greater variability was observed for populations from other hosts and a significant geographical structuring was found only for isolates from *Nicotiana* and *Solanum* spp. These differences were possibly related to the propagation systems for different crops. Isolates obtained from *Citrus* spp. are more probably distributed worldwide with infected nursery plants, whereas *Nicotiana* and *Solanum* spp. are propagated by seeds, which would not contribute to the spread of the pathogen and result in a greater opportunity for geographic isolation of different lineages. For ornamental species in nurseries, the high genetic variation is likely to result from mixtures of diverse pathogen genotypes through the trade of infected plant material from various geographic origins, the presence of several host plants in the same nursery, and genetic recombination through sexual reproduction of this heterothallic pathogen (Mammella *et al*., 2013; Biasi *et al*., 2016).

Restoration plantings or afforestation with nursery plants are pathways for the introduction and spread of exotic *Phytophthora* species in natural habitats and forests (Sims *et al*., 2019a, b). In a recent survey of protected natural areas and water courses crossing these areas in Sicily, 13 of 20 recovered *Phytophthora* species were exotic, while only seven, including *P. tyrrhenica* and *P. vulcanica* associated with *Fagaceae* hosts, could be regarded as endemic to Europe (Jung *et al*., 2017; Jung *et al*., 2019). Several species found in this survey, including *P. cactorum*, *P. citrophthora*, *P. megasperma*, *P. multivora*, *P. plurivora* and *P.* ×*cambivora*, are well-known invasive pathogens with wide host ranges and aggressiveness towards many cultivated and native European plant species. The presence of these pathogens indicates that they constitute threats to the homeostasis and resilience of these ecosystems, and protected natural areas are reservoirs of inoculum of potential pathogens for economically important crops. Garbelotto and Hayden (2012) highlighted the link between the ornamental plant industry and the introduction of *P. ramorum*, which causes lethal cankers on oak species native to California, into the wildlands in North America. *Phytophthora ramorum* is a quarantine pathogen also in the EPPO region (in the A2 list from 2013), has been repeatedly intercepted in nurseries of ornamentals in Italy, but was promptly eradicated (Ginetti *et al*., 2014). It has also been detected as an operational taxonomic unit (OTU) by metabarcoding analyses of soil samples sourced from chestnut stands in central Italy and ornamental nurseries in southern Italy (Vannini *et al*., 2013; Prigigallo *et al*., 2016). However, in both cases, the presence of the pathogen in samples was not confirmed by the isolation of living cultures. The official status of *P.*

ramorum in Italy, based on information updated to 2014, is "transient, under eradication". *Phytophthora nierderhauserii*, another invasive polyphagous species, has recently emerged in many countries (Abad *et al.*, 2014). The occurrence of this species in natural ecosystems in Australia, in vineyards (*Vitis vinifera*) in South Africa and in almond (*Prunus dulcis*) trees in California, Spain and Turkey, and its capability to infect shrubs and herbaceous ornamentals in several unrelated families indicates *P. nierderhauserii* has wide ecological adaptability and may threaten agricultural and natural ecosystems. There is evidence indicating that after the first detections of *P. niederhauserii* in Italy (Brasier, 2008; Cacciola *et al.*, 2009 a, b), this species has been spreading in nurseries of ornamental plants (Faedda *et al.*, 2013a; Prigigallo *et al.*, 2015; Aiello *et al.*, 2018). *Phytophthora tentaculata* is another emerging but less invasive, exotic *Phytophthora* species that has been reported in northern Italy and on origanum (*Origanum vulgare*) and on loof chicory (*Cichorium intibus*) in central Italy (Martini *et al.*, 2009; Garibaldi *et al.*, 2010). *Phytophthora capsici* remains a serious and economically important pathogen on bell pepper, tomato, eggplant and cucurbits in many countries. This pathogen occurs in protected crops and in open fields, causing severe losses (Hausbeck and Lamour, 2004). The type culture of *P. capsici* is from Italy and was deposited in 1927, suggesting this species was introduced into this country in the 20th century. The long-standing presence of *P. capsici* in Italy is reflected by high levels of genetic variability of the Italian population of this pathogen but its centre of origin remains unknown (Quesada-Ocampo *et al.*, 2011).

Fusarium oxysporum f. sp. *radicis-cucumerinum*, which causes wilting, root and stem rots on cucumber, was first observed in Greece and then in Spain in 2001 and Turkey in 2009, and has been recently reported in Italy on farms that have repeatedly grown cucumber in the same soil for 10 years (Garibaldi *et al.*, 2016). This pathogen can also infect other cucurbit crops, including melon (*Cucumis melo*), watermelon (*Citrullus lanatus*) and sponge gourd (*Luffa aegyptiaca*), while the interspecific hybrids of *Cucurbita maxima* × *C. moschata* and zucchini are not susceptible (Vakalounakis *et al.*, 2005).

Fusarium solani f. sp. *cucurbitae* is responsible for severe losses, causing root and stem rots, particularly on cucumbers in greenhouses. This pathogen is spreading in Spain, where it was reported for the first time in Europe (Gómez *et al.*, 2014). Significant economic damage caused by this pathogen can be expected in Italy in intensive cultivation systems as it can be transmitted by seeds (Vannacci, 1980), and has several cucurbit hosts, including *Cucurbita* hybrids (*C. maxima* × *C. moschata*) used as rootstocks for watermelon (Armengol *et al.*, 2008).

Fusarium wilt of lettuce (*Lactuca sativa*) caused by *F. oxysporum* f. sp. *lactucae* has recently become the most important disease of lettuce in cultivation areas in many countries, and its spread is favoured by seed transmission (Cabral *et al.*, 2018). Until recently, three races of *F. oxysporum* f. sp. *lactucae* were described and reported: race 1 in Europe, USA and South America and races 2 and 3 in Japan. However, in 2017, a new race, race 4, was detected in the Netherlands, identified through biological assays and molecular tools (Gilardi *et al.*, 2017). This new race is apparently spreading rapidly and has been observed in several European countries, including Belgium, the United Kingdom, Ireland (Taylor *et al.*, 2019) and Italy (Gilardi *et al.*, 2019). The presence of this new race is posing serious threats to growers and plant breeders. Before resistant varieties can be developed, preventative management measures, such as the use of healthy seeds and/or seed treatments, are required to reduce the risk of its rapid spread to new areas. Gilardi *et al.* (2017) speculated that race 4 may have evolved due to high selection pressure as a consequence of lettuce monoculture, or could have been introduced from a foreign source through infected seeds or seedlings.

Careful monitoring of the race situation in the field would be useful for the efficient use of host resistance for disease management. Specific molecular markers can also provide successful detection and identification of *formae speciales* and races of *F. oxysporum* from seeds, plants, and soil samples.

Three vegetative compatibility groups (VCGs), VCG-300, VCG-301 and VCG-302, corresponding, respectively, to races 1, 2 and 3, have been reported in *F. oxysporum* f. sp. *lactucae* (Pintore *et al.*, 2017). The Arizona, California and type 1 isolates from Taiwan all belong to the same VCG as race 1 isolates from Japan, and all the race 1 isolates from Arizona, California and Japan have identical mtSSU and EF-1α sequences and almost identical intergenic spacer (IGS) region sequences. This indicates a common origin of the three races. However, the possibilities of the pathogen being introduced from a foreign source through infected seeds or seedlings, or of evolution from non-pathogenic *F. oxysporum*, cannot be excluded. The isolates obtained from lettuce in the Netherlands belong to VCG-303 (Pintore *et al.*, 2017). Further studies are needed to elucidate why and how a new race of this pathogen has developed in Northern Europe.

Melon collapse, commonly considered a synonymous of Monosporascus root rot of melon and vine decline (MRRVD), one of the most important disease of melon and watermelon (Martyn and Miller, 1996;

Cohen *et al.*, 2000), has emerged in the last 15 years in Italy. The exotic Ascomycete *Monosporascus cannonballus* has been regarded as the main cause of the disease in several countries (Stanghellini *et al.*, 2003; Chilosi *et al.*, 2008). However, lines of evidence indicate that other soilborne pathogens, such as *Acremonium cucurbitacearum*, the most common fungal species associated to this disease in Spain (García-Jiménez *et al.*, 2000), *Olpidium* and *Plectosphaerella* species, *Macrophomina phaseolina* and *Rhizoctonia* species, are also involved in melon collapse (Bruton, 2000; García-Jiménez *et al.*, 2000; Stanghellini *et al.*, 2010; Stanghellini and Misaghi 2011; Ben Salem *et al.*, 2013; Felipe *et al.*, 2018). Recently in central Italy, *M. cannonballus, O. bornovanus*, and *O. virulentum* were recovered from a melon greenhouse soil with a history of severe infections of *Melon necrotic spot virus* (MNSV), which is vectored by *Olpidium* spp. In pathogenicity tests, all three fungi induced symptoms of root rot and vine decline, confirming a complex aetiology of MRRDV (Aleandri *et al.*, 2017). A binucleate *Rhizoctonia* AG-F was reported to be responsible for watermelon vine decline in Sicily (Aiello *et al.*, 2012). Carlucci *et al.* (2012) found several species of *Plectosphaerella* associated with melon collapse in southern Italy, including an already known species, *Pa. cucumerina* (= *Plectosporum tabacinum*), along with four new species, *Pa. citrullae*, *Pa. pauciseptata*, *Pa. plurivora* and *Pa. ramiseptata*. Carlucci *et al.* (2012) also showed that *A. cucurbitacearum* was a synonym of *Nodulisporium melonis*, and transferred it to the genus *Plectosphaerella* as *Plectosphaerella melonis* comb. nov. This increased to six the number of *Plectosphaerella* species reported on melon and watermelon in Italy. However, the roles of new *Plectosphaerella* species in MRRVD have not yet been clarified (Carlucci *et al.*, 2012). Some were reported to be responsible for root rot of other vegetable crops such as tomato, pepper, parsley and basil (Raimondo and Carlucci, 2018a and b). *Monosporascus cannonballus* has been found only on *Cucurbitaceae*, commonly in arid, hot climates (Stanghellini *et al.*, 1996). This pathogen is widespread in major melon-producing countries in North and Central America, Asia, North Africa, and Europe (Martyn and Miller, 1996; Cohen *et al.*, 2000). After the phase-out of methyl bromide fumigation and application of fungicides through irrigation systems, crop rotation with non-susceptible host plants, breeding for disease resistance and grafting on resistant rootstocks have been regarded as alternatives for the management of melon vine decline (Cohen *et al.*, 2000). Grafting on resistant rootstocks is an effective means for the management of a number of soil-borne diseases of vegetables (Cohen *et al.*, 2007; Davis *et al.*, 2008), and is increasingly used in Italy in commercial *Solanum* and cucurbit crops (Gilardi *et al.*, 2011; Colla *et al.*, 2012; Gilardi *et al.*, 2014a, b, c). However, no commercial rootstocks of melon and watermelon combine multiple resistance to melon collapse and Fusarium wilt with good production performance (Gilardi *et al.*, 2013).

CLIMATE CHANGE AND SEVERE WEATHER EVENTS

The role of climate change in the emergence of infectious plant diseases, its impact on endemic plant pathogens and the interactions between climate change and the introduction of exotic pathogens as a result of globalization have been the subjects of study and extended debate within the international scientific community (La Porta *et al.*, 2008; Pautasso *et al.*, 2012; Ramsfield *et al.*, 2016; Gilardi *et al.*, 2018b). Several studies have concerned effects of climate change on soil microbial communities and, in particular, on soil-borne pathogens (Dukes *et al.*, 2009; Manici *et al.*, 2014; Kubiak *et al.*, 2017; Gilardi *et al.*, 2018a). Climate change can have direct and indirect effects on plant disease epidemics, as climate affects host susceptibility, the survival of pathogen inoculum, the rate of disease progress and epidemic duration. Frequencies of extreme climatic conditions, such as droughts, floods and hurricanes, as well as damage caused by wind, snow and hail, are also likely to increase due to climate change (IPCC, 2019). According to projections, the increase in temperatures will favour expansion of the geographical ranges of mesophilic and thermophilic pathogens at their northern limits in the Northern hemisphere. More generally, warming is expected to cause pole-ward range shifts of plants and their pathogens affecting natural and managed ecosystems (Chakabortry, 2013).

Jung (2009) imputed the decline of European beech (*Fagus sylvatica* L.) in Central Europe to the interaction between *Phytophthora* spp. infections and climatic extremes. On the basis of a survey of natural parks and reserves in Sicily, it has been assumed that the interaction between climate change and root infections by *P.* × *cambivora* and other less frequent or less aggressive *Phytophthora* species (Jung *et al.*, 2017; Jung *et al.*, 2019) is the main driving factor of the decline of European beech in the Nebrodi regional park (Sicily, southern Italy). This is the extreme southern limit of the natural geographical range of European beech in Europe. *Phytophthora* × *cambivora* is also the prevalent species responsible for the resurgence of ink disease in central Italy. Although the effects of climate change on the complex interac-

tions between the diverse components of pathobiomes in agricultural and natural ecosystems are not fully known, there is abundant literature predicting altered geographic distribution of pathogens with changing climate suitability and host distribution (Shaw and Osborne, 2011). For example, a model for predicting the global distribution of *P. cinnamomi*, which is a generalist soil-borne pathogen with a very wide host range, has been developed. This pathogen is considered one of the 100 worst invasive alien species in many countries (Hardham and Blackman, 2018). Since it was first detected on avocado (*Persea americana*) (Cacciola *et al*., 1998), the number of records of this pathogen in Italian ornamental and forest nurseries, agricultural crops, plantation forests and native woodlands has been increasing (Scanu *et al*., 2013; Pilotti *et al*., 2014; Frisullo *et al*., 2018; Vitale *et al*., 2019). The model is based on the response of the pathogen to temperature and moisture, and incorporates extensive empirical evidence on the presence of *P. cinnamomi* in the soil (Burgess *et al*., 2017). Consistently with the model, the comprehensive global map of the *P. cinnamomi* distribution also includes Italian regions with temperate climates, where *P. cinnamomi* has been recently reported as the main stressor threatening the forest stands of evergreen Mediterranean oaks and the Mediterranean maquis vegetation (Scanu *et al*., 2015; Moricca *et al*, 2016; Frisullo *et al*., 2018). A study of diversity and distribution of *Phytophthora* species in chestnut (*Castanea sativa*) stands, and their association with ink disease in Europe, showed that *P. cinnamomi* had peculiar ecological requirements compared to the other species occurring in the chestnut rhizosphere (Vettraino *et al*., 2005). The pathogen was never detected from sites characterized by minimum and maximum temperatures, respectively, below 1.4°C and above 28°C. This confirms that climate change is a major driving factor conditioning the geographical distribution and emergence of this pathogen that is inhibited by low soil temperature. The rise in temperatures as a result of climate change, along with other factors, may have favoured the emergence of Pythium root rot caused by thermophilic *Pythium* species, in leafy vegetables crops in Northern and Southern Italy (Garibaldi *et al*., 2010; Garibaldi *et al*., 2015; Gilardi *et al*., 2018c).

The combined effects of temperature and atmospheric CO_2 concentration on the severity of infections by fungal pathogens in vegetable crops, including the soil-borne pathogen *F. oxysporum*, has been tested in controlled environment conditions simulating global warming (Ferrocino *et al*., 2013; Chitarra *et al*., 2015; Gullino *et al*., 2018). The information provided by these experiments can be useful for developing provisional models to forecast and counteract the effects of climate change on plant diseases caused by soil-borne pathogens.

A consequence of climate change is the increase in the frequency, extent, and intensity of extreme weather events, although these events are not necessarily linked to global warming. Climate change is believed to be responsible for the increasing frequency of medicanes, which are hurricanes of the tropical type occurring in the Mediterranean Sea. Extreme, severe weather events may trigger the emergence of soil-borne plant diseases as they predispose host plants to the infections and favour pathogen spread. The resurgence of mal secco disease (caused by *Plenodomus tracheiphilus*) in lemon (*Citrus ×limon*) orchards in the Syracuse province during the last few years may be imputed to severe hailstorms and the high susceptibility of 'Femminello Siracusano 2Kr' (Migheli *et al*., 2009). This is the prevalent cultivar in new plantings of this typical lemon growing area of Sicily. Medicanes were the drivers of epidemic outbreaks of citrus fruit brown rot and foliage blight in Sicily, caused by *P. citrophthora* (De Patrizio *et al*., 2012). This is a common soil-borne pathogen which has adapted to an occasional aerial lifestyle.

ENVIRONMENTAL AND ECOLOGICAL CONDITIONS

Environmental and ecological conditions, including warm temperatures, high relative humidity and conducive soil conditions, such as low pH, presence of residues of previous susceptible crops and overwatering, may be major drivers of the emergence of diseases caused by polyphagous soil-borne pathogens. The following case studies illustrate these interactions.

Damping-off, caused by *Pythium ultimum,* has been observed with increasing frequency in Italy on lettuce, wild rocket and lamb's lettuce at temperatures between 15 and 25°C (Gilardi *et al*., 2018a, b). *Pythium ultimum* has also been reported in northern Italy on coriander (*Coriandrum sativum*) for the first time in the world (Garibaldi *et al*., 2010a). The presence of new species of *Pythium* in Italy, such as *P. aphanidermatum* on spinach and on swiss chard, *P. irregulare* on lamb's lettuce and *Pythium* Cluster B2a (*P. dissotocum, P. coloratum, P. diclinum*, *P. dictyosporum*, *P. lutarium, P.* sp. 'Group F' and *P.* sp. *tumidum*) on lettuce is particularly important at warm temperatures (Garibaldi *et al*., 2015d; Gilardi *et al*., 2018b, c). Web blight and damping-off of seedlings, caused by the *Rhizoctonia solani* complex, have recently been found on many hosts of different families, including species of *Campanula* (C. *trachelium*, *C. rapunculodes* and *C. carpatica*), *Rebutia perplexa*, *Nigella dama-*

scena, *Lavandula officinalis*, *Origanum vulgare*, *L. stoechas*, *Rosmarinus officinalis*, *Satureja montana*, *Dodonea viscosa*, *Coprosma repens* and *C. lucida*, *Viburnum tinus*, *Murraya paniculata*, *Streptosolen jamesonii*, *Thyptomene saxicola*, *Chamaerops humilis*, *Passiflora mollissima* and *Tabebuia impetiginosa* (Garibaldi *et al.*, 2015a, c, e, g; Bertetti *et al.*, 2017; Aiello *et al.*, 2017b; Bertetti *et al.*, 2018a,b). Southern blight caused by *Sclerotium rolfsii* (teleomorph *Athelia rolfsii*) has been reported on numerous host plants grown either in pots or in fields, in greenhouses or in the open air, in northern and southern Italy. These host include *Dichondra repens*, potato, common bean, *Stevia rebaudiana*, *Hedera helix*, *Cannabis sativa*, ornamental *Citrus* species, *Convolvulus cneorum* and young seedlings of several other ornamental plants (Pane *et al.*, 2007b, c; Polizzi *et al.*, 2007; Pane *et al.*, 2008; Polizzi *et al.*, 2010; Garibaldi *et al.*, 2013). *Sclerotinia sclerotiorum*, a necrotrophic pathogen known to infect over 400 species of plants from 75 families (Grabowski, 2017), has been observed on many aromatic and ornamental plants, such as thyme, sage, borage, mint, rosemary, aquilegia, petunia, paris daisy, lavender, and gaillardia (Garibaldi *et al.*, 2008; Garibaldi *et al.*, 2015b, f; Garibaldi *et al.*, 2017). Several species of *Fusarium, Cylindrocarpon*-like asexual morphs (*Ilyonectria, Pleiocarpon)* and *Neocosmospora*, have been reported on ornamental plants, including *Agapanthus africanus*, *Bougainvillea glabra*, *Cordyline australis*, *Dasylirion longissimum*, *Eremophila* spp., *Philoteca myoporoides, Strelitzia reginae*, *V. tinus* as well as *Trachycarpus princeps* and various other *Arecaceae* species (Aiello *et al.*, 2014; Aiello *et al.*, 2017a; Guarnaccia *et al.*, 2019). Climatic conditions, farming practices, and conducive environmental conditions have been indicated as major drivers of the emergence of these soil-borne pathogens of ornamental plants.

PATHOGEN GENETIC RECOMBINATION OR MUTATION

Successful invasion by an exotic pathogen may depend on its evolutionary potential which allows it to emerge, adapt to new hosts and environments and persist in populations of host plants. It is generally assumed that asexual organisms may exhibit lower invasion success compared to sexually reproducing organisms, due to their inability to generate meiotic progeny which can rapidly adapt to new hosts and environments. Despite, mechanisms generating variation, such as genetic recombination and mutations, are not always associated with fitness benefits. There are several examples showing that clonality does not necessarily reduce invasiveness (Prospero and Cleary, 2017). One of the best documented cases of genetic recombination of an invasive soil-borne plant pathogen is that of *Heterobasidion irregulare* native to North America. This pathogen was accidentally introduced into Italy, and has become invasive in Latium (central Italy), where the Italian stone pine (*Pinus pinea*) is the sole or major pine species. In this invasion area *H. irregulare* prevails on the native sibling species *H. annosum*, and, unlike *H. annosum*, is also able to colonize as a saprophyte pure oak stands (Gonthier *et al.* 2012; Giordano *et al.* 2013; Garbelotto *et al.* 2013). There is evidence in the invasion area, where *H. irregulare* and *H. annosum* are in sympatry, of interspecific pathogen hybridization and introgression of genes, mostly from the native species into the invasive one, suggesting rapid, possibly adaptive, evolution of *H. irregulare* (Gonthier *et al.* 2007; Linzer *et al.*, 2008; Gonthier *et al.* 2015). Epidemiological consequences of this evolution are unpredictable, and no studies have determined how gene introgression may affect fitness and virulence of either the invasive or the native species. However, examples from other organisms, including several plant pathogens, indicate gene introgression is an important evolutionary mechanism, increasing adaptation and pathogenicity of the species involved (Brasier, 2001; Depotter *et al.*, 2016). Interspecific hybrids of *Phytophthora* occur frequently in natural ecosystems and may become invasive (Brasier *et al.*, 2004; Ioos *et al.*, 2006; Burgess, 2015). Hybridization and polyploidy are assumed to be the genetic mechanisms for adaptation to new hosts and speciation in these oomycetes (Bertier *et al.*, 2013). The interspecific hybrid nature, the mating reproduction system (both A1 and A2 mating types occur in Europe) and the intraspecific variability of *P.* ×*cambivora* may explain the ability of this heterothallic oomycete to adapt to different environmental conditions, ranging from rainforests of Southeast Asia, where the species probably originated, to temperate deciduous forests of central Europe and Southern Italy (Jung *et al.* 2017). In nurseries of ornamental plants, the sympatric occurrence of diverse *Phytophthora* species from different geographic origins favours genetic recombination through sexual reproduction between species that have evolved separately and have not developed premating barriers. A natural hybrid between *P. nicotianae* and *P. cactorum*, referred to as *P.* x *pelgrandis*, was reported in Italy as the causal agent of root rot of potted lavender (*L. stoechas*) plants. This interspecific hybrid showed a unique combination of morphological, biological and ecological characteristics inherited from both parental species, some of which may have epidemiological implications (Faedda *et al.* 2103b).

The frequency of natural *Phytophthora* interspecific hybrids in nurseries is possibly greater than expected from the low number of reports. Hybrids, may go unnoticed as they do not always have distinctive morphological traits. Sequencing of ITS-rDNA regions after PCR amplification with universal primers ITS6 and ITS4, often used for molecular identification of *Phytophthora* species, may fail to discriminate these species from their parents. In many cases, application of diverse molecular techniques is required to ascertain the hybrid nature of the isolates (Faedda *et al*., 2013b). Very recently a rapid High-Resolution Melting (HRM) diagnostic method has been proposed to distinguish *Phytophthora* hybrids from their parental species (Ratti *et al.* 2019).

The high genetic variability of soil-borne vegetable crop pathogens, such as *Fusarium oxysporum* and *P. capsici*, is a challenge and limits application of genetic resistance for the management of the diseases they cause. Evolution or accidental introduction of new pathogen physiological races have been indicated as possible causes of breakdown of rootstock resistance in grafted *Solanum* or cucurbit plants.

Genetic plasticity of *F. oxysporum* and the problem this poses for farmers and plant breeders are exemplified in *F. oxysporum* f. sp. *lactucae*, the cause of Fusarium wilt of lettuce. Until recently, three races (1, 2 and 3) of the pathogen had been identified by their ability to cause disease on differential lettuce cultivars, and using molecular tools. Race 4 was identified in the Netherlands and in Italy (Gilardi *et al*., 2019). Pathogenicity tests in controlled environmental conditions showed that none of the commercial lettuce cultivars popular in Italy are completely resistant to race 1 of *F. oxysporum* f. sp. *lactucae*, and only 57%of the tested cultivars were resistant to race 2, and 21% were resistant to race 3.

Almost all currently available commercial hybrids of muskmelon (*Cucumis melo*) possess FOM-1 and FOM-2 genes, conferring resistance to the physiological races 0, 1 and 2 of *F. oxysporum* f. sp. *melonis*. However, none of these hybrid hosts are resistant to the race 1-2 of this pathogen, which has occurred in all major production areas in Italy since the 1990s. No commercial hybrids of pepper are completely resistant to *P. capsici* due to the variability of this pathogen, which in Italy is also a major pathogen of tomato and cucumber grown in plastic greenhouses. *Fusarium oxysporum*, because of genetic variability and ability to evolve, encompasses *formae speciales* infecting aromatic flower plants (Gullino *et al.* 2012; Gullino *et al*., 2015). New *formae speciales* of this pathogen have been frequently reported in nurseries of ornamentals in Italy (Garibaldi *et al*., 2012; Matic *et al*., 2018; Ortu *et al.* 2018).

HOST SHIFTS AND EXPANSION OF HOST RANGES

Polyphagous soil-borne plant pathogens, including *S. sclerotiorum*, *S. rolfsii*, *P. cinnamomi*, *P. nicotianae*, *P. palmivora* and *P. niederhauserii*, infecting very many host plant species, usually widen their host ranges when they invade new areas rich in biodiversity. These areas include natural ecosystems, nurseries of ornamentals, and complex agricultural systems, so the pathogens come into contact with potential new host plants. General pathogens are better invaders than specialists, due to their non-selective ability to seek new hosts in a new environment (Navaud *et al*., 2018; Thines 2019). Although specialized pathogens may infect new hosts less frequently, the host range expansion of *formae speciales* of *F. oxysporum* to include plant species of the same family is quite common. A new *forma specialis* (f. sp.) of *F. oxysporum*, *F. oxysporum* f. sp. *papaveris*, was discovered in the Liguria region (northern Italy) on Iceland poppy (*Papaver nudicaule*) a plant native to Arctic regions of North America and Eurasia (Garibaldi *et al.* 2012; Ortu *et al.* 2015). The pathogen was initially thought to have been introduced through contaminated seeds (Bertetti *et al.* 2015). However, the high susceptibility of artificially inoculated *Chelidonium majus* and *P. rhoeas*, two species of *Papaveraceae* endemic in Italy, to *F. oxysporum* f. sp. *papaveris*, supports the hypothesis that this f. sp. was already present in Liguria, and shifted onto Iceland poppy from wild relatives (Bertetti *et al*., 2018a).

The report of *F. oxysporum* f. sp. *chrysanthemi* on orange coneflower (*Rudbeckia fulgida*) in Northern Italy, has expanded the list of hosts of this f. sp., including several other ornamental plants of the *Asteraceae* such as chrysanthemum, Paris daisy, African daisy, and gerbera (Matic *et al.* 2018).

INTRODUCTION, OR GEOGRAPHIC RANGE EXPANSION OF SUSCEPTIBLE HOST PLANTS

When an exotic plant is introduced into a new geographical area, there is justified concern about the risk of it being a vehicle for alien pathogens (Eschen *et al.* 2019), while its susceptibility to pathogens already established in the area is often overlooked. It is more likely for new hosts to be susceptible to native pathogens if components of the resident flora are closely related to the introduced host or if polyphagous pathogens are present in the area. One example is avocado (*P. americana*), whose culture is rapidly expanding in southern Italy. This tropical fruit tree is susceptible to white root

rot caused by *Rosellinia necatrix*, which is a well-known and widespread pathogen of olive and fruit trees in Italy (Schena *et al*., 2008; Pasini *et al*., 2016). White root rot is regarded as a major disease of avocado in Spain, the most important avocado producing country in the Mediterranean Basin. Selection for tolerant rootstocks was carried out, but this was probably not completely successful because integrated management strategies are being sought (Ruano-Rosa *et al*., 2018a). The disease is a serious threat to the avocado industry in Italy, that currently relies on *P. cinnamomi*–tolerant rootstocks for sustainable production. Molecular diagnostic methods to detect the pathogen in soil and host tissues are available (Schena *et al*., 2002; Schena *et al*., 2013), and these could be useful tools to prevent introduction with infected nursery plants or to select non-infested planting sites.

Verticillium wilt caused by *V. dahliae* and Phytophthora crown and root rot caused by *Phytophthora* spp. are common diseases in several traditional crops in Italy, and these pathogens have been recently reported on goji (*Lycium barbarum* **L.)**. This plant, producing edible fruit, is native to China, and was introduced into Italy and grown commercially from only a few years ago. Although goji can is a minor crop, it has become very popular due to the vaunted health benefits of its berries. Verticillium wilt of young plants of goji has been reported in Calabria (Ruano-Rosa *et al*. 2017), while crown and root rot caused by *P. nicotianae* have been reported in Apulia (Cariddi *et al*. 2018).

AVAILABILITY OF FUNGICIDES AND DEVELOPMENT OF FUNGICIDE RESISTANCE

Limited availability of fungicides and the development of fungicide resistance can be relevant as factors fostering emergence of soil-borne diseases in nurseries and agricultural systems. Insensitivity to metalaxyl among isolates of *P. capsici* causing Phytophthora blight of pepper has been reported in southern Italy since the 1990s (Pennisi *et al*., 1998). In a European-wide sample of 77 *P. ramorum* isolates collected in 2004, 24% were resistant to mefenoxam (Brasier, 2008). Phenylamide fungicides, including compounds such as metalaxyl, metalaxyl-M (mefenoxam) and benalaxyl, are frequently used against damping-off caused by *Pythium*. Resistance to phenylamides appeared shortly after their commercialization, in populations of various plant pathogenic oomycetes, including several *Pythium* spp. such as *P. aphanidermatum, P. dissotocum, P. heterothallicum, P. irregulare, P. cylindrosporum, P. splendens, P. torulosum* and *P. ultimum* (Moorman *et al*., 2002; Taylor *et al*., 2002).

A recent study by Matic *et al.* (2019), with 53 isolates of six *Pythium* species (*P. ultimum, P. aphanidermatum, P. irregulare* complex, *P. sylvaticum*, and *Pythium* ClusterB2a sp.) obtained from different vegetable hosts, showed that they were all sensitive to azoxystrobin, with small variations in their species-specific baseline sensitivity. As a consequence, this fungicide may be effectively applied regardless of the pathogen or host species involved. Conversely, precise *Pythium* species identification and sensitivity tests of isolates may be crucial for reliable use of mefenoxam, as baseline sensitivity to this fungicide varies greatly among species, and resistant isolates may occur in field populations of a sensitive species (Matic *et al*., 2019).

A relevant example of the emergence of diseases due to restrictions in the use of fungicides is the emergence or re-emergence of endemic soil-borne pathogens of vegetable crops after the phasing out of methyl bromide. Without soil fumigation, soil-borne pathogens considered minor, such as *C. coccodes* on tomato and pepper, have emerged, and well-known major pathogens, such as *P. capsici* on pepper and tomato, *S. sclerotiorum*, *R. solani*, *F. oxysporum* f. sp. *lactucae* and *Verticillium dahliae* on lettuce, and *S. sclerotiorum* and *R. solani* on *Solanum* or cucurbit hosts, have re-emerged (Garibaldi *et al*., 2008; Gilardi *et al*. 2014a,b). The implications of phasing-out of methyl bromide for the management of diseases of vegetable crops in Europe have been addressed by exhaustive reviews (Lazarovits and Subbarao, 2010; Colla *et al*., 2012).

In the last 10 years, cases of resistance to diverse classes of fungicides have been reported in populations of *Calonectria* and *Phytophthor*a species in nurseries of ornamentals in Southern Italy (Vitale *et al.* 2009; Guarnaccia *et al.* 2014; Aiello *et al.* 2018). These cases document the failures to control already established soil-borne polycyclic diseases as results of excessive and improper use of fungicides to control diseases of ornamental plants. The emergence of fungicide resistance in nursery populations of pathogens that have wide host ranges, such as *Phytophthora* and *Calonectria*, is a potential threat for other agricultural systems, because these fungicides are part of integrated pest management (IPM) strategies for many horticultural crops. More ecological approaches, such as the use of pathogen-suppressive soil mixtures or substrates (Hoitink *et al*.,1997; Hoitink and Boehm, 1999; Raviv, 2008; Pugliese *et al*., 2012; Cesarano *et al*., 2017a; Pascual *et al*., 2018; De Corato *et al*., 2019), and systems-based methods, can be alternatives to the intensive use of fungicides for the management of polycyclic soil-borne diseases in ornamental plant nurseries. Systems-based approaches are

disease management strategies that have evolved from IPM concepts, and these incorporate agricultural practices fostering disease suppressive soil microbial communities into the cropping system design (Chellemi *et al.*, 2016).

CHANGES OF CROPPING SYSTEMS

Shifts in farming practices and agricultural techniques, such as propagation methods, soil management types, planting densities and irrigation systems, or the substitution of cultivars or rootstocks, may trigger the emergence or re-emergence of soil-borne plant diseases. The disease consequences of the introduction and the large-scale use of grafted plants in intensive and soilless vegetable crops in Italy, fostered by the phasing-out of methyl-bromide, have changed cropping systems, and this can be a major driving factor of the emergence or resurgence of soil-borne fungal diseases (Gilardi *et al.*, 2013; Gilardi *et al.*, 2014 a, b, c,). Although most rootstocks of *Solanum* or cucurbit crops possess multiple resistances, none tolerate all the potential soil-borne pathogens of a particular crop. Resistance of different rootstocks to a single pathogen also varies greatly, and in some cases resistance may be overcome by high inoculum pressure and environmental conditions conducive for the disease. This has occurred for infections from *V. dahliae* on eggplant grafted on *S. nigrum*, and for *P. nicotianae* on tomato grafted on tomato hybrid rootstocks 'Beaufort' and 'He Man' (*Solanum lycopersicum* x *S. hirsutum*) (Gilardi *et al.*, 2011).

Change and intensification of cultivation systems may favour emergence of soil-borne fungal diseases in traditional tree crops. This is the case with Verticillium wilt (*V. dahliae*) and Phytophthora root rot (*Phytophthora* spp.) in olive orchards, as a consequence of the expansion of intensive and super-intensive planting systems (Cacciola *et al.*, 2011a; Jiménez-Diaz *et al.*, 2012). Mixed infections of both diseases have also been reported in Italy (Lo Giudice *et al.*, 2010). Verticillium wilt is the most serious disease of olive in Spain (López-Escudero and Mercado-Blanco, 2011), the most important world olive-producing country. In Italy, Verticillium wilt is regarded as a major disease of olive in Apulia (Nigro *et al.*, 2005), the most important olive-producing region, and is increasingly becoming a serious problem in new plantings. In Calabria and Sicily this disease occurs sporadically. Verticillium wilt also occurs on old trees, but symptoms are usually less severe and transient, as mature trees may recover spontaneously. Particular attention has been paid to irrigation as a factor favouring the increasing incidence and severity of Verticillium wilt in young, intensive and super-intensive olive plantations (Pérez-Rodríguez *et al.* 2016; Santos-Rufo *et al.*, 2017). However, numerous other factors are involved in the emergence of this disease, including infected planting (rooted-cuttings) and propagation material, establishment of new orchards in infested soils, spread of virulent *V. dahliae* strains of the D (defoliating) pathotype, and susceptibility of many popular cultivars, such as 'Arbequina' and 'Arbosana', which are widely used in super-intensive plantings. These and additional factors contributing to the emergence of Verticillium wilt in olive orchards are discussed in detail in the review on this disease by López-Escudero and Mercado-Blanco (2011). In Italy, evaluation of susceptibility of olive cultivars to the disease, and the search for resistant rootstocks, have potential for management of this disease (Bubici and Cirulli, 2012).

Pomegranate (*Punica granatum*) is another traditional fruit tree, native to the region extending from modern-day Iran to northern India. This plant has been cultivated since ancient times throughout the Middle East, Caucasus and Mediterranean region for its edible fruit. Recent intensive cultivation of this deciduous shrub, and establishment of modern commercial orchards for increasing yields, are posing new disease problems that could be serious constraint to expansion of this fruit crop in southern Italy. Crown and root rots caused by *Coniella* (syn. *Pilidiella*) *granati* are emerging soil-borne diseases in irrigated pomegranate orchards. This pathogen has been recently reported in Apulia, Basilicata, and Calabria regions (Pollastro *et al.*, 2016), and occurs in many other pomegranate-producing countries, including China, Iran, Spain, Greece and Turkey. *Coniella granati* may also cause pomegranate fruit rots. No effective management strategy is available for crown and root rot caused by this pathogen.

MONOCULTURE

It is commonly assumed that continuous cultivation of one crop, or crops of the same family, in the same soil results in increased inoculum of soil-borne pathogens, and increased incidence of root diseases, with consequent detrimental effects on crop yields. There is extensive evidence that reduction of biodiversity due to monoculture leads to development of detrimental soil conditions, that limit the cultivation of the same crop. This phenomenon, known in agriculture since ancient times but not fully explained, has been called "soil sickness". More recently, plant ecologists have preferred the more

comprehensive term negative plant-soil feedback (NPSF). This concept stresses the negative feedback between plant and soil, and includes agro-ecosystems and natural plant communities (Cesarano *et al.*, 2017b). Soil sickness is a serious concern for staple, cash, vegetable, forage, flower, ornamental and fruit tree crops (Bonanomi *et al.*, 2007; Bonanomi *et al.*, 2011a,b), including major crops such as wheat, maize, rice, sugarcane, alfalfa, soybean, grape, peach, apple, olive, citrus, tea and coffee. Three fundamental hypotheses have been put forward to explain soil sickness. These are: i) soil nutrient depletion or imbalance; ii) release of autotoxic compounds as root exudates or during decomposition of crop residues; and iii) build up of inoculum of soil-borne pathogens and corresponding modification in soil microbiomes, i.e. shifts in the soil microbial community structure from beneficial, including mychorrizae, to detrimental microorganisms (Cesarano *et al.*, 2017b). The effectiveness of soil sterilization or soil treatments with fungicides in restoring crop productivity has been regarded as the most convincing proof supporting the hypothesis that plant pathogens are major determinants of soil sickness (Cesarano *et al.*, 2017b). In Italy, the build-up of inoculum of several soil-borne fungal pathogens has been implicated in apple replanting problems and in the black root rot complex of strawberry (Manici *et al.*, 2003; 2005). Soil sickness due to *Phytophthora* spp. is causing the decline of lentil (*Lens culinaris*) crops on the island of Ustica (Puglisi *et al.*, 2016), a small island in the Tyrrhenian sea 38 km north of Sicily. The local landrace of lentil, appreciated by consumers and recently recognized as a Slow Food Presidium, is grown as a monocrop.

CONCLUSIONS

Based on published reports, most of the recently emerging diseases in Italy have been caused by pathogens introduced from other countries. Although emerging disease occurrence is partly distorted by the greater attention paid to discoveries of exotic pathogens and the rapidity with which the news of the emergence of new pathogens is delivered, this can mainly be imputed to the failure of the current phytosanitary system to prevent the introduction of alien pathogens, and confirms the need to reinforce this system. The EU regulation 2016/2031, which becomes fully effective in December 2019, demonstrates that there is awareness of the problem, and has addressed biosecurity by introducing major changes in phytosanitary regulations. This regulation aims to prevent introduction, establishment, and spread of harmful organisms for plants, and to coordinate and harmonize efforts made by different countries throughout the European Union. Similarly, an efficient phytosanitary certification system for propagation materials and nursery plants is the only effective means to prevent the spread of introduced and native pathogens within individual countries. These systems may be mandatory or voluntary, and must include forestry and landscape nursery plants as there is considerable evidence that these plants may be "Trojan horses" for the introduction and spread of invasive soil-borne plant pathogens in natural and forestry ecosystems. These ecosystems are particularly vulnerable, as eradication or mitigation of the effects of harmful pathogens in these ecosystems are not feasible or is more problematic than in agricultural systems.

Another way to reduce the risk of introduction and spread of exotic pathogens is to develop modern and self-sufficient national nursery production systems. In this regard Italy, which was able to meet domestic demand for nursery plants in strategic sectors, such as citrus, olive, fruit and nut trees crops, increasingly relies on imports from other countries. Plant material for ornamental, forestry, and staple and horticultural crops such as potato and strawberry, is also imported into Italy.

Application of phytosanitary regulations and the success of phytosanitary certification systems depend largely on the availability of adequate diagnostic methods, which have to be continuously updated to accommodate the rapid evolution of nomenclature and molecular taxonomy of fungi (Rossman and Palm-Hernandez, 2008; Groenewald *et al.*, 2011; Stielow *et al.*, 2015). These systems must be specific, sensitive, rapid, reproducible and practical. In the last 20 years, a large number of molecular methods for the detection of soil-borne fungal plant pathogens have been published in the scientific literature (Schena *et al.*, 2013). However, very few of these methods have been validated or applied routinely on large-scales, while some lack specificity which is a basic requirement (Blomquist *et al.*, 2005; Kunadiya *et al.*, 2017). Development of new and more specific molecular diagnostic methods is facilitated by the availability of whole genomes for a growing number of fungi and oomycetes (Feau *et al.*, 2019). Innovative and promising approaches for diagnoses of soil-borne pathogens include multiplex assays for genus- and species-specific detection of *Phytophthora* in environmental samples (Scibetta *et al.*, 2012; Bilodeau *et al.*, 2014), mRNA-based protocols to circumvent the problem of false positives due to the detection of DNA of non-viable propagules (Chimento *et al.*, 2012; Kunadiya *et al.*, 2019), isothermal amplification assays for *in situ* detection of *Phytophthora* spp. in plant tissues (Miles *et al.*, 2014), and PCR-based meth-

ods for the identification of *formae speciales* and races of *F. oxysporum* from seeds, plants and soil samples (Pasquali *et al.*,2008; Mbofung and Pryor, 2010; Srinivasan *et al.*, 2010; Lievens *et al.*, 2012; Gilardi *et al.,* 2017; Thomas *et al.*, 2017). Recently, a loop-mediated-isothermal amplification (LAMP) assay using a panel of target and non-target species was developed for detection of *Fusarium oxysporum* f. sp. *lactucae* in soil, lettuce seeds and plants. This assay is a significant advantage over the traditional methods, which do not allow clear discrimination of the *formae speciales* of *F. oxysporum* (Franco Ortega *et al.*, 2018). The taxonomy of *F. oxysporum* is evolving, so this will affect evaluation and interpretation of results obtained with these new diagnostic methods. Very recently, this taxonomy has been substantially modified and several cryptic species (so far 15) have been resolved within this species complex (Lombard *et al.*, 2019).

Next-generation sequencing approaches, which identify microorganisms in terms of operational taxonomic units, remain too expensive to be used as routine diagnostic methods, and the results are not precise enough to be used for quarantine or certification purposes. Conversely, as shown for aerial plant pathogens (Mosca *et al.*, 2014; Abdelfattah *et al.*, 2016), high-throughput sequencing could be an appropriate tool for studying the complexity and ecological functions of soil microbiota, its role in soil suppressiveness and negative plant-soil feedback, its interactions with soil-inhabitant pathogens, and effects of soil management practices on microbial population dynamics and functionality (Bonanomi *et al.*, 2016; Gómez Expósito *et al.*, 2017; Schlatter *et al.*, 2017; Ampt *et al.*, 2018).

Climate change, whose most evident effect is the rise in temperatures, has often been presumed to be responsible for the emergence of soil-borne diseases, simply because they were caused by thermophilic or mesophilic pathogens. This over simplification causes direct and indirect effects of climate change to be underestimated, and disease emergence drivers, such as severe weather events or conducive environmental conditions, which here have been considered distinct, may themselves be a consequence of climate change. Natural ecosystems are generally more vulnerable to the effects of climate changes, while in agricultural systems these effects can be mitigated by active interventions, such as irrigation during dry periods or the use of genotypes resistant to biotic or abiotic stress factors. Conversely, other disease emergence drivers, such as the genetic mutation of pathogens, prevail in agricultural systems due to the selective pressure exerted by the genetic uniformity of the host on the pathogen. In general, host genetic uniformity and susceptibility have crucial roles in determining invasion success and spread dynamics of plant pathogens after introduction and establishment, so monocultures of genetically similar or identical plants are severely impacted by invasive pathogens. It is generally assumed that biodiversity of natural ecosystems give them resilience against invasive exotic pathogens, but if the invader is a polyphagous pathogen, such as *P. cinnamomi* or *P. ramorum*, the result is a loss of biodiversity. It is, therefore, not easy to predict and evaluate the impacts of emerging diseases, as the emergence driving factors are numerous and complex, often interacting with each other.

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Research Paper

Race structure and distribution of *Pyrenophora tritici-repentis* in Tunisia

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Summary. Tan spot, caused by *Pyrenophora tritici-repentis* (Ptr), is a widespread foliar disease of wheat, which is becoming important in North Africa particularly in Tunisia. To assess the pathogenic variation of Ptr in Tunisia, 84 single conidium isolates of Ptr were characterized from durum wheat cultivars, sampled during the 2017–2018 cropping season. The virulence of isolates were assessed, under controlled conditions, on a standard differential set of six wheat genotypes. Ptr races 2, 4, 5, 6, 7 and 8 were identified, the first such information available for Tunisia. Race 2, commonly found in North America, South America and Asia, was identified for the first time in North Africa, at a low frequency of 5%. Races 5 and 7 were the most frequent, representing, respectively, 39% and 43% of the isolates tested. Only 8% of the isolates were classified as race 8, while 4% were identified race 6. Race 6 was only detected at the experimental station in the North Western region of Tunisia and in a nearby farm field. Only one Ptr isolate was avirulent on all six differential genotypes, and was therefore designated race 4. The identification of six races of Ptr on durum wheat demonstrates the high diversity of the pathogen population in Tunisia.

Keywords. Physiological races, *Pyrenophora tritici-repentis*, durum wheat, tan spot.

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INTRODUCTION

The fungus *Pyrenophora tritici-repentis* (Died.) Drechs., anamorph *Drechslera tritici-repentis* (Died.) Shoemaker (synonym *Helminthosporium tritici-repentis* Died.) causes tan spot, a foliar disease that affects bread wheat (*Triticum aestivum* L.), durum wheat (*T. turgidum* L. var. *durum*) and several grass species in many areas of the world (Morrall and Howard, 1975; Hosford *et al.,* 1975; Hosford, 1982; Krupinsky, 1982; De Wolf *et al.,* 1998; Ciuffetti and Tuori, 1999; Singh *et al.,* 2010).

Tan spot was first identified in Canada in 1939 (Conners, 1939), in the United States of America in New York State in 1940 (Barrus, 1942), in Australia in 1950 (Valder and Shaw, 1952) and in Mexico in 1982 (Gilchrist *et al.,* 1984). The disease was subsequently reported as the fastest spreading disease in the Southern Cone region of South America (Kohli *et al.,* 1992), and as a damaging disease in Argentina, Brazil, Paraguay and Australia (Annone, 1998; Kohli and Diaz, 1998; Loughman *et al.,* 1998). Since the 1970s, tan spot has been considered a serious problem that has caused significant yield losses in wheat crops (Watkins *et al.,* 1978; Hosford, 1982; Rees *et al.,* 1982; 1983; Shabeer and Bockus, 1988; Sykes and Bernier, 1991). In the late 1990s, tan spot was considered to be one of the main wheat diseases in Central Asia (Postnifova and Khasanov, 1998: Lamari *et al.,* 2005), and to be increasing on wheat grown in the Mediterranean region (Nasrellah and Mergoum, 1997; Benslimane *et al.,* 2006, 2011; M.S. Gharbi, *personal communication*). The increase in tan spot severity and incidence were reported to be due to changes in pathogen virulence, wide adoption of no-till and conservation tillage practices without suitable crop rotations, and the cultivation of susceptible cultivars (Rees, 1982; Rees and Platz, 1983; Brennen and Murray, 1988; Lamari and Bernier, 1989a; Mehta and Gaudencio, 1991; Kohli *et al.,* 1992). Furthermore, increased tan spot severity is associated with the survival of the pathogen on seeds, crop residues, and grass hosts (De Wolf *et al*., 1998; Singh *et al.,* 2010).

Typical symptoms of tan spot appear as brown necrotic lesions surrounded by chlorotic haloes, with small black points in the centre of the lesions. In some cases, extensive chlorosis occurs throughout host leaves. Lesions can coalesce, resulting in death of leaves (Lamari and Bernier, 1989a). Tan spot also affects kernels (red smudge) (Canadian Grain Commission. 1991), resulting in kernel discolouration and affecting seedling emergence, seedling vigour, yield, and grain quality. Seed infections could provide inoculum for epidemics and dispersal of pathogenic strains to new geographic areas (Vanderpool, 1963; Schilder and Bergstrom, 1995; Fernandez *et al.,* 1997; Bergstrom and Schilder, 1998; Fernandez *et al.,* 1998).

To date, eight races of *P. tritici-repentis* (Ptr) have been identified and characterized, based on their ability to induce necrosis and/or chlorosis on a wheat differential set. This includes four hexaploid wheats, 'Glenlea', 6B365, 6B662 and 'Salamouni', and two tetraploid wheats, 'Coulter' and 4B-160 (Lamari *et al.,* 1995, 1998; Strelkov *et al.,* 2002; Strelkov and Lamari, 2003; Lamari *et al.,* 2003; Singh *et al.,* 2010). Each race produces a unique necrotrophic effector (NE) (singly or in combination), which are designated Ptr ToxA, Ptr ToxB and Ptr ToxC (Lamari and Bernier, 1989c; Orolaza *et al.,* 1995; Ciuffetti *et al.,* 1998; Effertz *et al.,* 2002). The NEs are largely responsible for the necrosis and chlorosis symptoms associated with tan spot and serve as pathogenicity factors (Strelkov and Lamari, 2003). Races 2, 3 and 5 each produce a single NE, respectively Ptr ToxA, Ptr ToxC and Ptr ToxB, and are therefore considered the 'basic' races, while races 1, 6, 7 and 8 produce more than one NE each and are considered 'composite' races. Race 1 produces Ptr ToxA and Ptr ToxC, race 6 produces Ptr ToxB and Ptr ToxC, race 7 produces Ptr ToxA and Ptr ToxB, race 8 produces all three NEs (Strelkov *et al.,* 2002; Lamari *et al.,* 2003), and race 4 does not produce any active NE and is therefore avirulent.

The race structure of Ptr has been determined for several regions. In North America, 90% of the isolates have been classified as races 1 or 2 (Strelkov *et al.,* 2002; Singh *et al.,* 2007; Lamari and Strelkov, 2010; Aboukhaddour *et al.,* 2013), while races 3, 4 and 5 represent only 10% of the North American races. In the Southern Cone Region of South America, only races 1 and 2 were identified (Gamba *et al.,* 2012). Similarly, limited surveys in central Asia indicated the presence of races 1 and 2 (Lamari *et al.,* 2005). Moreover, races 1, 2, 3, 5, 7 and 8 were found in the Caucasus and the Fertile Crescent regions (Strelkov and Lamari, 2003; Lamari *et al.,* 2003, 2005; Lamari and Strelkov, 2010). In North Africa, all races except races 2 and 3 have been reported (Lamari *et al.,* 1995; Benslimane *et al.,* 2011; Gamba *et al.,* 2017). In addition to the eight well-characterized races, there have been some suggestions of the existence of other races, but no complete description has yet been published (Manning *et al.,* 2002; Meinhardt *et al.,* 2003; Andrie *et al.,* 2007; Ali *et al.,* 2010; Benslimane, 2018).

The aim of the present study was to examine the race structure and distribution of Ptr populations in the major wheat growing regions of Tunisia.

MATERIALS AND METHODS

Survey and fungal isolation

Surveys were carried out in the main wheat growing regions of Tunisia in the 2017–2018 cropping season. Each survey sample consisted of 40 leaves exhibiting typical tan spot symptoms, collected randomly from six commercial durum wheat fields (*Triticum durum*) in three regions, designated Coastal (CR), Northern (NR) and North Western (NWR), and from durum wheat growing at two experimental stations, Kodia at Bousalem and Oued Beja at Beja, at the NWR. Wheat growth

Table 1. The *Pyrenophora tritici-repentis* race structure and distribution in Tunisia.

Origin (location)	Coastal Region (CR)	Northern Region (NR)	North Western Region (NWR)	
			FF	ES
Race 2	13 %	0 %	5 %	5 %
Race 4	0 %	0 %	2 %	0 %
Race 5	38 %	34 %	46 %	29 %
Race 6	0 %	0 %	2 %	9 %
Race 7	25 %	58 %	40 %	48 %
Race 8	25 %	8 %	5 %	9 %

FF, Farmer field; ES, Experimental station.

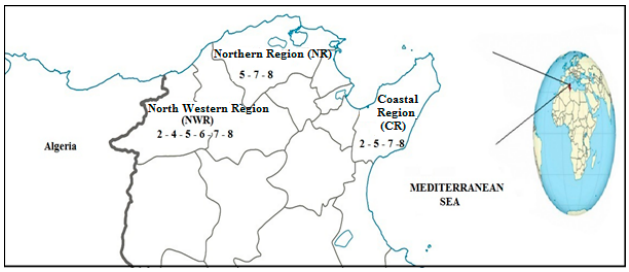

Figure 1. Races of *Pyrenophora tritici-repentis* characterized from durum wheat grown in three regions of Tunisia, North Africa.

stages at the time of the survey ranged from the beginning of stem elongation (ZGS 30) to the milk stage (ZGS 77) (Zadoks *et al.,* 1974). Leaf samples were collected and kept at room temperature overnight to dry. Fungal isolation and inoculum production were performed as described by Lamari and Bernier (1989a). Leaves were cut into 1 to 2 cm pieces, surface sterilized in 30% alcohol for 20 sec then 1% sodium hypochlorite solution for 2 min, and then washed three times, for 1 min each, with sterile distilled water. The leaf fragments were the placed in 9 cm-diam. Petri dishes each containing two layers of sterile filter paper moistened with sterile distilled water to maintain high humidity. These plates were incubated under fluorescent light for 24 h at 21°C to promote the production of conidiophores. The Petri dishes cultures were then incubated for 18 to 24 h at 15°C to induce conidial production. After incubation, leaf fragments were examined using 40× binocular magnifiers and single conidia identified as Ptr were transferred to V8-PDA medium (150 mL of V8 juice, 10 g of Potato Dextrose Agar, 3 g of $CaCO_3$, 10 g of water agar, and 850 mL of distilled water) and incubated at 20°C until the colony reached approx. 4 cm in diameter. In total, 84 single conidium isolates were obtained from the three production regions, and these isolates were subsequently phenotypically characterized on the wheat differential set (Table 1, Figure 1).

Inoculum production and inoculation

The Ptr cultures were incubated on V8-PDA medium in the dark for 7 to 8 days at 20°C, until they reached approx. 4 cm in diameter. Plates were then flooded with sterile distilled water, the mycelium in each plate was flattened with the bottom of a flamed test tube, and excess water was poured out. The cultures were subsequently incubated for 24 h under light at room temperature (20–22°C), followed by 24 h at 15°C in the dark. Conidia were then harvested by flooding the Petri dishes with sterile distilled water and dislodging the conidia with a wire loop. The inoculum concentration was adjusted to 3,000 conidia mL^{-1} using a haemocytometer (Hausser Scientific Company), and a drop of Tween 20 was added (polyoxyethylene sorbitanmonolaurate) per 100 mL to reduce surface tension in the conidium suspensions.

Wheat seedlings (see below) at the two-leaf stage were sprayed with conidium suspensions to run off, using a hand sprayer. Precautions were taken to avoid cross-infection of isolates. The inoculated seedlings were incubated in a dew chamber for 24 h (16 h light then 8 h darkness) at 20°C and 90% relative humidity (Lamari and Bernier, 1989a). All experiments were conducted at the CRP Wheat Septoria Precision Phenotyping Platform laboratory in Tunis. The seedlings were evaluated for symptom development 7 d after inoculation. Tan spot severity was assessed using the 1 to 5 scale developed by Lamari and Bernier (1989a), where: 1 = small, dark-brown to black spots, without any surrounding chloroses or tan necroses; 2 = small dark-brown to black spots, with very little chloroses or tan necroses; 3 = small, dark-brown to black spots, completely surrounded by distinct chlorotic or tan necrotic rings, not coalescing; 4 = small, dark-brown to black spots, completely surrounded by tanned chlorotic or necrotic zones, sometimes coalesced; and 5 = most lesions consisting of coalescing chlorotic or tan necrotic tissue. Scores equal to or greater than 3 indicated susceptibility, recorded as necrosis (N) and/or chlorosis (C), while those less than 3 indicate a resistant (R) reaction of the genotype to the tested isolate.

Plant material

The differential set consisted of six wheat genotypes, including the four hexaploid wheats 6B365, 'Glenlea',

Table 2. Reaction types[a] of the eight races of *Pyrenophora tritici-repentis* on six international wheat differential genotypes.

Wheat genotype	Race 1	Race 2	Race 3	Race 4	Race 5	Race 6	Race 7	Race 8
'Glenlea'	N[a]	N	R	R	R	R	N	N
6B662	R	R	R	R	C	C	C	C
6B365	C	R	C	R	R	C	R	C
'Salamouni'	R	R	R	R	R	R	R	R
'Coulter'	N	N	N	N	N	N	N	N
4B-160	C	R	N	R	N	-	-	-

[a] N, Necrosis; C, chlorosis; R, resistance. Adapted from (Lamari and Bernier, 1989a; Singh *et al*., 2010; Lamari and Strelkov, 2010).

6B662 and 'Salamouni', and the two tetraploid wheats 4B-160 and 'Coulter'. These were proposed by Lamari *et al.* (1995) to characterize the eight known races of Ptr (Table 2). Seeds of each genotype (five per pot) were sown into 10 cm diam. pots filled with a mix of ⅔ peat moss and ⅓ soil, and then kept in a growth chamber at 21°C (day) and 19°C (night) with a 16 h photoperiod. All treatments were replicated three times.

RESULTS

Race assessments for the differential host genotypes identified Ptr races 2, 4, 5, 6, 7 and 8 occurring in Tunisia (Table 3). Race 2 induced severe necrosis in 'Glenlea' and 'Coulter', but was avirulent on 4B-160, 6B662, 6B365 and 'Salamouni'. Race 5 was recovered from all locations, and induced severe chlorosis only on 6B662, and necrosis on 'Coulter' and 4B160, but was avirulent on 6B365 'Glenlea' and 'Salamouni'. Race 6 induced extensive chlorosis on 6B365 and 6B662 and necrosis on 'Coulter,' but was avirulent on 'Glenlea' and 'Salamouni'. Race 7 was avirulent on 'Salamouni' and 6B365, but induced extensive chlorosis on 6B662 and extensive necrosis on 'Coulter'. Race 8 was avirulent on 'Salamouni', but induced extensive chlorosis on 6B662 and 6B365 and extensive necrosis on 'Glenlea' and 'Coulter'. None of the races exhibited virulence on 'Salamouni'. All the Ptr isolates produced clear symptoms on the differential set, in accordance with the designated race structure; no new virulence types were observed. The distribution of Ptr races in Tunisia is shown in Figure 1, while the different responses of the differential host lines following inoculation are illustrated in Figure 2.

The 84 Ptr isolates tested grouped into six races: (2, 4, 5, 6, 7 and 8), of which four (5%) were classified as race 2; 33 (39%) were characterized as race 5; three (4%) were race 6, 36 (43%) were race 7, and seven (8%) were classified as race 8. One isolate was avirulent on all six differential genotypes and was designated as race 4 (Table 1). Races 5, 7 and 8 were found in all the three regions (CR, NR and NWR) surveyed. Race 6 was detected only in the NWR, while race 2 was found in the CR and NWR. Only one isolate from the NWR was race 4.

Races 2, 4, 5, 6, 7 and 8 were identified from the NWR, which represents the main durum wheat growing region in Tunisia. Races 2, 5, 7 and 8 were identified from a single farm field in the CR, while races 5, 7 and 8 were detected in two farm fields in the NWR. Races 5, 7 and 8 were prevalent across the surveyed regions, with race 8 predominating in the CR, race 7 in the NR, and race 5 in the NWR particularly in the farm fields (Table 1). Races 5 and 7 were predominant in all regions surveyed; representing, respectively, 38% and 25% in the CR, 34% and 58% in the NR, 46% and 40% in farm fields in the NWR, and 29% and 48% at the experimental stations in the NWR. In the CR, race 5 was the most common; in the NWR this was race 7. Races 7 and 8 were equally prevalent (25% of isolates) in the CR. Although race 8 was found in all regions, it was most prevalent in the CR, followed by the NR and NWR. Race 2 was identified in both CR (13%) and NWR (5%), but not in the NR. Races 4 and 6 were only present in the NWR with different frequencies in farm fields compared with the experimental stations. The presence of race 4 in a farm field but not at the experimental stations could have been due to the presence of grasses around this particular farm field, as grasses are likely alternative hosts of Ptr (Ali and Francl, 2003). Race 7 was present at a slightly higher frequency at the experimental stations compared with the farm fields, possibly due to the greater diversity (including levels of Ptr resistance) of wheat germplasm growing at test sites compared to single varieties likely being grown in farm fields.

DISCUSSION

This is the first study that has characterized the race structure of Ptr in Tunisia. Race 7 was the predominant

Table 3. Reactions of six differential wheat lines to 84 isolates of *Pyrenophora tritici-repentis*, their regional origin and race designation.

Isolate	Origin	Glenlea	6B662	6B365	Salamouni	Coulter	4B-160	Race
TSPPTR1	CR[a]	R[b]	C	R	R	N	N	5
TSPPTR2	CR	R	C	R	R	N	N	5
TSPPTR3	CR	N	R	R	R	N	R	2
TSPPTR4	CR	N	C	R	R	N	-	7
TSPPTR5	CR	N	C	R	R	N	-	7
TSPPTR6	CR	R	C	R	R	N	N	5
TSPPTR7	CR	N	C	C	R	N	-	8
TSPPTR8	CR	N	C	C	R	N	-	8
TSPPTR9	NR	R	C	R	R	N	N	5
TSPPTR10	NR	N	C	R	R	N	-	7
TSPPTR11	NR	R	C	R	R	N	N	5
TSPPTR12	NR	N	C	R	R	N	-	7
TSPPTR13	NR	N	C	R	R	N	-	7
TSPPTR14	NR	N	C	R	R	N	-	7
TSPPTR15	NR	N	C	R	R	N	-	7
TSPPTR16	NR	N	C	C	R	N	-	8
TSPPTR17	NR	N	C	R	R	N	-	7
TSPPTR18	NR	N	C	R	R	N	-	7
TSPPTR19	NR	R	C	R	R	N	N	5
TSPPTR20	NR	N	C	R	R	N	-	7
TSPPTR21	NWR	R	C	R	R	N	N	5
TSPPTR22	NWR	R	C	R	R	N	N	5
TSPPTR23	NWR	R	C	C	R	N	-	6
TSPPTR24	NWR	N	C	R	R	N	-	7
TSPPTR25	NWR	N	C	C	R	N	-	8
TSPPTR26	NWR	N	C	R	R	N	-	7
TSPPTR27	NWR	N	C	C	R	N	-	8
TSPPTR28	NWR	R	C	C	R	N	-	6
TSPPTR29	NWR	N	C	R	R	N	-	7
TSPPTR30	NWR	N	C	R	R	N	-	7
TSPPTR31	NWR	N	R	R	R	N	R	2
TSPPTR32	NWR	N	C	R	R	N	-	7
TSPPTR33	NWR	R	C	R	R	N	N	5
TSPPTR34	NWR	N	C	R	R	N	-	7
TSPPTR35	NWR	R	C	R	R	N	N	5
TSPPTR36	NWR	N	C	R	R	N	-	7
TSPPTR37	NWR	N	R	R	R	N	R	2
TSPPTR38	NWR	R	C	R	R	N	N	5
TSPPTR39	NWR	N	C	R	R	N	-	7
TSPPTR40	NWR	N	R	R	R	N	R	2
TSPPTR41	NWR	R	C	C	R	N	-	6
TSPPTR42	NWR	N	C	R	R	N	-	7
TSPPTR43	NWR	N	C	R	R	N	-	7
TSPPTR44	NWR	N	C	R	R	N	-	7
TSPPTR45	NWR	N	C	R	R	N	-	7
TSPPTR46	NWR	N	C	R	R	N	-	7
TSPPTR47	NWR	N	C	R	R	N	-	7
TSPPTR48	NWR	N	C	R	R	N	N	5

(Continued)

Table 3. (Continued).

Isolate	Origin	Glenlea	6B662	6B365	Salamouni	Coulter	4B-160	Race
TSPPTR49	NWR	R	R	R	R	R	R	4
TSPPTR50	NWR	N	C	R	R	N	-	7
TSPPTR51	NWR	N	C	R	R	N	-	7
TSPPTR52	NWR	N	C	R	R	N	-	7
TSPPTR53	NWR	N	C	R	R	N	-	7
TSPPTR54	NWR	N	C	C	R	N	-	8
TSPPTR55	NWR	N	C	R	R	N	-	7
TSPPTR56	NWR	N	C	R	R	N	-	7
TSPPTR57	NWR	N	C	R	R	N	-	7
TSPPTR58	NWR	N	C	R	R	N	-	7
TSPPTR59	NWR	R	C	R	R	N	-	5
TSPPTR60	NWR	R	C	R	R	N	-	5
TSPPTR61	NWR	R	C	R	R	N	-	5
TSPPTR62	NWR	R	C	R	R	N	-	5
TSPPTR63	NWR	R	C	R	R	N	-	5
TSPPTR64	NWR	R	C	R	R	N	-	5
TSPPTR65	NWR	R	C	R	R	N	-	5
TSPPTR66	NWR	R	C	R	R	N	-	5
TSPPTR67	NWR	R	C	R	R	N	-	5
TSPPTR68	NWR	N	C	C	R	N	-	8
TSPPTR69	NWR	R	C	R	R	N	-	5
TSPPTR70	NWR	R	C	R	R	N	-	5
TSPPTR71	NWR	R	C	R	R	N	-	5
TSPPTR72	NWR	R	C	R	R	N	-	5
TSPPTR73	NWR	R	C	R	R	N	-	5
TSPPTR74	NWR	R	C	R	R	N	-	5
TSPPTR75	NWR	R	C	R	R	N	-	5
TSPPTR76	NWR	R	C	R	R	N	-	5
TSPPTR77	NWR	N	C	R	R	N	-	7
TSPPTR78	NWR	N	C	R	R	N	-	7
TSPPTR79	NWR	R	C	R	R	N	-	5
TSPPTR80	NWR	R	C	R	R	N	-	5
TSPPTR81	NWR	N	C	R	R	N	-	7
TSPPTR82	NWR	R	C	R	R	N	-	5
TSPPTR83	NWR	N	C	R	R	N	-	7
TSPPTR84	NWR	N	C	R	R	N	-	7

[a] CR, Coastal Region; NR, North Region; NWR, North West Region
[b] N, susceptible necrosis; C, susceptible chlorosis; R, resistance.

race in Tunisia, and this was expected since it is predominant in neighbouring Algeria, where 40% of isolates tested were also race 7 (Benslimane *et al.,* 2011). This race was identified in Bejaia, Algeria, the closest wheat growing region to the NWR of Tunisia, where race 7 was identified mostly from durum wheat. Isolates of race 7 of the pathogen in Syria and Azerbaijan were primarily found on tetraploid wheat hosts (Lamari *et al.,* 2005).

The presence of races 5 and 6 in Tunisia was expected, since race 5 had previously been found in eastern regions of Algeria, and in Morocco (Lamari *et al*., 1995; Strelkov *et al*., 2002; Benslimane *et al.,* 2011; Gamba *et al.,* 2017). Almost 95% of isolates tested from the farm fields in the NWR, located 335 km from Guelma, Algeria, were race 5, which was previously reported in Algeria by Benslimane *et al.* (2011). In addition, Lamari *et al.* (2005) identified race 5 from tetraploid wheat in the countries along the Silk Road, including Syria and Azerbaijan. Results of the present study are consistent with previous studies by Lamari and Bernier (1989b) and

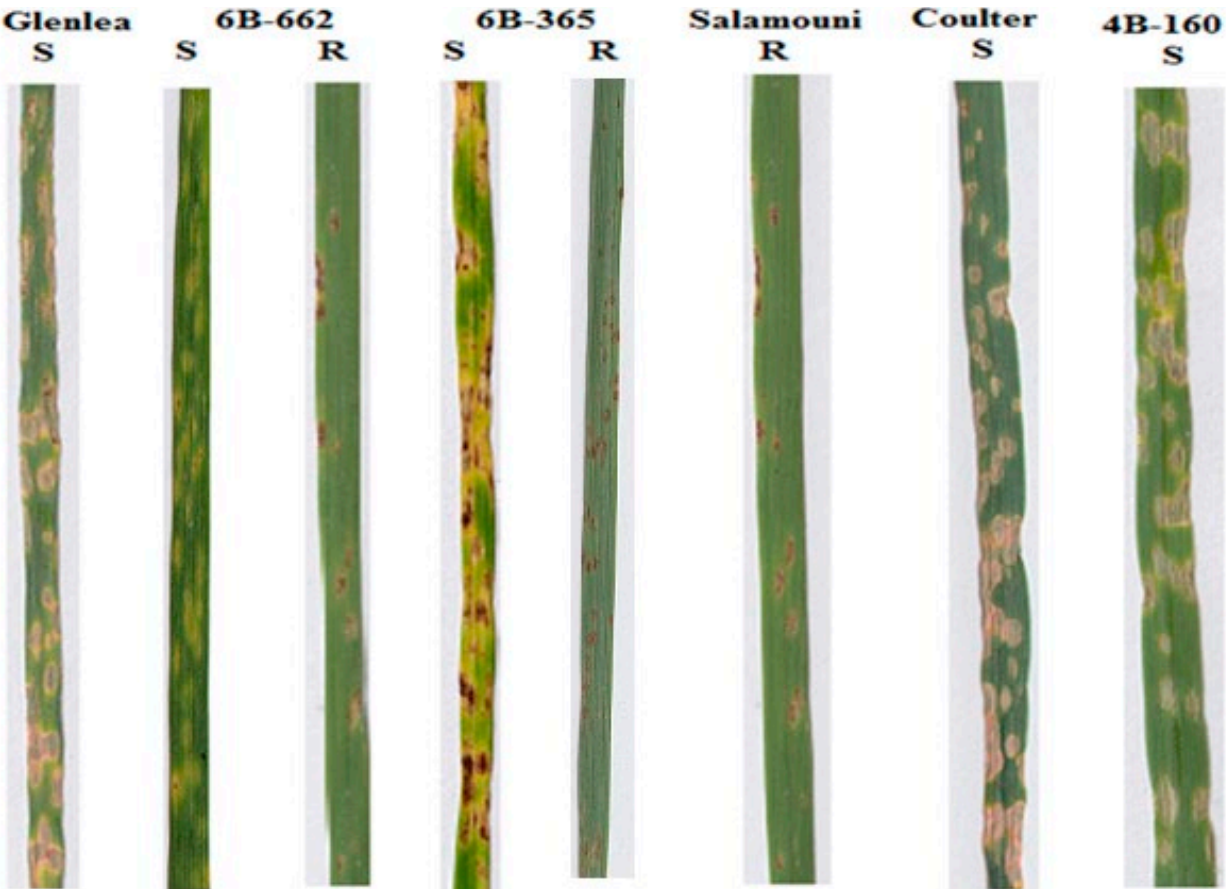

Figure 2. Representative resistant (R) and susceptible (S) reactions of wheat cvs. 'Glenlea', 'Salmouni', 'Coulter' and lines 6B662, 6B365 and 4B-160, to inoculation with isolates of *Pyrenophora tritici-repentis*. 'Glenlea' developed tan necrosis (S) when inoculated with Tunisian isolates classified as races 2, 7 or 8. Line 6B662 developed chlorosis (S) in response to inoculation with isolates of races 5, 6, 7 or 8. Line 6B365 developed chlorosis (S) when inoculated with Tunisian isolates classified as races 6 or 8. 'Coulter' developed necrosis (S) when inoculated with races 2, 5, 6, 7 or 8. Line 4B-160 developed necrosis (S) when inoculated with race 5. 'Salamouni' was resistant (R) to all identified isolates.

Lamari *et al.* (1998). Since all of our isolates were from durum wheat, the current results support the hypothesis that chlorosis-inducing isolates, which lack the ability to produce the Ptr ToxA, are associated with durum wheat.

Race 6 was found only in the NWR, at the Kodia experimental station and a farm field 10 km distant from the station. It is possible that this race was spread from the experimental station to the farm field by wind, since Ptr can be dispersed by wind-blown ascospores up to 200 km (Maraite *et al.,* 1992; Francl, 1997). Race 6 was also reported to occur in the western and central regions of Algeria, and in Morocco (Benslimane *et al.*, 2011; Gamba *et al.,* 2017).

Race 8 was found only in the central areas of Algeria, but was found in all regions of Tunisia at a low frequency (8%) (Benslimane *et al.*, 2011). Unlike Algeria and Morocco, where race 1 represented, respectively, 41% and 6% of Ptr isolates, this race was not identified in the present study. This was probably because all isolates were obtained from durum wheat, while in Algeria most isolates identified as race 1 were derived from bread wheat (Benslimane *et al.,* 2011).

Race 4 was represented by only one isolate from the NWR. This low frequency was reported in other regions, such as Canada (1%), North Dakota (5%) and one single isolate from Algeria (Lamari *et al.,* 1998; Ali and Francl, 2003; Benslimane *et al.,* 2011). The occurrence of limited isolates of race 4 could be linked to the sampling protocol used in the present study, since most of the samples were collected from growing wheat plants and not from crop debris or wild grasses. In addition, avirulent isolates do not form lesions on living hosts, and are therefore unlikely to be present when fungi are isolated from leaf samples. Several studies have shown that Ptr can survive on, and be isolated from, non-cereal grasses. The race 4 isolate we detected could have originated

from a grass host, as was shown in the study of Ali and Francl (2003), where 98% of the isolates obtained from non-cereal grass hosts were identified as race 4. Moreover, race 4 might be non-persistent in wheat fields since it would not compete with races that are virulent on wheat.

Race 2 has been reported from North America, the Southern Cone Region of South America, Central Asia, the Caucasus and the Fertile Crescent, Baltic states and Romania (Strelkov *et al.,* 2002; Lamari *et al.,* 2003, 2005; Singh *et al.,* 2007; Lamari and Strelkov, 2010; Lamari *et al.,* 2010; Gamba *et al.,* 2012; Aboukhaddour *et al.,* 2013; Momeni *et al.,* 2014; Abdullah *et al.,* 2017). However, the present study is the first to report the presence of Ptr race 2 in North Africa. This race was detected in the CR and NWR of Tunisia, albeit at overall low frequency (5%).

Occurrence of a greater number of Ptr races in the NWR than the other two regions could be due to the widespread use of zero tillage in the NWR. In contrast, in the NR and CR, cereal-legume crop rotations are common, likely resulting in little opportunity for the pathogen to survive in wheat stubble or on alternative hosts. Regarding races 2 and 8, their high frequency in the CR could be due to monoculture of the durum wheat cultivar 'Maali-cv' that is less cultivated in the other two regions of Tunisia. This also suggests that Ptr in the CR is very diverse. Our results are similar to those of Lamari *et al.* (2005), where five races were found within a single field in Azerbaijan.

Previous studies have reported that Ptr race 1 has been identified primarily from hexaploid wheat. In contrast, all samples in the present study were taken from tetraploid (durum) wheat. Isolates identified as race 1 originating from countries along the Silk Road likewise were mostly (70%) from hexaploid wheat, while only 22% were from tetraploid wheat (Ali and Francl, 2003; Lamari *et al.,* 2005; Benslimane *et al.,* 2011).

Leaf spot and blight diseases such as tan spot oversummer or over-winter on wheat stubble, which is retained in farm fields where zero tillage is practiced. Crop debris and stubble could act as major sources of primary inoculum of the pathogens and media allowing for sexual recombination. However, there are no reports to date that demonstrate ascospore race associations. Isolates from stubble may give different race structures following sexual reproduction during the resting stage.

Aung (2001) and Aboukhaddour *et al.* (2011) suggested that host genotypes could promote changes in pathogen population structures. In Tunisia, where cereal growers practice conservation agriculture or fallows between cereal crops, considerable straw is left on the soil, which can harbor leaf spot pathogens such as Ptr. Ascospores produced from previous crop debris can readily spread during early subsequent crop development to initiate infections (Morrall and Howard, 1975). Knowledge of when ascospores are first released would be a major asset for growers, enabling them to apply preventative measures to reduce early plant infections. Knowledge of Ptr race structure could also provide breeders with the opportunity to incorporate effective resistance genes (if available) into new varieties. Ideally, newly-developed varieties should carry several different resistance genes to maximize resistance durability.

The present study has demonstrated that the Ptr population in Tunisia is diverse. As such, and to develop effective and durable resistance, it would be prudent to test breeders' lines against all virulent races present in the region(s), to determine the resistance status of the lines, and make selections accordingly. Future studies should include Ptr isolates obtained from bread wheat and from wheat stubble and wild grasses, in order to fully characterize the races present. This would be useful in the eventual development of control measures that include choice of resistant cultivars along with appropriate fungicide disease management strategies.

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Research Paper

Resistance of wild barley (*Hordeum spontaneum*) and barley landraces to leaf stripe (*Drechslera graminea*)

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Summary. Wild barley (*Hordeum spontaneum*) and barley landraces are important sources of genetic variation for disease resistance. Thirty wild barley (*H. spontaneum*) genotypes and 30 barley landraces were evaluated for susceptibility to two *Drechslera graminea* isolates. Virulence differences were observed between the isolates, while the responses of the host genotypes to the isolates also varied. Of the *H. spontaneum* genotypes, 23% and 63%, respectively, were resistant to the Yozgat *D. graminea* isolate, and Eskişehir *D. graminea* isolates. On the other hand, 43% and 90% of the barley landraces were resistant to Yozgat and Eskişehir *D. graminea* isolates, respectively. *Hordeum spontaneum* genotypes 13, 24, 27, 29, 54, 86, and 91 exhibited resistance to both *D. graminea* isolates, while genotypes 14 and 32 showed intermediate reactions to the Yozgat isolate and resistant reactions to the Eskişehir isolate. Barley landraces 21, 37, 38, 39, 40, 73, 98, 128, 139, 153, 159,167, and 171 showed resistant reactions, and barley landrace 8 showed an intermediate reaction to both isolates. Barley landraces 3, 20, 24, 71, 101, 103, 104 and 160 exhibited intermediate responses to the Yozgat isolate and a resistant response to the Eskişehir isolate. Using resistant barley genotypes would reduce the need for pesticides for control of leaf stripe, and be an environmentally preferred strategy for disease control. The disease resistance present in wild barley and barley landraces are important for expanding the genetic basis of cultivated barley (*H. vulgare*). The resistant and intermediate genotypes identified in this study could be used as resistance sources in barley breeding, or landraces could be used directly for commercial barley production.

Keywords. Disease resistance, *Pyrenophora graminea, Hordeum vulgare.*

INTRODUCTION

Barley (*Hordeum vulgare* L.) is the second most cultivated cereal crop, after wheat, in Turkey, constituting 22% of the cereal production area. In this country, 7.1 million tons of barley are produced per year, with an average yield of 293 kg ha^{-1} (Tuik, 2017). It is believed that approx. 10,000 years ago, the first area where barley was cultivated was in the Fertile Crescent Region, located between the Mediterranean and Arab peninsulas, and bordered by

the Tigris and Euphrates valleys (Harlan and Zohary, 1966; Nesbitt, 1995; Willcox, 1995; Ladizinski, 1998). Throughout history, this region has been considered as one of the richest centres of plant diversity (Zohary and Hopf, 2000). Turkey is uniquely situated in terms of plant genetic diversity, as it has an abundance of plant species and endemism due to a combination of geomorphologic, topographic, and seasonal diversity (Fao, 2015). Turkey is located at the intersection point between Mediterranean and Near East gene centres, and this area is one of the most significant genetic centres for barley (Kün, 1996).

Barley landraces (*H. vulgare* L. subsp. *vulgare*) are heterogeneous plant species grown by farmers and are populations exposed to natural and artificial selection (Brown, 2000), and barley landraces are genetically closer to modern varieties compared to wild barley (Thomas *et al*., 1998). Local barley varieties are the main sources of seed used in regions of low annual rainfall and where 'traditional' agriculture is practiced (Ceccarelli and Grando, 2000). Wild barley (*H. spontaneum* C. Koch) is accepted as the progenitor of cultivated barley (*H. vulgare*), and its habitat is in the Fertile Crescent Region. This plant is indigenous to the area between the south and southeast of Turkey and the area between North Africa and southwest Asia (Harlan and Zohary, 1966; Nevo, 1992; Von Bothmer *et al*., 1995). *Hordeum spontaneum* is often found in secondary habitats such as Mediterranean scrub lands or roadsides (Zohary and Hopf, 2000).

Wild barley genotypes and barley landraces are important resources for genetic variation, as they are highly adapted to abiotic and biotic stresses and can therefore be cultivated under unfavorable conditions (Allard and Bradshaw, 1964; Yitbarek *et al*., 1998; Ellis *et al*., 2000; Ceccarelli and Grando, 2000; Karakaya *et al*., 2016a). This genetic variation provides a potential source of disease resistance alleles for breeding programmes (Allard and Bradshaw, 1964; Ceccarelli, 1996). Turkey is one of the most important genetic centres for barley, as landraces are widely planted and wild barley genotypes grow under natural conditions (Helbaek, 1969; Kün, 1996; Pourkheirandish and Komatsuda, 2007; Karakaya *et al*., 2016a; Ergün *et al*., 2017).

The causal agent of barley leaf stripe is the fungus *Drechslera graminea* (Rabenh. ex Schlecht.) Shoemaker (=*Helminthosporium gramineum* Rabh.) (teleomorph: *Pyrenophora graminea* (S. Ito & Kurib.). This fungus is a single-cycle, seed borne pathogen that causes reductions in barley yields and quality throughout world cereal production areas. The pathogen survives as mycelium within host pericarps and grows into developing seedlings via the coleorhizae when the barley seeds germinate (Platenkamp, 1976). Subsequently, the pathogen grows systemically in developing host plants (Çetinsoy, 1995; Mathre, 1997; Aktaş 2001). The first symptoms of disease occur as yellow stripes on seedling leaves, and these progress to chlorotic and necrotic stripes areas along the leaves. As results of the disease, sterile spikes and stunting also occur in affected plants (Tekauz and Chiko, 1980; Zad *et al*., 2002). Severe infections result in drying out and premature death of plants (Mathre, 1997). Yield losses due to leaf stripe have been reported from various countries (Porta-Puglia *et al*., 1986; Arabi *et al*., 2004). The disease is present in Turkish barley fields, causing yield losses between 3% and 15% (Mamluk *et al*., 1997). In 2012 and 2013, it was found that 40% of the surveyed barley fields in Central Anatolia were affected by leaf stripe (Karakaya *et al*., 2016b). Kavak (2004) emphasized that in addition to yield losses, quarantine issues can be important, because the pathogen is readily seed transmitted. While barley leaf stripe can be controlled through the use of seed treatment fungicides, growing of resistant varieties would minimize the need for pesticides and be an economic and environmentally friendly method for controlling the disease.

Research has shown a diversity of morphological characters and virulence levels for populations of *D. graminea* (Gatti *et al*., 1992; Jawhar and Arabi, 2006; Karakaya *et al*., 2017). McDonald and Linde (2002) emphasized that pathogen populations that vary genetically can quickly evolve and overcome plant resistance. Significant virulence diversity and the possible results of a shrinking genetic basis of cultivated barley have been studied by plant pathologists and plant breeders (Jensen, 1988; Ulus and Karakaya, 2007; Çelik *et al*., 2016). Plant breeders need sustainable sources of disease resistance for effective long-term disease control.

In this study, 30 barley landraces and 30 wild barley (*H. spontaneum*) genotypes were selected from a collection maintained by the Gene Bank of the Central Research Institute for Field Crops located in Ankara, Turkey. These host germplasm lines were assessed for their resistance to leaf stripe using two isolates of *D. graminea*.

MATERIALS AND METHODS

*Barley landraces, wild barley (*H. spontaneum*) genotypes and* Drechslera graminea *isolates*

Thirty *H. spontaneum* genotypes and 30 barley landraces were obtained from the Gene Bank of the Field Crops Central Research Institute, in Ankara, Turkey.

These plant lines had been collected from different regions of Turkey, and seeds of these genotypes had been multiplied from single spikes and maintained in the Gene Bank.

The virulent Yozgat isolate of *D. graminea* and the moderately virulent Eskişehir isolate were compared. The virulence of these isolates was previously determined by Çelik *et al*. (2016) and Karakaya *et al*. (2017). The isolates were maintained at the Mycology Laboratory of Ankara University, Faculty of Agriculture, Department of Plant Protection, Turkey. Barley cultivars 'Çumra 2001' and 'Larende' were used, respectively, as resistant and susceptible controls (Çelik *et al*., 2016). The disease responses of these 30 landraces and 30 wild barley genotypes used in the present study against *Pyrenophora teres* f. *teres*, *P. teres* f. *maculata* and *Rhynchosporium commune* had been determined in previous studies (Çelik Oğuz *et al*., 2017b; Çelik Oğuz *et al*., 2019; Azamparsa *et al*., 2019).

Treatments and disease evaluations

The sandwich method described by Mohammad and Mahmood (1974) was used to inoculate the barley landraces and wild barley genotypes. Seeds were surface sterilized with a 1% NaOCI solution for 3 min and then rinsed with sterile water. Cultures of *D. graminea* were grown on potato dextrose agar medium in Petri dishes at 22 ± 2°C for 10 d. Fifteen seeds of each host landrace or wild genotype were placed on the surface of half of a *D. graminea* culture followed by folding the other half of the culture over the seeds under sterile conditions. Cultures folded as 'sandwiches' were kept at 22°C for 4 d. After germination, seeds were incubated at 4°C for an additional 5 d. Three replications were used for each host line and isolate combination. Following treatment, the incubated seeds were taken from the sandwich cultures using sterile forceps and planted in pots containing growth medium (soil, sand, and animal manure at 3:1:1 w:w:w). The resulting plants were grown under greenhouse conditions of 15 ± 2°C at night, 22 ± 2°C during the day, using a 15/9 h light/dark regime. Pots were arranged on a greenhouse bench in a completely randomized fashion. Disease ratings were taken at 45 and 60 d after planting of inoculated seeds and were recorded separately. The responses of the plants to the two *D. graminea* isolates were evaluated using the scale developed by Tekauz (1983). Scale values were: 1 = infection < 5% (Resistant, R); 2 = infection 5–17% (Intermediate, I); and 3 = infection > 17% (Susceptible, S).

Data analyses

The percentage of leaf barley stripe was calculated using the following equation (Dumalasova *et al*., 2014).

% Disease incidence = No. of infected plants ÷ Total no. of plants ×100.

Separate analyses of variances were performed for disease assessed at 45 and 60 d after planting for isolate, genotype and isolate*genotype interaction effects (Tables 1-4).

Biplot analysis was performed for the isolates and disease percentage values to assess the disease responses of each genotype tested to the two *D. graminea* isolates used (MSTAT, Michigan State University).

RESULTS

At 45 d after planting of inoculated seeds, there were 20 wild barley (*H. spontaneum*) genotypes showing susceptible reactions to the virulent Yozgat isolate of *D. graminea*, three genotypes with intermediate reactions and seven genotypes with resistant reactions to this isolate. At 60 days after planting, 21 genotypes were susceptible, two were intermediate and seven were resistant to the Yozgat isolate. Five genotypes were susceptible to

Table 1. Analysis of variance for the resistance of 30 *Hordeum spontaneum* genotypes 45 d after planting following seed inoculation with two isolates of *Drechslera graminea.*

Source	DF	SS	MS	F	P
H. spontaneum genotypes	29	66151.3	2281.1	1105.86	<0.001
Isolates	1	46217.7	46217.7	22406.17	<0.001
*H. spontaneum**isolates	29	33414.9	1152.2	558.60	<0.001
Error	120	247.5	2.1		
Total	179	146031.4			

Table 2. Analysis of variance for the resistance of 30 *Hordeum spontaneum* genotypes 60 d after planting following seed inoculation with two isolates of *Drechslera graminea.*

Source	DF	SS	MS	F	P
H. spontaneum genotypes	29	72401.8	2496.6	1842.90	<0.001
Isolates	1	52473.2	52473.2	38733.54	<0.001
*H. spontaneum**Isolates	29	36521.1	1259.3	929.60	<0.001
Error	120	162.6	1.4		
Total	179	161558.6			

Table 3. Analysis of variance for the resistance of 30 barley landraces 45 d after planting following seed inoculation with two isolates of *Drechslera graminea*.

Source	DF	SS	MS	F	P
Barley landraces	29	13938.64	480.64	706.65	<0.001
Isolates	1	5261.77	5261.77	7736.00	<0.001
Barley landraces*Isolates	29	7961.87	274.55	403.65	<0.001
Error	120	81.62	0.68		
Total	179	27243.90			

Table 4. Analysis of variance for the resistance of 30 barley landraces 60 d after planting following seed inoculation with two isolates of *Drechslera graminea*.

Source	DF	SS	MS	F	P
Barley landraces	29	13551.12	467.28	687.01	<0.001
Isolates	1	6646.66	6646.66	9772.10	<0.001
Barley landraces*Isolates	29	7878.26	271.66	399.41	<0.001
Error	120	81.62	0.68		
Total	179	28157.66			

the moderately virulent Eskişehir isolate, six genotypes had intermediate reactions, and 19 were resistant to this isolate. The numbers of genotypes showing susceptible, intermediate, and resistant reactions to the Eskişehir isolate remained unchanged at 60 d after planting (Table 5).

At 45 d after planting, seven barley landraces exhibited susceptible reactions to the Yozgat isolate of *D. graminea*, eight landraces showed intermediate reactions, and 15 ladraces were resistant to the isolate. At 60 d after planting, eight barley landraces were susceptible to the Yozgat isolate, nine landraces were intermediate, and 13 landraces showed resistant reactions to this isolate. At 45 d after planting, three landraces showed intermediate reactions and 27 landraces showed resistant reactions to the Eskişehir isolate. At the 60 day assessment, the reactions of the genotypes to the Eskişehir isolate were the same as those assessed at 45 d (Table 6).

The susceptible control barley cultivar 'Larende' exhibited susceptible reactions to both *D. graminea* isolates, and the resistant control cultivar 'Çumra 2001' was resistant to the two isolates.

Separate analyses of variance revealed statistically significant ($P < 0.01$) differences among the *H. sponatenum* genotypes and barley landraces and between the two *D. graminea* isolates, both at 45 and 60 d after planting of inoculated seeds. Significant ($P < 0.01$) isolate*genotype interactions were also detected (Tables 1-4).

Disease resistance evaluations require clear understanding of host/pathogen interactions. Visual analyses of these interactions are possible with biplot analyses. Low Component 1 negative values, and Component 2 values close to zero in biplots clearly illustrate the resistance of genotypes to disease (Yan and Falk 2002). In the biplot analyses, *H. spontaneum* genotypes 13, 24, 27, 29, 54, 86, 91, and the resistant control cultivar 'Çumra 2001' were grouped together, and representing the most resistant genotypes of those studied (Figure 1). The wild barley genotypes 32 and 14 showed intermediate responses to the Yozgat isolate, but they were resistant to the Eskişehir isolate. These two genotypes were closest to the point where the resistant genotypes were placed. Genotypes 1, 52, 62, 107, and the susceptible control cultivar 'Larende' which showed a susceptible reaction to both isolates, were between the two isolates in the biplot. The wild barley genotype 4, which was susceptible to both *D. graminea* isolates, was closer to the Eskişehir isolate biplot line because it was more susceptible to the Eskişehir isolate than to the Yozgat isolate (Table 1, Figure 1).

Barley landraces 39, 21, 38, 139, 98, 40, 159, 73, 167, 171, 37, 128, 153 and the resistant control cultivar 'Çumra 2001' were the genotypes that were most resistant to *D. graminea*. These landraces exhibited resistant reactions to both *D. graminea* isolates, and they were all at the same point on the biplot graph (Figure 2). Landraces 160, 24, 103, 20, 101, 104, 71, and 3 exhibited intermediate reactions to the Yozgat isolate and resistant reactions to the Eskişehir isolate. No genotypes were susceptible to both pathogen isolates, except for the susceptible control cultivar 'Larende'. Barley landraces 148 and 74 were susceptible to the Yozgat isolate and exhibited intermediate responses to the Eskişehir isolate (Figure 2).

DISCUSSION

The present study is the first evaluation of resistance to leaf stripe for these 30 barley landraces and 30 wild barley genotypes. Differences in host reactions to inoculation with *D. graminea* were detected. Virulence differences between two isolates of the pathogen were also evident. Overall, the barley landraces were more resistant to *D. graminea* than the *H. spontaneum* genotypes examined in this study. Other reports from Turkey and elsewhere have also shown variable levels of resistance in barley to *D. graminea*. Mueller *et al.* (2003) carried out a study using 612 barley accessions, and determined that they exhibited different reactions to natural infections by *D. graminea* under organic agriculture conditions.

Table 5. Reactions of 30 wild barley (*Hordeum spontaneum*) genotypes following inoculation with two isolates of *Drechslera graminea*. For disease values, the scale of Tekauz (1983) was used.

D. graminea, Yozgat isolate					*D. graminea*, Eskişehir isolate				
Hordeum spontaneum genotype	45 d after planting		60 d after planting		*Hordeum spontaneum* genotype	45 d after planting		60 d after planting	
	Mean disease percent	Scale value	Mean disease percent	Scale value		Mean disease percent	Scale value	Mean disease percent	Scale value
1	100	3 (S)	100	3 (S)	1	22.2	3 (S)	22.2	3 (S)
4	25	3 (S)	25	3 (S)	4	33.3	3 (S)	33.3	3 (S)
5	16.6	2 (I)	33.3	3 (S)	5	0	1 (R)	0	1 (R)
6	25	3 (S)	25	3 (S)	6	0	1 (R)	0	1 (R)
8	40	3 (S)	40	3 (S)	8	0	1 (R)	0	1 (R)
9	66.6	3 (S)	83.3	3 (S)	9	12.5	2 (I)	12.5	2 (I)
13	0	1 (R)	0	1 (R)	13	0	1 (R)	0	1 (R)
14	14.2	2 (I)	14.2	2 (I)	14	0	1 (R)	0	1 (R)
16	55.5	3 (S)	55.5	3 (S)	16	0	1 (R)	0	1 (R)
24	0	1 (R)	0	1 (R)	24	0	1 (R)	0	1 (R)
27	0	1 (R)	0	1 (R)	27	0	1 (R)	0	1 (R)
29	0	1 (R)	0	1 (R)	29	0	1 (R)	0	1 (R)
32	12.5	2 (I)	12.5	2 (I)	32	0	1 (R)	0	1 (R)
33	28.5	3 (S)	28.5	3 (S)	33	0	1 (R)	0	1 (R)
38	66.6	3 (S)	66.6	3 (S)	38	0	1 (R)	0	1 (R)
44	33.3	3 (S)	33.3	3 (S)	44	16.6	2 (I)	16.6	2 (I)
45	71.4	3 (S)	71.4	3 (S)	45	11.1	2 (I)	11.1	2 (I)
49	42.8	3 (S)	42.8	3 (S)	49	14.2	2 (I)	14.2	2 (I)
52	71.4	3 (S)	100	3 (S)	52	28.5	3 (S)	28.5	3 (S)
54	0	1 (R)	0	1 (R)	54	0	1 (R)	0	1 (R)
62	75	3 (S)	75	3 (S)	62	50	3 (S)	50	3 (S)
66	50	3 (S)	50	3 (S)	66	0	1 (R)	0	1 (R)
70	40	3 (S)	40	3 (S)	70	0	1 (R)	0	1 (R)
76	71.4	3 (S)	71.4	3 (S)	76	0	1 (R)	0	1 (R)
80	87.5	3 (S)	87.5	3 (S)	80	11.1	2 (I)	11.1	2 (I)
86	0	1 (R)	0	1 (R)	86	0	1 (R)	0	1 (R)
91	0	1 (R)	0	1 (R)	91	0	1 (R)	0	1 (R)
93	75	3 (S)	75	3 (S)	93	10	2 (I)	10	2 (I)
99	75	3 (S)	75	3 (S)	99	0	1 (R)	0	1 (R)
107	57.1	3 (S)	57.1	3 (S)	107	28.5	3 (S)	28.5	3 (S)
Larende	80	3 (S)	80	3 (S)	Larende	60	3 (S)	60	3 (S)
Çumra 2001	0	1 (R)	0	1 (R)	Çumra 2001	0	1 (R)	0	1 (R)
	40.01*		41.09*			7.93*		9.31*	

*Significant at $P < 0.01$ (Tables 1 and 2).

More than 30% of the accessions were resistant to *D. graminea*. In the same study, a small group of accessions was selected and tested using the sandwich inoculation method for reactions to two aggressive *P. graminea* isolates. They found that the accessions BGRC 5592, HOR 333, HOR 11475, and OU J362 showed resistant reactions. Similarly, the sandwich method was applied in the present study, and we determined that 23% of the wild barley genotypes and 43% of the barley landraces were resistant to both isolates of *D. graminea*.

Arabi *et al.* (2004) tested ten widely cultivated barley varieties against a virulent *D. graminea* isolate (Sy3) in southern Syria. Differential reactions were observed among the varieties, and as the level of disease increased, there were decreases in crop yield, kernel weight, and plant biomass. It has also been reported

Table 6. Reactions of 30 barley landraces following inoculation with two isolates of *Drechslera graminea*. For disease valuaes, the scale of Tekauz (1983) was used.

D. graminea, Yozgat isolate					*D. graminea,* Eskişehir isolate				
Barley landrace	45 d after planting		60 d after planting		Barley landrace	45 d after planting		60 d after planting	
	Mean disease percent	Scale value	Mean disease percent	Scale value		Mean disease percent	Scale value	Mean disease percent	Scale value
3	12.5	2 (I)	12.5	2 (I)	3	0	1 (R)	0	1 (R)
8	16	2 (I)	16	2 (I)	8	12.5	2 (I)	12.5	2 (I)
12	20	3 (S)	20	3 (S)	12	0	1 (R)	0	1 (R)
18	22.2	3 (S)	22.2	3 (S)	18	0	1 (R)	0	1 (R)
20	0	1 (R)	16.6	2 (I)	20	0	1 (R)	0	1 (R)
21	0	1 (R)	0	1 (R)	21	0	1 (R)	0	1 (R)
22	12.5	2 (I)	25	3 (S)	22	0	1 (R)	0	1 (R)
24	14.2	2 (I)	14.2	2 (I)	24	0	1 (R)	0	1 (R)
37	0	1 (R)	0	1 (R)	37	0	1 (R)	0	1 (R)
38	0	1 (R)	0	1 (R)	38	0	1 (R)	0	1 (R)
39	0	1 (R)	0	1 (R)	39	0	1 (R)	0	1 (R)
40	0	1 (R)	0	1 (R)	40	0	1 (R)	0	1 (R)
71	16.6	2 (I)	16.6	2 (I)	71	0	1 (R)	0	1 (R)
73	0	1 (R)	0	1 (R)	73	0	1 (R)	0	1 (R)
74	50	3 (S)	50	3 (S)	74	14.2	2 (I)	14.2	2 (I)
83	37.5	3 (S)	37.5	3 (S)	83	0	1 (R)	0	1 (R)
90	37.5	3 (S)	37.5	3 (S)	90	0	1 (R)	0	1 (R)
98	0	1 (R)	0	1 (R)	98	0	1 (R)	0	1 (R)
101	16.6	2 (I)	16.6	2 (I)	101	0	1 (R)	0	1 (R)
103	12.5	2 (I)	12.5	2 (I)	103	0	1 (R)	0	1 (R)
104	16.6	2 (I)	16.6	2 (I)	104	0	1 (R)	0	1 (R)
128	0	1 (R)	0	1 (R)	128	0	1 (R)	0	1 (R)
139	0	1 (R)	0	1 (R)	139	0	1 (R)	0	1 (R)
148	50	3 (S)	50	3 (S)	148	11.1	2 (I)	11.1	2 (I)
153	0	1 (R)	0	1 (R)	153	0	1 (R)	0	1 (R)
159	0	1 (R)	0	1 (R)	159	0	1 (R)	0	1 (R)
160	0	1 (R)	11.1	2 (I)	160	0	1 (R)	0	1 (R)
162	28.5	3 (S)	28.5	3 (S)	162	0	1 (R)	0	1 (R)
167	0	1 (R)	0	1 (R)	167	0	1 (R)	0	1 (R)
171	0	1 (R)	0	1 (R)	171	0	1 (R)	0	1 (R)
Larende	66.6	3 (S)	66.6	3 (S)	Larende	33.3	3 (S)	33.3	3 (S)
Çumra 2001	0	1 (R)	0	1 (R)	Çumra 2001	0	1 (R)	0	1 (R)
	12.10*		14.68*			1.26*		2.22*	

*Significant at $P < 0.01$ (Tables 3 and 4).

that a decrease in plant biomass affects vital activities of plants such as photosynthesis and respiration (Mathre, 1997). In Turkey, the reactions of 1,216 barley lines to barley leaf stripe were assessed and it was found that 25 lines were resistant and eight were intermediate to resistant to the disease (Albustan *et al*., 1999). Ulus and Karakaya (2007) assessed the resistance of 15 widely used barley varieties to five *D. graminea* isolates, and determined that the barley cultivars 'Çumra 2001' and 'Yerçil 147' were resistant to all five isolates, and that the isolate Dg3 was the most virulent. Bayraktar and Akan (2012) reported that barley cultivars 'Durusu', 'Balkan 96 (Igri)', 'Çumra 2001' and 'Anadolu 98' were resistant to the 13 *D. graminea* isolates they tested, and that isolate 1003 was the most virulent.

Çelik *et al*. (2016) evaluated the reactions of three barley cultivars and 20 barley landraces to ten *D. graminea* isolates, and found that one barley landrace

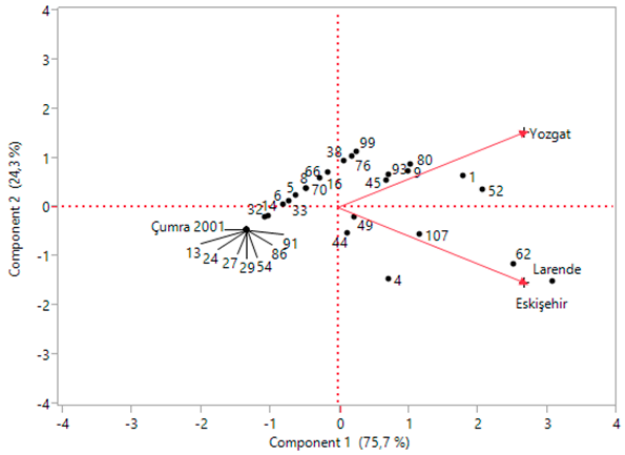

Figure 1. Biplot based on PCA analysis of the mean disease incidences of wild barley (*H. spontaneum*) genotypes inoculated with two isolates of *Drechslera graminea*.

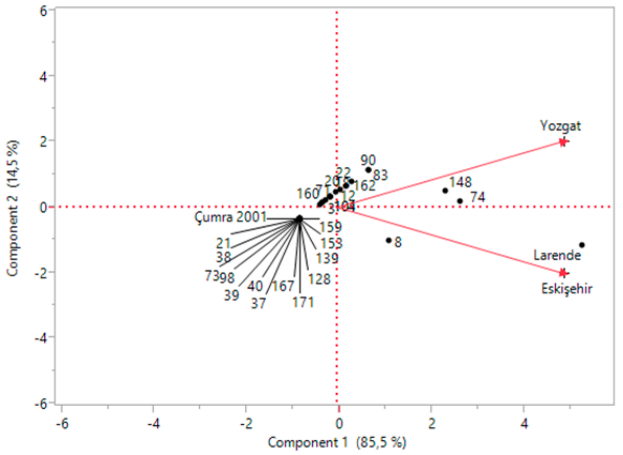

Figure 2. Biplot based on PCA analysis of the mean disease incidences of barley landraces inoculated with two isolates of *Drechslera graminea*.

was resistant to eight *D. graminea* isolates and had intermediate reactions to the other two isolates. The barley variety 'Çumra 2001' was resistant to all ten isolates. These authors also reported virulence differences among the isolates. In their study, average disease incidence for the Yozgat isolate was 40.2%, and for the Eskişehir isolates was 15.4%. Çelik Oğuz *et al.* (2017a), also found virulence differences among *D. graminea* isolates, and that only one of the 23 hulless barley lines they tested was resistant to three isolates of the pathogen. Karakaya *et al.* (2017) tested the same three isolates on 25 Iranian barley landraces and found similar virulence differences among the isolates. In their study, no Iranian barley landraces were resistant to all three isolates.

Turkey is a major genetic centre for cultivated and wild barleys, and there are barley genotypes in this country that are resistant to different abiotic and biotic stresses (Vavilov, 1951; Kün, 1996; Afanasenko *et al.*, 2000; Jakob *et al.*, 2014; Çelik *et al.,* 2016; Karakaya *et al.*, 2016a). Barley landraces and wild barley genotypes show great variation in agronomic traits as well as reaction to biotic stress factors. Resistance to different diseases has been reported in barley landraces and wild barley (*H. spontaneum*) genotypes (Azamparsa *et al.*, 2019; Karakaya *et al.*, 2017; Çelik and Karakaya, 2017; Çelik Oğuz *et al.*, 2017b; Çelik Oğuz *et al.*, 2019). Resistance among barley genotypes originating from the Middle East has been reported, with Anatolian landraces being superior compared to those from other origins, in terms of yield, drought, and disease tolerance (Chakrabarti, 1968; Khan and Boyd, 1969; Gökgöl, 1969).

In the present study, *H. spontaneum* genotypes 13, 24, 27, 29, 54, 86, and 91 were resistant to two *D. graminea* isolates. Genotypes 24, 27, and 54 were also resistant to virulent isolates of both forms of *Pyrenophora teres* (Çelik Oğuz *et al.*, 2019). *Hordeum spontaneum* genotype 13 was found to be resistant to virulent isolates of *P. teres* f. *maculata* (which causes the spot form of net blotch), while *H. spontaneum* genotype 29 was resistant to virulent isolates of *P. teres* f. *teres* (which causes the net form of net blotch) (Çelik Oğuz *et al.*, 2019). In addition, all seven of these wild barley genotypes showed resistance to up to four of six *Rhynchosporium commune* isolates (Azamparsa *et al.*, 2019).

Barley landraces 21, 37, 38, 39, 40, 73, 98, 128, 139, 153, 159, 167, and 171 were resistant to both *D. graminea* isolates examined in the present study. Among these landraces, landrace 40 was also resistant to virulent isolates of both forms of *P. teres* (Çelik Oğuz *et al.*, 2017b). Barley landraces 98, 167, and 171 were found to be resistant to virulent isolates of *P. teres* f. *maculata* (causing the spot form of net blotch), while barley landraces 21 and 153 were resistant to virulent isolates of *P. teres* f. *teres* (causing the net form of net blotch) (Çelik Oğuz *et al.*, 2017b). In addition, barley landraces 21, 38, 40, 218, 139, 153 and 167 showed resistance to up to three of six *R. commune* isolates (Azamparsa *et al.*, 2019).

The disease resistance of different *H. spontaneum* and barley landraces to other diseases has also been determined in other studies. For example, Kopahnke (1998) evaluated the reactions of wild barley and barley landraces to *P. teres* and found that 143 genotypes exhibited resistant reactions to all isolates tested. Jana and Bailey (1995) found resistance among the *H. spontaneum* genotypes and cultivated barley landraces obtained from Turkey and Jordan to the fungal pathogens *P. teres*

f. *maculata*, *P. teres* f. *teres*, and *Cochliobolus sativus*. The percentage of *H. spontaneum* accessions resistant to these pathogens (10.5%) was greater compared to that of the cultivated accessions (1.3%). Fetch *et al*. (2003) determined the reactions of 116 *H. spontaneum* genotypes originating from Jordan and Israel to six fungal pathogens. They showed that 98% of the genotypes from Jordan and 77% of the genotypes from Israel were resistant to Septoria leaf blotch, 70% and 90%, respectively from the two countries, were resistant to leaf rust, 72% and 78%, respectively, were resistant to net blotch, 58% and 70%, respectively, were resistant to powdery mildew, 53% and 46%, respectively, were resistant to spot blotch, and 2% and 26%, respectively from the two countries, were resistant to stem rust.

Wild barley (*H. spontaneum*) has greater genetic variation than cultivated barley (Saghai-Maroof *et al*., 1994; Provan *et al*., 1999; Nevo, 2004), and it is possible to crossbreed *H. spontaneum* with cultured barley (*H. vulgare*). Useful traits including disease resistance can be transferred to cultivated barley from *H. spontaneum* (Çelik and Karakaya, 2017), so wild barley is a significant potential genetic source for barley genetic improvement. Wild barley populations in the Middle East also possess considerable genetic variation (Nevo, 1992). It has been suggested that *H. spontaneum* genotypes should be preserved under *in situ* and *ex situ* conditions for barley improvement programmes, including those selecting for enhanced disease resistance (Nevo, 1992; Ceccarelli and Grando, 2000; Nevo, 2012). *Hordeum spontaneum* genotypes may show different resistance reactions based on their origins, and resistance genes can vary depending on geographic conditions (Sato and Takeda, 1997). *Hordeum spontaneum* populations from the Fertile Crescent Region, including parts of the Levant (eastern Mediterranean, including Turkey and Israel) and Iran are genetically variable for adaptation capability and population sustainability (Nevo, 2004; Jakob *et al*., 2014). In the present study, *H. spontaneum* genotypes resistant to *D. graminea* isolates were observed. Seven and two of the *H. spontaneum* genotypes showed resistant reactions to the Yozgat isolate and two genotypes were of intermediate resistance. On the other hand, 19 of *H. spontaneum* genotypes were resistant to the Eskişehir isolate while six genotypes were of intermediate resistance to this isolate. The heterogenous nature of wild barley (*H. spontaneum*) resistance to diseases has been reported previously (Çelik and Karakaya, 2017; Karakaya *et al*., 2016a). In a survey carried out in 2015, a total of 40 *H. spontaneum* populations in their natural habitat were examined, and it was determined that nine of these were disease-free. In these fields, the fungal pathogens *R. commune*, *Blumeria graminis* f. sp. *hordei*, *D. teres* f. *teres*, *D. teres* f. *maculata*, *Ustilago nigra*, *U. nuda*, *Puccinia hordei*, and *D. graminea* were identified (Karakaya *et al*., 2016a).

The region between the south of the Fertile Crescent and the Himalayan mountains was the first area in which barley was domesticated (Azhaguvel and Komatsuda, 2007; Morrell and Clegg, 2007; Saisho and Purugganan, 2007). In recent years, barley varieties and yields in the area of Fertile Crescent have been under serious threat because of climate change and environmental pollution originating from human activities. Barley landraces provide gene resources that can be used to decrease the negative impacts of climate change (Mzid *et al*., 2016). It is known that wild barleys and barley landraces have wide variation in terms of resistance to diseases (Simmonds, 1987; Çelik and Karakaya, 2017; Çelik Oğuz *et al*., 2017b; Azamparsa *et al*., 2019). In conventional agriculture systems, barley leaf stripe is controlled through treating seed with fungicides. However, European Union regulations state that under certified organic production practices, barley leaf stripe can only be controlled using hot water treatments. But, these may not always be fully effective. Barley stripe, which is very important under organic agriculture conditions, is prevalent in Northern Germany due to the cool and humid climatic conditions found there (Mueller *et al*., 2003). Genetic resistance can be transferred from wild relatives to cultivated crops to decrease the use of chemicals (Laurei *et al*., 1992).

In summary, barley leaf stripe is an important disease that can cause significant yield losses when no disease management practices are utilized. In the present study, new sources of resistance to *D. graminea* have been identified. The wild barley genotypes and barley landraces identified here could be used in plant breeding programmes to develop leaf stripe resistant genotypes, which would be ecosystem-friendly and also enhance farmer profitability.

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Research Paper

Isolation of atypical wheat-associated xanthomonads in Algeria

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Summary. Bacterial leaf streak and black chaff are important bacterial diseases of wheat, which have been reported to be caused by *Xanthomonas translucens*. In 2016, symptoms of bacterial leaf streak and black chaff were observed in Algeria, at experimental wheat breeding stations and in farmers' fields under sprinkler irrigation on two wheat cultivars, 'Hiddab' and 'Simeto'. Yellow *Xanthomonas*-like bacterial colonies were isolated from plant material, including leaves, spikes and post-harvest crop residues. Initial characterisation using biochemical, physiological and pathogenicity tests identified the bacteria as *Xanthomonas*. Diagnostic PCR targeting the 16S-23S rRNA intergenic region indicated that the strains were *X. translucens*, a clade-1 xanthomonad. However, partial DNA sequences of the housekeeping genes *gyrB* and *rpoD* revealed that the strains belong to clade 1, but likely represent a new *Xanthomonas* species that has not been previously described on wheat or other *Gramineae*. The most closely related strain, NCPPB 2654, was isolated from a bean plant in the United Kingdom in 1974. Further characterization is required to clarify the taxonomic status of the Algerian *Xanthomonas* isolates from wheat, and to determine their host ranges and impacts on plant cultivation.

Keywords. *Xanthomonas*, bacterial leaf streak, black chaff, wheat.

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INTRODUCTION

Wheat is a major crop worldwide, affected by some bacterial diseases, among which bacterial leaf streak (BLS) is the most important. BLS is caused by *Xanthomonas translucens* (ex Jones *et al.* 1917) (Vauterin *et al.*, 1995). When symptoms occur on the wheat plant glumes, the disease caused by

X. translucens is called black chaff (BC) (Duveiller *et al*., 1997). BLS is widely distributed in the world and the disease is prevalent in most regions where small-grain cereals are cultivated (Paul and Smith, 1989; Duveiller and Maraite, 1994). However, these diseases have not been extensively studied in North Africa, despite sporadic reports of occurrence in countries close to Algeria, including Libya (Bragard *et al*., 1995), Morocco and Tunisia (Sands and Fourest, 1989).

Yield losses caused by BLS are generally not considered problematic. However, losses of up to 40% have been recorded under conditions that are conducive for the pathogen (Duveiller *et al*., 1997). Algeria cultivates cereals on an area of 3.3 million ha, 1.3 million of which are irrigated and thus likely to be vulnerable to BLS. Furthermore, more than 20% of yield may be lost if 50% of flag leaf area is affected by the disease (Duveiller and Maraite, 1993). BLS generally appears late in the growing season in regions with temperate climate or in warmer environments characterized by cool nights and frequent temperature variations (Duveiller *et al*., 2002). The symptoms are usually more obvious after heading (Wiese, 1987). All aerial parts of host plants can be affected, but the leaves and glumes are more often affected than the other parts. In cases of severe damage, the seeds can be blackened and wrinkled (Zillinsky, 1983). BC is characterized by many black longitudinal stripes on the upper portions of the glumes (Smith, 1917), that can be identified by greasy appearance. Yellow bacterial droplets exude along the lesions, particularly in wet weather.

Xanthomonas species are known for their ability to adhere to and colonize host leaf surfaces as epiphytes before invading the intercellular spaces (Boulanger *et al*., 2014; Dutta *et al*., 2014; Zarei *et al.,* 2018). Xanthomonads have evolved several strategies for successful infection, including mechanisms to suppress host plant resistance and access nutrients from host cells (Büttner *et al.,* 2010; Fatima and Senthil-Kumar, 2015; Jacques *et al*., 2016). However, much less is known for *X. translucens*, which belongs to xanthomonad clade 1, while most functional research has been carried out with clade-2 xanthomonads (Parkinson *et al*., 2007). The importance of the bacterial type III secretion system and TAL effectors for pathogenicity has been demonstrated (Wichmann *et al*., 2013; Peng *et al*., 2016; Falahi Charkhabi *et al*., 2017; Pesce *et al.,* 2017).

Since no data are available on bacterial diseases of small grain cereals in Algeria, the objective of this study was to assess the presence of wheat-associated xanthomonads in this country, to verify their ability to cause BLS on cereals and to determine their identity. This research was based on morphological, biochemical and physiological characterization of bacterial isolates, complemented by pathogenicity tests on host plants and DNA-based molecular diagnostics.

MATERIALS AND METHODS

Bacterial strains

Bacteria were isolated from symptomatic wheat leaf and spike samples and from post-harvest plant residues originating from fields that had shown symptoms of BLS and BC in the previous growing season (Table 1) (Kar-

Table 1. Details of Algerian bacterial isolates obtained from wheat.

Isolate	Gene	GenBank Accession number	Sample	Symptoms[a]	Wheat cultivar	Location	Year
X1	*gyrB* *rpoD*	MF142045 MF142046	Leaf	BLS	'Simeto'	El Goléa	2016
X2	*gyrB*	MF142047	Leaf	BLS	'Simeto'	El Goléa	2016
X3	*gyrB*	MF142048	Leaf	BLS	'Simeto'	El Goléa	2016
X4	*gyrB*	MF142049	Leaf	BLS	'Simeto'	El Goléa	2016
X5	*gyrB*	MF142050	Crop residue	No visible symptoms	'Hiddab' (HD1220)	Algiers experimental station	2016
X8	*gyrB* *rpoD*	MF142051 MF142052	Spike	BC	'Simeto'	El Goléa	2016
X12	*gyrB* *rpoD*	MF142053 MF142054	Crop residue	No visible symptoms	'Hiddab' (HD1220)	Algiers experimental station	2010
X13	*gyrB* *rpoD*	MF142055 MF142056	Crop residue	No visible symptoms	'Hiddab' (HD1220)	Algiers experimental station	2010
X16	*gyrB*	MF142057	Spike	BC	Breeding line	Algiers experimental station	2016
X17	*gyrB*	MF142058	Spike	BC	Breeding line	Algiers experimental station	2016

[a]BLS = bacterial leaf streak, BC = black chaff.

avina *et al.*, 2008). Samples (10 g each) of plant organs or post-harvest stubble residues were each ground to a fine powder and mixed with 50 mL phosphate-buffered saline (PBS). The resulting cell suspension was shaken for 20 min and allowed to settle for 3 h. Series of ten-fold dilutions were prepared from the suspension supernatants and appropriate amounts of each dilution were plated on standard nutrient agar medium and on semi-selective Wilbrink's medium supplemented with 0.75 g L^{-1} of boric acid, 10 mg L^{-1} of cephalexin and 75 mg L^{-1} of cycloheximide (WBC medium) to reduce fungal growth (Duveiller, 1990). For comparison, *X. translucens* pv. *undulosa* strain UPB753, which had been isolated from wheat in Brazil (Bragard *et al.*, 1995), was used as a reference strain. Pure cultures were obtained upon cultivation at 30°C. Based on morphological characteristics, *Xanthomonas*-like bacterial strains were assigned a designation number and kept at 4°C on glucose, yeast extract-calcium carbonate agar (GYCA) tubes for short-term conservation and at -80°C in 20% glycerol for long-term storage.

Phenotypic characterization

Established tests were used to identify the bacteria (Bradbury, 1986; Van den Mooter and Swings, 1990; Schaad *et al.*, 2001). These included formation of mucoid colonies on GYCA medium, Gram reaction using the KOH test, oxidase test using tetramethyl-*p*-phenylenediamine dihydrochloride reagent, oxidative and fermentative metabolism of glucose, hydrolysis of Tween 80, aesculin and starch, production of levane sucrase, production of catalase, liquefaction of gelatine, H_2S production from cysteine, nitrate reduction, growth at 35°C, and growth on 2% and 5% NaCl media.

DNA manipulations

For rapid tests, bacterial cells were lysed by brief boiling and then directly used for PCR, as described previously (Maes *et al.*, 1996). For PCR amplification of housekeeping genes, DNA was isolated using the Wizard® Genomic DNA Purification Kit (Promega Corp.), following the manufacturer's instructions.

PCR amplification of the alanine-specific tRNA gene in the 16S-23S rRNA intergenic region was performed as described previously (Maes *et al.*, 1996), with modifications. The reaction mixture was prepared in a total volume of 25 µL comprising 5 µL of 5 × PCR buffer, 2.5 µL $MgCl_2$ (25 mM), 0.75 µL dNTP mixture (25 mM), 0.5 µL of each PCR primer at 10 µM (T1, 5'-CCGCCATAGGGCGGAGCACCCCGAT; T2, 5'-GCAGGTGCGACGTTTGCAGAGGGATCTGCAAATC), 2.5 µL DNA sample (50 ng μL^{-1}), 0.2 µL Taq polymerase (Promega), and 13.05 µL distilled water. PCR was performed with the following conditions: 90°C for 2 min, 29 cycles of 93°C for 30 s, 50°C for 30 s, 72°C for 30 s, and a final extension for 10 min at 72°C. The PCR products were separated by electrophoresis on 2% agarose gel in TAE buffer, stained with Midori green (Nippon Genetics Europe) and visualized under UV light.

Previously published MLSA primers were used for PCR amplification and partial DNA sequencing of two housekeeping genes, *gyrB* and *rpoD* (Fargier and Manceau, 2007). PCR amplifications were performed as recommended (Mhedbi-Hajri *et al.*, 2013) in a 50 µL reaction mixture containing 1 × GoTaq® buffer, 200 µM dNTP, 0.5 µM of each primer, 0.4 U of GoTaq® DNA polymerase (Promega), and 3 ng of genomic DNA, with an initial denaturation at 94°C for 2 min, 30 cycles of denaturation for 1 min at 94°C, annealing for 1.5 min at 60°C, extension for 1.5 min at 72°C, and a final extension for 10 min at 72°C. 8 µL of PCR products reaction mixtures were analysed by electrophoresis on 1.5% agarose gel in TAE buffer, stained with Midori green direct and visualized under UV light. The remaining amplified PCR products were purified with the Wizard® PCR clean-up kit (Promega) and sequenced with reverse and forward primers using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Both forward and reverse sequences were aligned and manually edited before deposition in the GenBank database (Table 1).

Pathogenicity tests

Bacterial cells were suspended in sterile solution of 0.90% (w/v) of NaCl and the concentration of cells was adjusted to 1×10^9 CFU mL^{-1} for hypersensitive reaction tests on tobacco plants of the variety Xanthi and to 1×10^7 CFU mL^{-1} for pathogenicity tests. Sterile saline solution served as negative controls in the pathogenicity assays.

All strains were tested for pathogenicity, by inoculation on the sensitive wheat cultivar 'Acsad 885', using three different assays. First, after injection of sterile water into plant leaf sheath at 2.5 cm above soil level, three-leaf stage seedlings were puncture inoculated with a sterile needle that had been passed through a bacterial colony ("pricking inoculation") (Bragard and Maraite, 1992). Second, using a needle-less plastic syringe, bacterial suspensions were infiltrated through the upper leaf surfaces until appearance of liquid-soaked areas of about 2 cm length ("leaf infiltration") (Bragard and Maraite, 1992). Third, whole leaves were immersed into bacterial

solutions for 20 sec ("dip inoculation") (Darsonval *et al.*, 2009). Plants were incubated at 28°C and 95% relative humidity with a photoperiod of 16h/8h (day/night), and symptoms were scored over time.

To re-isolate bacteria from infected plant material, symptomatic leaf segments were cut into small pieces in sterile physiological saline and plated on standard and semi-selective media.

Bioinformatic analyses

To link the different strains with their respective taxa among the species of *Xanthomonas*, corresponding *gyrB* and *rpoD* gene portions were retrieved from GenBank and PAMDB databases (https://www.ncbi.nlm.nih.gov, http://www.pamdb.org) (Almeida *et al.*, 2010; Sayers *et al.*, 2019). For recently described species and pathovars that are not represented in PAMDB, such as "*Xanthomonas pseudalbilineans*", *X. maliensis*, *X. floridensis*, *X. nasturtii*, and *X. prunicola*, corresponding sequences were extracted from the genome sequences (Supplementary Table 1) (Pieretti *et al.*, 2015; Triplett *et al.*, 2015; Hersemann *et al.*, 2016b; Vicente *et al.*, 2017; López *et al.*, 2018).

Multiple sequence alignments were performed using the MUSCLE algorithm (https://www.ebi.ac.uk/Tools/msa/muscle/) (Edgar, 2004). Phylogenetic trees were generated using the phylogeny.fr pipeline, with default parameters (http://www.phylogeny.fr) (Dereeper *et al.*, 2008). Newick files were generated and the tree was manipulated using the iTOL website (https://itol.embl.de) (Letunic and Bork, 2011) to improve visualization.

RESULTS

Isolation of bacteria from wheat

BLS symptoms were observed in various plots of the Algiers experimental station, at the tillering and at the heading crop growth stages (Figure 1A). Additionally, severe disease symptoms of BC were observed at the heading stage (Figure 1B). Affected varieties in the Algiers area included wheat 'Hiddab' (HD1220), and also several breeding lines and cultivars grown at the Algiers experimental station for studies under the pedoclimatic conditions of the region, to verify their performance, efficiency, specific features and disease resistance. Symptoms were also observed on the cultivar 'Simeto', grown under the sprinkler irrigation at El Ménéa (El Goléa), South Algeria. This is an area characterized by cool nights and high day temperatures. Furthermore, during the 2016 agricultural campaign, the disease was sporadically present in plots of the Algiers experimental station, and more widespread in plots under pivot irrigation, where 'Simeto' was sown. Yellow colonies on media, resembling *Xanthomonas*, were easily isolated from the plant symptoms. In order to evaluate if post-harvest material could serve as reservoirs for infections, crop residues from plots at the Algiers multiplication station that had shown symptoms, were analysed as well, as described previously (Karavina *et al.*, 2008). *Xanthomonas*-like bacteria were also isolated from this material.

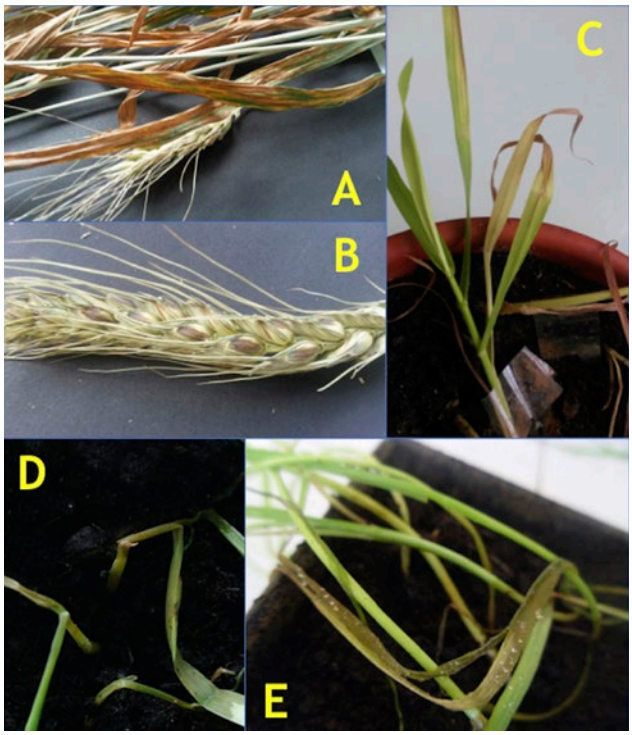

Figure 1. Disease symptoms on wheat. (A) Symptoms of bacterial leaf streak at the heading stage, (B) symptoms of black chaff at the heading stage, (C) chlorotic to necrotic lesions from leaf infiltration 7 d after inoculation, (D) disease symptoms 8 d after pricking inoculation of wheat seedlings at the three-leaf stage, and (E) water-soaked spots associated with bacterial exudates 8 d after dip inoculation of leaves.

Phenotypical characterization of bacteria isolated from wheat

Among all the strains obtained from the different origins, 30 were retained (Table 2) with consistent biochemical and physiological test responses corresponding to those obtained with the *X. translucens* reference strain (Bradbury, 1986; Van den Mooter and Swings,

Table 2. Biochemical and physiological tests used to identify bacterial isolates from wheat, and a reference strain (*Xanthomonas* translucens pv. *undulosa* strain UPB753 (Bragard *et al.*, 1995)).

Test	Algerian isolates	Reference strain
Mucoid and yellow colonies on GYCA medium	+	+
Gram staining	-	-
Oxidase	-	-
Metabolism of glucose	oxidative	oxidative
Tween 80 hydrolysis	+	+
Aesculin hydrolysis	+	+
Starch hydrolysis	+	+
Levane sucrase	+	+
Catalase	+	+
Liquefaction of gelatine	+	+
H_2S production from cysteine	+	+
Nitrate reduction to nitrite	-	-
Growth at 35 °C	+	+
Growth in 2% NaCl	+	+
Growth in 5% NaCl	-	-
Hypersensitive reaction on tobacco	+	+
Pathogenicity on wheat cv. Acsad 885	+	+
Diagnostic PCR (Maes *et al.*, 1996)	+	+

1990; Schaad *et al.*, 2001). All these strains grew as mucoid and yellow pigmented colonies, and they were negative for Gram staining, oxidase activity and nitrate reduction. The strains could hydrolyse aesculin, gelatine and starch and produced catalase, levane sucrase, lipase and hydrogen sulphide from cysteine. Bacteria grew at 35°C and in nutrient broth supplemented with 2% sodium chloride, but not when supplemented with 5% sodium chloride. All strains triggered hypersensitive reactions on tobacco. These analyses indicated that the wheat-associated bacteria belonged to the genus *Xanthomonas* and may be related to *X. translucens*.

Pathogenicity assays with xanthomonads isolated from wheat

Three inoculations methods were applied to evaluate the pathogenicity of the bacterial strains. Upon leaf infiltration of a susceptible cultivar, chlorotic to necrotic lesions developed within 7 d after infection (Figure 1C). When pricking three-leaf stage wheat seedlings, similar symptoms were observed 8 d after inoculation (Figure 1D). Dip inoculation of wheat leaves resulted in symptoms that were clearly visible after 8 d, including typical water-soaked spots associated with bacterial exudates from the plant organs (Figure 1E).

Bacteria were re-isolated from infected plant material. Morphological, biochemical and physiological characterization confirmed the identity of the re-isolated bacteria with the inoculum, thus fulfilling the Koch's postulates.

DNA-based diagnostics of xanthomonads isolated from wheat

In order to evaluate whether the strains belong to *X. translucens*, they were subjected to a protocol that had been developed for specific detection of this species, based on a discriminatory region in the 16S-23S intergenic region, which encodes two tRNAs (Maes *et al.*, 1996). A DNA fragment with a size of 139 bp was amplified with PCR primers T1 and T2 for all 30 strains.

To further characterize ten representative strains from wheat, a portion of the *gyrB* gene that was previously used in multiple locus sequence analysis (MLSA) was amplified by PCR and sequenced, using previously developed primers (Fargier and Manceau, 2007; Young *et al.*, 2008). All sequences were identical. Homologous sequences of type, pathotype or other *Xanthomonas* strains were retrieved from the PAMDB database (Almeida *et al.*, 2010). In addition, sequences for those of *Xanthomonas* species that were not available at PAMDB, but had been included in a previous *gyrB*-based phylogenetic study, including sequences for undescribed species ("slc" species-level clades) (Parkinson *et al.*, 2009), were used for comparison. Sequences were aligned using MUSCLE and manually trimmed to 528 bp. A phylogenetic tree was calculated using the Phylogeny.fr pipeline (Figure 2). This analysis revealed that the Algerian sequences clustered with other sequences from clade-1 xanthomonads, including *Xanthomonas hyacinthi*, *X. theicola* and *X. translucens*. The closest sequence corresponded to strain NCPPB 2654 from species-level clade 5. This strain was isolated in 1974 by F. W. Catton, from navy bean (*Phaseolus vulgaris*) in the United Kingdom. No other sequence information is available for this species-level clade.

This result prompted us to partially sequence another housekeeping gene *rpoD*, that was previously used in MLSA studies (Young *et al.*, 2008). Multiple sequence alignment of four 870-bp sequences from representative Algerian strains revealed their identity with each other. A phylogenetic tree was generated including representative haplotypes for clade-1 xanthomonads from PAMDB. In addition, sequences from three additional *X. translucens* pathotype strains and two sequences belonging to the recently suggested clade-1 species "*X. pseudalbilineans*" were included (Pieretti *et al.*, 2015; Hersemann

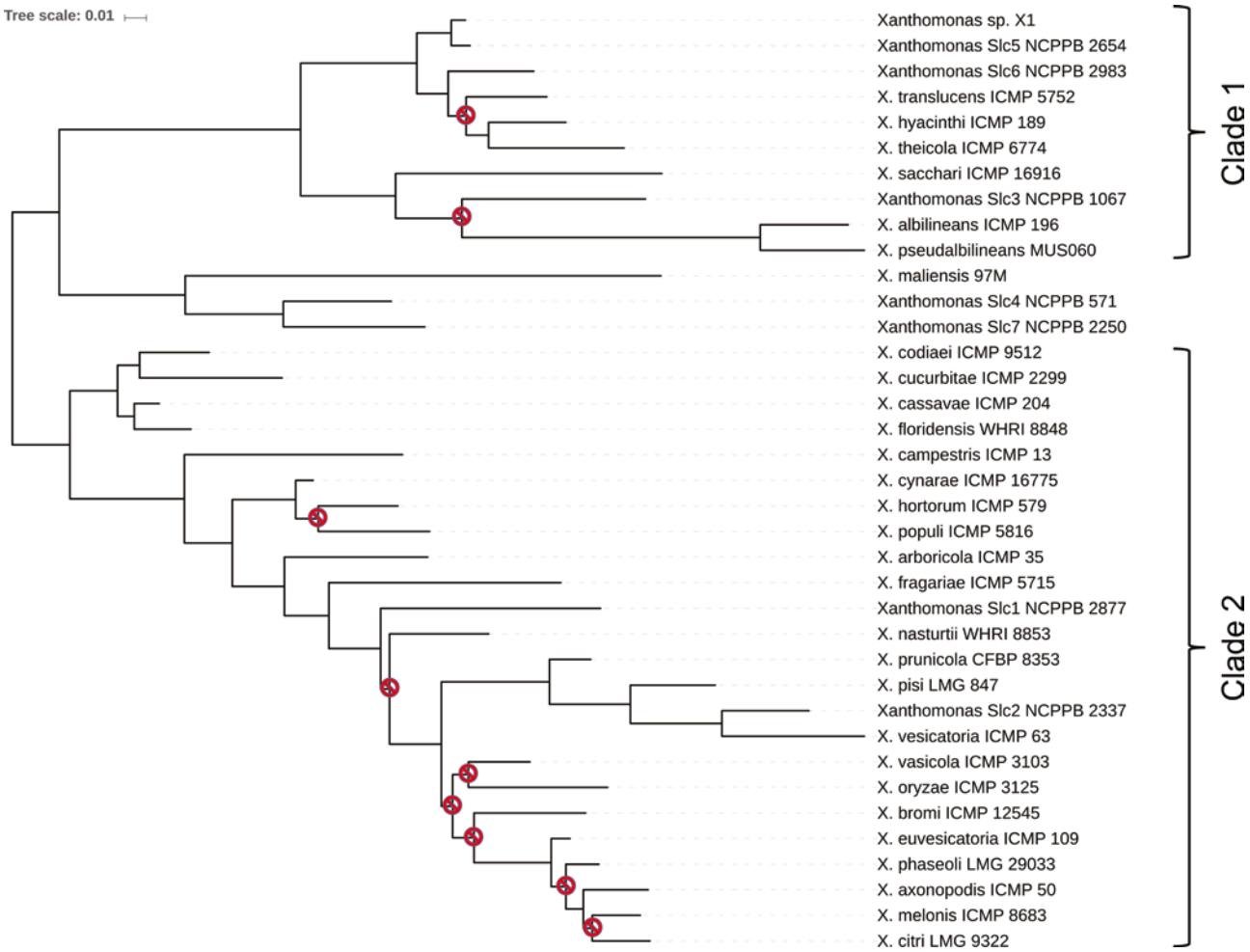

Figure 2. *gyrB*-based phylogenetic classification of the Algerian wheat-associated xanthomonads. Representative strains of all species following taxonomic revisions of *Xanthomonas gardneri* and *X. axonopodis* species complex are included (Constantin *et al.*, 2016; Timilsina *et al.*, 2019). Taxonomically unassigned species-level clades (Slc 1–7) are indicated (Parkinson *et al.*, 2009). All sequences were trimmed to the portion that is available in GenBank for the *Xanthomonas phaseoli* type strain (528 bp, Accession number KT585789). The tree was constructed with the Phylogeny.fr pipeline, using default parameters, and graphically edited using the iTOL suite. All nodes were supported by bootstrap values greater than 0.75, except for those marked with a stop symbol. The scale of branch lengths is indicated at the top left.

et al., 2016b). This analysis confirmed that the Algerian strains belong to clade-1, but did not cluster with any of the six described species. This indicates that the Algeria strains from wheat belong to another species, with strain NCPPB 2654 as the likely founder (Figure 3).

DISCUSSION

Diseased wheat plants were reported from several plots in Algeria, with symptoms on the leaves and spikes as well as melanotic areas on the glumes, that were similar to those described for leaf streak and black chaff (Duveiller *et al.*, 2002). These diseases result from bacterial infections, often in high temperature and high humidity conditions, and the observed symptoms have long been described for these diseases (Smith, 1917; Johnson and Hagborg, 1944). The strains characterized in the present study were mostly obtained from sprinkler-irrigated fields or from breeding stations where genotype behaviour was being assessed.

All strains obtained from affected wheat samples, including leaves, spikes and crop residues, had phenotypic characteristics that corresponded to those described in the literature for *X. translucens*. Moreover, inoculation of wheat seedlings at the three-leaf stage caused water-soaked lesions within 8 d after inoculation, accompanied by signs of necrosis around the inoculat-

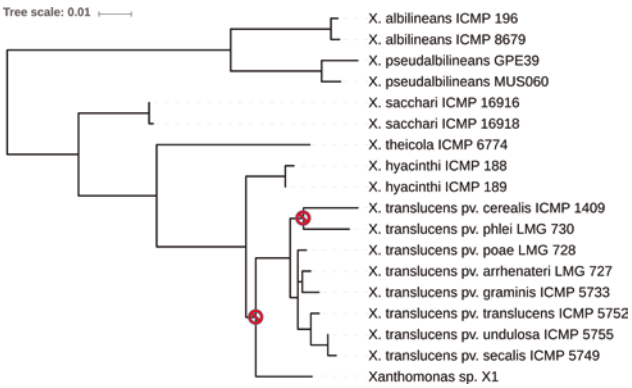

Figure 3. *rpoD*-based phylogenetic classification of the Algerian wheat-associated xanthomonads. Sequences were trimmed to the size of the sequences that were retrieved from PAMDB (855 bp for *X. translucens* and the Algerian strain). The phylogenetic tree was constructed with the Phylogeny.fr pipeline, using default parameters, and graphically edited using the iTOL suite. All nodes were supported by bootstrap values greater than 0.75, except for those marked with a stop symbol. The scale of branch lengths is indicated at the top left.

ed areas and ultimately causing the death of the leaves. When the pricking inoculation method was used, initial local necrosis expanded with time and affected whole leaf blades, concomitantly showing typical exudate droplets. The inoculation techniques mimicked temperature and moisture conditions conducive for the disease (Duveiller and Maraite, 1993; Duveiller *et al*, 1997). These results confirmed that the Algerian *Xanthomonas*-like strains were pathogenic on wheat cultivar 'Acsad 885'. Bacteria could also be isolated from crop residues 3 to 4 weeks after harvest, indicating that the pathogen can survive on plant material. This may serve as reservoirs for new infections in the next cropping cycle. Similar observations have been made for *X. translucens*, which was found to survive on crop debris for more than 30 months under laboratory conditions, and for less than 8 months under field conditions (Malavolta Jr. *et al*., 2000). Occurrence on, and isolation from, wheat plants and the symptoms observed in the fields and upon artificial inoculation strongly indicated that the strains were *X. translucens*.

Initial molecular characterization of the strains using a PCR assay that was developed for detection of *X. translucens*, including pathovars infecting small-grain cereals (pvs. *cerealis*, *hordei*, *secalis*, *translucens* and *undulosa*) and those infecting forage grasses (pvs. *arrhenatheri*, *graminis*, *phlei*, *phleipratensis* and *poae*), further supported that the Algerian strains were *X. translucens* (Maes *et al*., 1996). This assay targets the 16S-23S intergenic region, which encodes two tRNAs in all xanthomonads, one for alanine (UGC anticodon) and one for isoleucine (GAU anticodon). The diagnostic primers T1 and T2 anneal immediately upstream and downstream of the tRNA (Ala) gene. Most xanthomonads have short regions of 14 to 19 bp between the transcribed sequences for the tRNA (Ala) and the tRNA (Ile) (Gonçalves and Rosato, 2002), and therefore lack the target region for the T2 primer. However, *X. translucens*, two other clade-1 species (*X. hyacinthi*, *X. theicola*) and two clade-2 species (*Xanthomonas codiaei*, *Xanthomonas melonis*) have longer regions of 75 to 79 bp, that are fairly conserved and might allow annealing of the T2 primer under less stringent conditions (data not shown). Since the region corresponding to the T1 primer is less similar for *X. codiaei* and *X. melonis*, the PCR should not amplify the diagnostic DNA fragment of 139 bp, as had been confirmed for *X. melonis* (Maes *et al*., 1996). However, that the PCR could amplify the diagnostic DNA fragment for bacteria of the *X. hyacinthi*-*X. theicola*-*X. translucens* subclade cannot be excluded. When the assay was developed by Maes and co-workers, all xanthomonads that were known as pathogens on *Gramineae* (i.e. *X. albilineans*, *X. axonopodis*, *X. bromi*, *X. oryzae* and *X. vasicola*), and representative strains of most described species of *Xanthomonas*, were included, although some species of clade-1 were not tested (e.g. *X. hyacinthi* and *X.theicola*). Yellow disease of hyacinth, a monocot of the *Asparagaceae*, was the first disease described to be caused by *Xanthomonas* (Van Doorn and Roebroeck, 1993). Infection of asparagus by *X. translucens* has been reported (Rademaker *et al*., 2006). Therefore, the taxonomic status of the Algerian strains remained uncertain based on the diagnostic PCR.

Since we felt that the diagnostic PCR is not able to unambiguously identify strains of *X.translucens*, two housekeeping genes that are included in MLSA schemes were analysed (Young *et al*., 2008). Partial sequences of both genes, *gyrB* and *rpoD*, clustered with sequences from other clade-1 xanthomonads, such as *X. hyacinthi*, *X. theicola* and *X. translucens*, but were distant enough to question whether they belonged to any of the described species. The *gyrB* gene has been used for exhaustive phylogenetic analyses of *Xanthomonas*, including strains from species-level clades that still await precise taxonomic assignment (Parkinson *et al*., 2007, 2009). The partial *gyrB* gene sequences from the Algerian isolates were very similar to that of strain NCPPB 2654, which was isolated from a navy bean plant in the United Kingdom (https://www.fera.co.uk/ncppb). According to the NCPPB website, this strain has a fatty acid profile typical of *Xanthomonas*, but is not pathogenic on bean pods. It would be interesting to know wheth-

er this strain was isolated near a wheat field, and to test this strain for pathogenicity on wheat plants. Likewise, the next similar sequence corresponded to strain NCPPB 2983, which was deposited as *Xanthomonas campestris* pv. *phormiicola* and belongs to species-level clade 6. This strain was isolated in Japan from New Zealand flax (*Phormium tenax*), a member of the *Asparagales* (*Asphodelaceae*). Three species related to the Algerian strains, *X. hyacinthi*, *X. translucens* and *X. campestris* pv. *phormiicola*, are able to colonize plants in the *Asparagales*, and future work will evaluate whether the Algerian strains from wheat can infect these plants as well.

In conclusion, this is the first description of wheat-pathogenic xanthomonads from Algeria, which were atypical in that they most likely do not belong to *X. translucens*. Further characterization, ideally including whole-genome sequencing, will clarify their taxonomic status and their host range (Peng *et al*., 2016; Langlois *et al*., 2017). It will also be important to compare these strains with other bacterial pathogens of cereals, and to elucidate whether candidate type III effectors, phytohormones and/or toxins are involved in pathogenicity and host adaptation (Royer *et al*., 2013; Gardiner *et al*., 2014; Hersemann *et al*., 2016a, 2016b, 2017; Triplett *et al*., 2016; Nagel and Peters, 2017).

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Research Paper

Control of bottom rot in hydroponic lettuce, caused by strains of *Botrytis cinerea* with multiple fungicide resistance

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Summary. For two consecutive growing periods, fungicide-resistant *Botrytis cinerea* strains were detected in high proportions in glasshouse-grown lettuce, but at variable frequencies. Pre-transplanting fungicide sprays applied on two successive occasions reduced disease severity and increased the number of healthy plants without leaving detectable residues above accepted MRLs at harvest. In some instances, the disease was further decreased when pre-transplanting applications were combined with one or two further sprays applied soon after transplanting. The fungicide mixture of fludioxonil + cyprodinil was the most effective against the disease and provided better control of *B. cinerea* isolates *in situ*. These treatments gave satisfactory disease control despite the predominance of multi-fungicide resistant *B. cinerea* populations.

Keywords. Fungicides, grey mould, fludioxonil, cyprodinil, chlorothalonil.

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INTRODUCTION

Bottom rot of butterhead lettuce (*Lactuca sativa* L.) caused by *Botrytis cinerea* Pers. :Fr. is the most common disease problem in hydroponic lettuce production in Greece, during the late autumn to early spring period. Infections can start in nurseries and spread systemically (endophytically) in plants, without early visible symptoms (Sowley *et al.*, 2010). *Botrytis* head rots are less common and, in most cases, follow the appearance of 'tip burn' symptoms, due to inadequate transport of calcium into emerging leaves. These rots can be avoided by keeping calcium in balanced nutrient solutions, using cultivars which are less susceptible to 'tip burn', and manipulating the environment (Morgan, 1999; 2012). Good ventilation practices reducing excess of moisture combined with application of fungicides give adequate control of infections caused by *Botrytis cinerea* (Dik and Wubben, 2007).

In addition to multi-site fungicides such as thiram, compounds with site-specific modes of action against grey mould in lettuce crops are currently registered in Greece. These include anilinopyrimidines (cyprodinil

and pyrimethanil), the phenylpyrrole fludioxonil, the succinate dehydrogenase inhibitor (SDHI) boscalid and the quinone outside inhibitor (QoI) pyraclostrobin. Two commercial fungicide formulations with widespread use against *B. cinerea* in lettuce are Signum® (26.7% boscalid + 6.7% pyraclostrobin; BASF) and Switch® (25% fludioxonil + 37.5% cyprodinil; Syngenta). However, the use of fungicides for *B. cinerea* control in various crops has been associated with the development of fungicide resistance (Hahn, 2014). High levels of resistance against site-specific fungicides are the result of gene mutations at positions encoding their target sites. For example, the point mutations G143A, H272R, and F412S, which lead to changes in the target proteins CytB, SdhB, and Erg27, confer high resistance of the pathogen to, respectively, the QoI, SDHI, and hydroxyanilide fungicide classes (Leroux, 2007).

Multi-drug resistance (MDR) is another mechanism associated with fungicide resistance in *B. cinerea*. This involves mutations leading to over-expression of efflux transporters such as the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS), allowing weak resistance towards fungicides with unrelated modes of action (Kretschmer *et al.*, 2009). Very often, MDR and specific fungicide resistance types are coupled (Leroch *et al.*, 2013; Fernández-Ortuño *et al.*, 2014; Rupp *et al.*, 2016). The presence of *B. cinerea* strains with multiple fungicide resistance to all site-specific classes of fungicides have been reported in different parts of the world, especially for small fruits (Weber, 2011; Amiri *et al.*, 2013; Fernández-Ortuño *et al.*, 2014). Recent surveys made on lettuce crops in Greece and Germany have also demonstrated increasing threats from emergence of multiple fungicide resistance in *B. cinerea* populations (Chatzidimopoulos *et al.*, 2013; Weber and Wichura, 2013).

Current trends in agriculture demand fewer chemical applications, while maintaining profitable high-quality production with low pesticide residues. The limited number of registered fungicide formulations against bottom rot of lettuce forces growers to make repeated seasonal sprays with the one fungicide. Some studies have also shown that pesticide residues are detected in greater amounts in leafy vegetables compared to other crops (Skovgaard *et al.*, 2017). Multiple applications may compromise reduced pesticide strategies, which aim to delay the development of resistance and reduce pesticide residues.

The present study was undertaken: (i) to evaluate the efficacy and timing of applications with current botryticides against multi-resistant *B. cinerea* strains; (ii) to detect and measure possible fungicide residues at harvest; and (iii) to determine effects of different compounds against selected resistant isolates of the pathogen *in situ*.

MATERIALS AND METHODS

Host plant material

The 2-year experiments were carried out in a commercial lettuce glasshouse located at Krokion, Magnesia, Greece. The glasshouse was surrounded by cereal crops and olive trees, which were unlikely to be sources of *B. cinerea* inoculum. Pelletized lettuce seeds (*Lactuca sativa* 'Penelope'; 'butterhead' type, Rijk Zwaan), pre-treated with thiram were used in all tests. Seeding, germination and emergence of nursery plants took place in 4 × 4 cm horticultural cells filled with a peat-based substrate. The young seedlings were transplanted at the 4- to 5-leaf stage, about 5 weeks after sowing, into Hortiplan hydroponic gutters (nutrient film technique, NFT). For plant nutrition, a dense aqueous (bore water) solution was prepared, composed of (mg L^{-1}): Ca 200, Mg 40, K 210, P (PO_4^{3-}) 50, N (NH_4^+) 25, N (NO_3^-) 165, Fe 5, Mn 0.5, Cu 0.1, Zn 0.1, B 0.5, Mo 0.05. The pH of the nutrient solution was maintained between 5.5 and 5.8, and conductivity between 1.5 and 2.0 mS (Resh, 2012). The aqueous solution was supplied to plant every 15 min during daytime.

Experimental design and treatments

Experiments were organized in randomized blocks with three replicates for each treatment. Each plot consisted of 50 plants spaced 20 cm apart in one row. Fungicide applications was carried out using a hand-operated sprayer at 1,120 L ha^{-1} at 10 to 20-day intervals. To minimize the effects on neighbouring treatments, plots were separated with a plastic frame (100 × 50 cm), during the spray applications. The last application was made at least 4 weeks before harvest. Plants sprayed with water were used as experimental controls.

The fungicides used, at the standard recommended labelled rates for vegetable crops, were as follows: chlorothalonil (Daconil® 50 SC, Syngenta Ltd) at 3 mL L^{-1}; fenhexamid (Teldor ® 50 WG, Bayer CropScience) at 1.5 g L^{-1}; boscalid + pyraclostrobin (Signum® 26.7 + 6.7 WG, BASF SE) at 1.5 g L^{-1}; and fludioxonil + cyprodinil (Switch® 25 + 37.5 WG, Syngenta Crop Protection AG) at 0.5 g L^{-1}.

In the first trial (2012-2013 season), lettuce was sown on November 8, 2012, was transplanted into the NFT system on December 7, and was harvested on January 24, 2013. In this trial, the effectiveness of chlorothalonil and the mixture of fludioxonil + cyprodinil was evaluated in two, three or four spray programmes (Table 1). Fungicides were applied on two occasions during the

Table 1. Comparison of fungicide spray programs against bottom rot in hydroponic lettuce (Trial 1).

Applications			Disease		AUDPC	Healthy plants at harvest (%)	Fungicide residues[a] (mg Kg^{-1})	Phenotypes[b] recovered (%)		
Pre-Transplanting (2)	Post-Transplanting (1 or 2)		Severity (%)	Incidence (%)				I	II	W
Fludioxonil+ cyprodinil	-	-	0.6 c[c]	5.3 d	4.19 b	92.67 b	<LoQ[d]	0	0	0
	Fludioxonil+ cyprodinil	-	0.4 c	2.7 d	2.80 b	85 b	Fludioxonil:<LoQ Cyprodinil:0.053	0	0	0
	Fludioxonil+ cyprodinil	Fludioxonil+ cyprodinil	0.1 c	0.7 d	0.66 c	93.67 b	Fludioxonil:0.06 Cyprodinil:0.2	0	0	0
Chlorothalonil	-	-	20.7 a	40.7 b	267.14 a	64.67 ab	<LoQ	33	0	67
	Chlorothalonil	-	14.2 ab	26.0 c	201.86 a	64.67 ab	0.061	50	0	50
	Chlorothalonil	Chlorothalonil	9.2 bc	25.3 c	120.26 a	73.33 b	0.073	75	0	25
Control			21.8 a	55.3 a	261.91 a	37.33 a	-	62	15	23

[a] Maximum residue levels as determined by the European Community. Fludioxonil: 15; Cyprodinil:15; Chlorothalonil: 0.01; Fenhexamid: 40; Boscalid: 30; Pyraclostrobin: 2.
[b] *B. cinerea* phenotypes: I=QoI^{R}Bos^{R}Ani^{R}Ben^{HR}Dic^{MR}, II=Hyd^{R}QoI^{R}Bos^{R}Ani^{R}Phen^{MR}Ben^{HR}Dic^{MR}, W=Wild type.
[c] Means followed by the same letter in each column do not significantly differ (P = 0.05, Student-Newman-Keuls).
[d] LoQ: limit of quantification (0.005 mg Kg^{-1}).

nursery stage (November 19, December 1), followed by one or two more applications after transplanting (December 13 and 24).

In the second trial (2013–2014 season), lettuce was sown on October 17, 2013, transplanted on November 23, and harvested on January 27, 2014. Following the results from the first trial, the addition of fungicides from unrelated chemical groups was also evaluated in two-, three- or four-spray programmes. Two basal applications were carried out during the nursery stage (October 27 and November 17) with either chlorothalonil or the fludioxonil + cyprodinil mixture. After transplanting, one (December 6) or two (December 30) more applications were made with either fenhexamid, fludioxonil + cyprodinil, boscalid + pyraclostrobin or chlorothalonil, in alternating applications (Table 2).

Efficacy of the fungicide programmes was evaluated at harvest by recording the number of healthy plants from each plot. The disease incidence and severity were also recorded every week by counting the number and estimating the proportion (%) lesion area on plants infected by *B. cinerea* in each treatment. The sample size for all assessments was 50 plants per plot. At the end of each trial, the area under the disease progress curve (AUDPC) was calculated, based on the formula:

$$AUDPC = \sum_{i=1}^{N_i-1} \left(\frac{y_i + y_{i+1}}{2}\right)(t_{i+1} - t_i)$$

where t = the time of each assessment; y = the percent disease severity at each assessment; and n = the number of assessments.

Fungicide residues on lettuce heads were determined at harvest, in randomly collected samples of three plants from each treatment. Following EU directions, highly sensitive and selective multi residue methods were used to detect multiple fungicides (Waziha *et al.*, 2018). Chlorothalonil and fenhexamid residues were determined using gas chromatography with an electron capture detector. Boscalid, pyraclostrobin, cyprodinil and fludioxonil were analyzed using liquid chromatography with tandem mass spectrometry, following acetonitrile extraction/partitioning. Analyses were carried out at the Cadmion accredited Analytical Laboratory, 202 00 Kiato Korinthia, Greece.

Airborne inoculum monitoring

Portable air samplers (Burkard Manufacturing Co Ltd) were used, containing 9 cm Petri plates with selective medium (Edwards and Seddon, 2001), as slightly modified by Chatzidimopoulos *et al.* (2014b). The medium was enriched with a discriminatory concentration of each fungicide and was used to entrap *B. cinerea* propagules in the air. Only fungicide-resistant isolates were able to grow on these media. The different fungicides were dissolved in dimethyl sulphoxide and were added to different plates with the selective medium. The final con-

Table 2. Comparison of fungicide spray programs against bottom rot caused by *Botrytis cinerea* in hydroponic lettuce (Trial 2).

Applications			Disease		AUDPC	Healthy plants at harvest (%)	Fungicide residues[a] (mg Kg^{-1})	Phenotypes[b] recovered (%)		
Pre-Transplanting (2)	Post-Transplanting (1 or 2)		Severity (%)	Incidence (%)				I	II	W
Fludioxonil+ cyprodinil	-	-	0.8 b[c]	2.67 b	7.35 b	90.00 a	<LoQ[d]	0	0	100
	Fludioxonil+ cyprodinil		1.2 b	7.33 b	9.72 b	97.33 a	<LoQ	0	100	0
	Fenhexamid	-	0.7 b	0.67 b	11.64 b	95.33 a	<LoQ	0	100	0
	Boscalid+ pyraclostrobin	-	0.9 b	2.67 b	9.58 b	96.67 a	<LoQ	50	50	0
	Chlorothalonil	-	1.1 b	2.67 b	15.71 b	98.00 a	<LoQ	100	0	0
	Fenhexamid	Fludioxonil+ cyprodinil	2.4 b	10.67 b	21.92 b	94.00 a	Fludioxonil:0.43 Cyprodinil:0.74 Fenhexamid<LoQ	25	75	0
	Fludioxonil+ cyprodinil	Fenhexamid	0.9 b	1.33 b	11.93 b	92.00 a	Fludioxonil <LoQ Cyprodinil<LoQ Fenhexamid:5.95	0	100	0
Chlorothalonil	-	-	1.7 b	3.33 b	23.94 b	83.33 a	<LoQ	100	0	0
	Chlorothalonil	-	0.6 b	1.33 b	6.26 b	96.67 a	<LoQ	0	0	0
	Fenhexamid	-	2.1 b	4.67 b	33.22 b	89.33 a	<LoQ	25	75	0
	Boscalid+ pyraclostrobin	-	0.8 b	1.33 b	13.38 b	95.33 a	<LoQ	75	25	0
	Fludioxonil+ cyprodinil	-	1.7 b	2.00 b	26.84 b	93.33 a	<LoQ	0	75	25
	Fenhexamid	Fludioxonil+ cyprodinil	3.0 b	4.67 b	45.34 b	89.33 a	Fludioxonil:0.43 Cyprodinil:0.74 Fenhexamid<LoQ	0	0	0
	Fludioxonil+ cyprodinil	Fenhexamid	1.6 b	3.33 b	20.62 b	92.00 a	Fludioxonil <LoQ Cyprodinil<LoQ Fenhexamid:5.95	0	100	0
Control			14.2 a	28 a	182.58 a	88.00 a		60	20	20

[a] Maximum residue levels as determined by the European Community. Fludioxonil: 15; Cyprodinil:15; Chlorothalonil: 0.01; Fenhexamid: 40; Boscalid: 30; Pyraclostrobin: 2.
[b] *B. cinerea* phenotypes: I=QoI^{R}Bos^{R}Ani^{R}Ben^{HR}Dic^{MR}, II=Hyd^{R}QoI^{R}Bos^{R}Ani^{R}Phen^{MR}Ben^{HR}Dic^{MR}, W=Wild type.
[c] Means followed by the same letter in each column do not significantly differ (P = 0.05, Student-Newman-Keuls).
[d] LoQ: limit of quantification (0.005 mg Kg^{-1}).

centration of the solvent in the growth medium did not exceed 1%. The discriminatory doses used were: 1 mg L^{-1} fenhexamid from the hydroxyanilide (Hyd) class; 10 mg L^{-1} pyraclostrobin from QoI class (strobilurins) plus 100 mg L^{-1} salicylhydroxamic acid; 10 mg L^{-1} boscalid (Bos) from the SDHI class (carboxamides); 10 mg L^{-1} cyprodinil from the anilinopyrimidine (Ani) class; 1 mg L^{-1} fludioxonil from the phenylpyrrole (Phen) class, and 3 mg L^{-1} iprodione from dicarboximide (Dic) class. Plates containing no fungicide were used as experimental controls.

The air samplers were operated simultaneously for 60 min near midday once every 10 d during the experimental periods. To avoid the formation of holes in the medium, the agar layer inside each Petri dish was thick (each dish contained approximately 20 mL of medium). Typical *B. cinerea* sporulating colonies developed on the sampling media, following 6 d of incubation at 20°C in the dark. The number of colonies on each fungicide-containing medium was expressed as the proportion of the total number of colonies on the control plates.

Isolation of the pathogen and definition of the resistance phenotype

Botrytis cinerea was isolated from plants bearing lesions at the stem bases, during the harvest. Infected

plant tissues were transferred in separate moist polyethylene bags to the laboratory and stored at room temperature for 24 h. From each sample a single isolate was made onto sterilized potato dextrose agar (PDA) media by slight touching a flamed wire loop onto a freshly sporulating *Botrytis* lesion. In order to identify the phenotype, each isolate was tested for sensitivity response to the fungicides fenhexamid, chlorothalonil, pyraclostrobin, boscalid, cyprodinil, fludioxonil, carbendazim (50% WP, Cequisa SA) or iprodione, with the point inoculation method (Chatzidimopoulos *et al*., 2013). The same procedure was followed to determine the phenotype of airborne trapped inocula after 7 d of growth in plates containing *B. cinerea* selective medium, in a sample of 100 collected colony forming units (CFUs). The samples were collected at random from control plates and plates amended with fungicides, among different sampling dates.

In situ *pathogenicity assays*

Lettuce plants were grown in 9 cm diam. plastic pots containing peat substrate, in a growth chamber (Sanyo MLR-350HT) with 10 h light period at 18°C, until the 14th true leaf unfolded. Leaf blades (approx. 4 × 4 cm) were excised from the upper half of each plant and immersed in aqueous fungicide suspensions at the same rates used in the field trials. Control leaf blades were immersed in sterilized water. The leaves were allowed to dry for 30–40 min and were then placed in Petri dishes containing water agar (1.5%) with adaxial surfaces uppermost. Three *B. cinerea* isolates, each from the three dominant phenotypes in the glasshouse, were used as inoculum. Mycelium discs (5 mm diam.) were removed from the periphery of 3-d-old colonies grown on PDA, and were aseptically placed upside down over the leaf blades. The Petri dishes with the inoculated leaves were then placed in the growth chamber conditions described above. After 72 h incubation, the mean diameters of the lesion on each leaf blade (minus the 5 mm of mycelial plug) was determined using a measuring rod. Three replicates per isolate/fungicide treatment were made.

Data analyses

Data from the glasshouse trials were analysed by one-way ANOVA and Student-Newman-Keuls test. Tukey's Honestly Significant Difference test was used to assess differences of mean values from the pathogenicity trials. To meet the assumptions of ANOVA, percentage and count values were logarithmically transformed to base 10 where necessary. P-values ≤ 0.05 were considered statistically significant. Statistical analyses were performed using ARM software (Revision 2017.4, Gylling Data Management, Inc.).

RESULTS

Control of disease

In the untreated control lettuce plants, small, hardly visible, brown lesions appeared at the base of the petioles of the bottom leaves, 14 d after transplanting. *Botrytis cinerea* invaded the basal stems *via* the senescent cotyledons or leaf petioles within 3 weeks after transplanting, at the 13 true unfolded leaf growth stage. Early assessments at 1 to 3 weeks after transplanting indicated that when the infection was initiated at an earlier stage of development, the disease progressed more rapidly and eventually the plant stem bases rotted within 10 to 14 d. All the plants which showed early symptoms of infections within this period eventually rotted. The overall disease severity ranged from 0.11 to 0.3 %, and incidence from 2 to 8 %, in both trials at 14 d after transplanting. When the infection was initiated at a later stage, the stem rot progressed very slowly. The disease was more severe in the first trial than the second. By the time of the last assessment at harvest, disease severity and incidence were up to 21.8 % and 55.3 % for trial 1 and up to 14.2 % and 28 % for trial 2 (Tables 1 and 2).

In first trial, two pre-transplanting applications of fludioxonil + cyprodinil reduced disease incidence and severity compared with unsprayed plants (Table 1). Disease control was further improved when one or two more applications were made after transplanting. The AUDPC values for these treatments were very low ranging from 0.66 to 4.19 and a significantly increased number of healthy plants was observed at harvest compared to the untreated control. Chlorothalonil, although providing some control of the disease, was the least effective fungicide. Although disease incidence and severity incidence were reduced, the mean AUDPC values were high, ranging from 120.26 to 267.14 for all treatments, and these were not significantly different from the untreated control. However, the programme with two post-transplanting sprays of chlorothalonil significantly increased the number of healthy plants at harvest compared to the untreated control. With the exception of post-transplanting applications with chlorothalonil, no fungicide residues exceeding the maximum residue level (MRL) defined by the European Community (EC) were detected at harvest. These are 0.01 mg kg^{-1} for chlorothalonil, 15 mg kg^{-1} for fludioxonil and 15 mg kg^{-1} for cyprodinil. For the pre-transplanting applications alone, the residues at

harvest were below the adopted analytical reporting limits of quantification (LoQs) of 0.005 mg kg^{-1} (Table 1).

In the second trial, with lower disease pressure, two applications of fungicide at the pre-transplanting stage, with either fludioxonil + cyprodinil or chlorothalonil, decreased disease incidence and severity. One or two more post-transplanting fungicide applications with alternating treatments did not improve disease control (Table 2). All the treatments were of high efficacy, and the disease progress (AUDPC) was significantly reduced compared to the untreated control (Table 2). Although increased numbers of healthy plants were observed from most treatments compared to the untreated control, the differences between treatments were not statistically significant. With the exception of two cases (four-spray programmes), in which the fungicides fenhexamid and fludioxonil + cyprodinil were applied at 27 d before harvest, no fungicide residues were detected. Residues of fenhexamid were 5.95 mg kg^{-1} and of fludioxonil + cyprodinil were 0.43 + 0.74 mg kg^{-1}. However, the residue amounts were much less than the European MRLs, at 40 mg kg^{-1} of fenhexamid, 15 mg kg^{-1} of fludioxonil and 15 mg kg^{-1} of cyprodinil (Table 2).

Fungicide resistant airborne inoculum

Fungicide resistant *B. cinerea* inocula in the air of the lettuce glasshouse were detected at all the sampling dates, for both of the trials. The numbers of trapped CFUs in each of the six fungicide-amended substrates are shown in Figure 1. From the beginning until the end of the experimental periods, iprodione-, pyraclostrobin- and cyprodinil-resistant CFUs were detected at frequencies comparable to the CFUs trapped in control plates. CFUs resistant to fenhexamid (Fen^R) were trapped on all sampling dates but at variable frequencies. An increase of the Fen^R population was observed during the second trial. However, the total numbers of CFUs trapped in the media from the mid-December to mid-January were generally low. Boscalid resistant (Bos^R) inocula were detected at variable frequency during both trials. The Bos^R populations reached peaks in the middle of both growing periods. By contrast, with the exception of the late sampling dates during the second trial, fludioxonil resistant ($Phen^{MR}$) CFUs were rarely trapped (Figure 1).

Approximately 90% of the isolates recovered from the control plates exhibited multiple resistance to fungicides (Figure 2). The three prevalent resistant phenotypes in decreased frequency were: $QoI^RBos^RAni^R$-$Ben^{HR}Dic^{MR}$ (phenotype I; 57% frequency), Hyd^RQoI^R-$Bos^RAni^RPhen^{MR}Ben^{HR}Dic^{MR}$ (phenotype II; 33%) and wild type (phenotype W; 10%). Only phenotype-II CFUs were detected in media amended with fenhexamid or fludioxonil (Figure 2). In media amended with pyraclostrobin, cyprodinil, iprodione and boscalid, the most

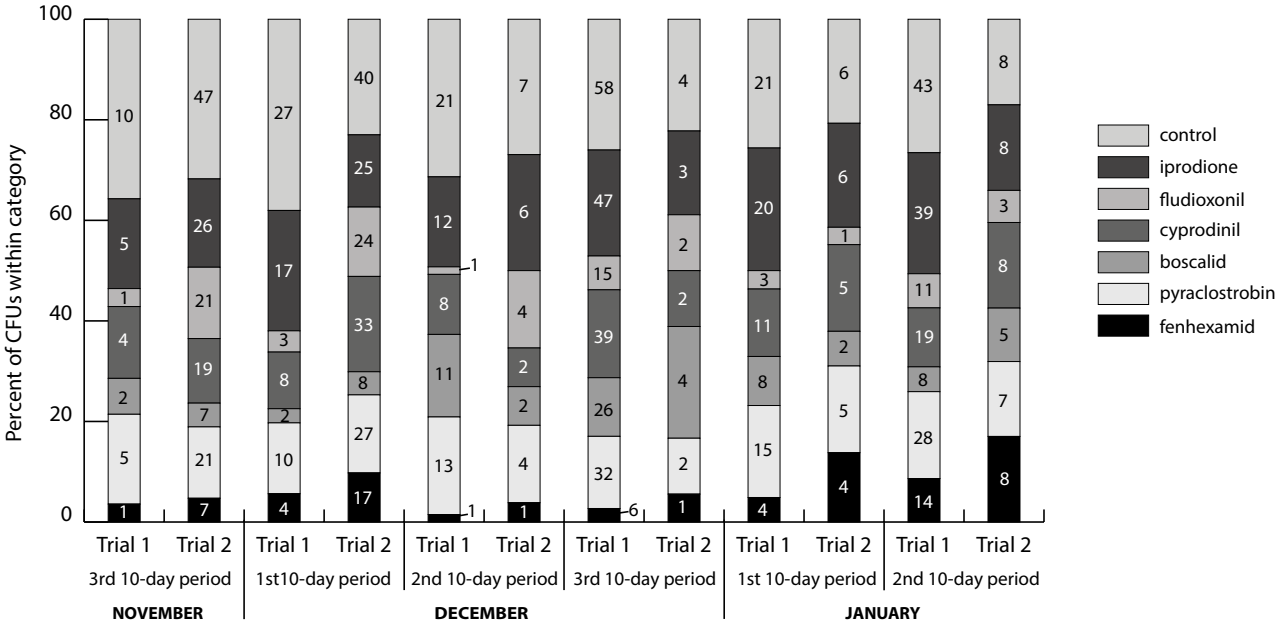

Figure 1. Number of airborne *B. cinerea* conidia trapped in different fungicide amended media during 2012-13 (Trial 1) and 2013-14 (Trial 2) growing periods. The number of CFUs on fungicide enriched selective media are indicated in each bar and expressed as % of the total number of CFUs on all the plates including controls (top bars). The discriminatory concentrations used were: 1 mg L^{-1} fenhexamid, 10 mg L^{-1} pyraclostrobin + 100 mg L^{-1} SHAM, 10 mg L^{-1} boscalid, 10 mg L^{-1} cyprodinil, 1 mg L^{-1} fludioxonil and 3 mg L^{-1} iprodione.

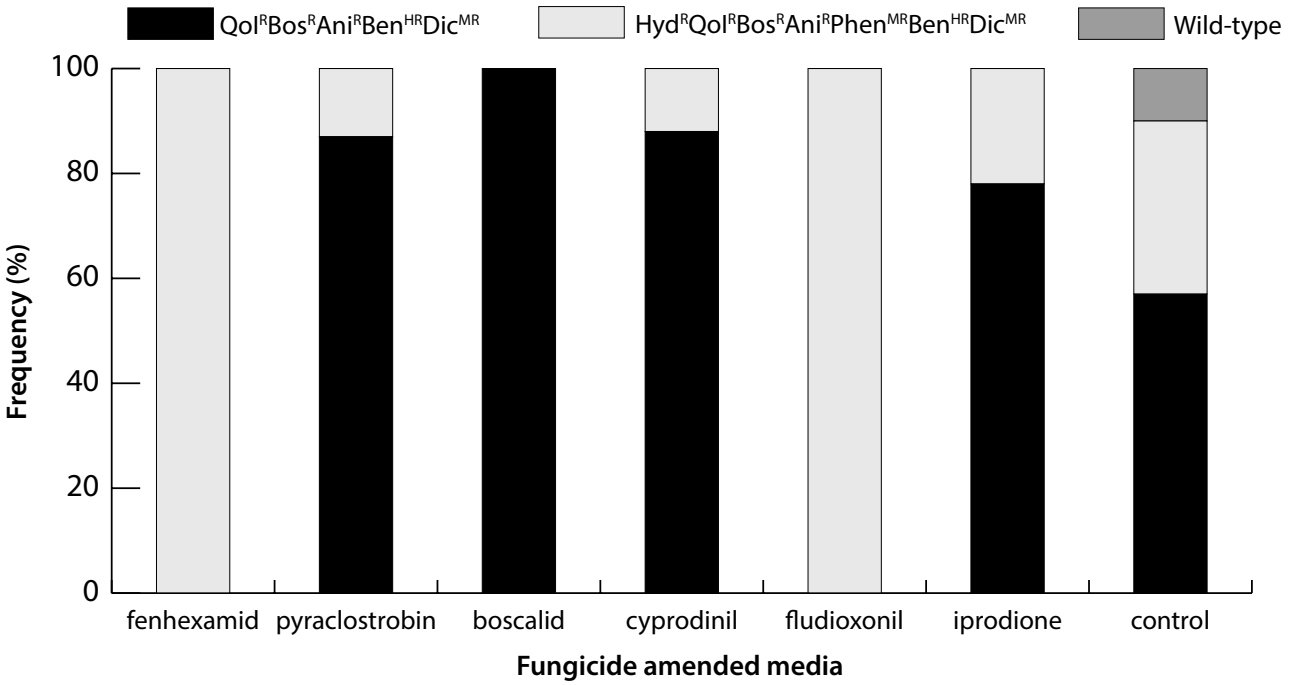

Figure 2. *Botrytis cinerea* phenotypes recovered from fungicide amended selective media. The sensitivity response of each CFU to the discriminatory doses of the fungicides, was examined by the point inoculation method.

prevalent phenotype was type I at frequencies ranging from 78 to 100%. CFUs of the phenotype II were detected at lower frequencies (12 to 22%) on media containing pyraclostrobin, cyprodinil and iprodione (Figure 2). Those three were the only phenotypes detected on all media tested.

Detection of fungicide resistant strains in diseased plants

All the isolates recovered from infected plants were classified in the following three phenotypes: QoI^{R}-$Bos^{R}Ani^{R}Ben^{HR}Dic^{MR}$ (phenotype I; 58%), $Hyd^{R}QoI^{R}$-$Bos^{R}Ani^{R}Phen^{MR}Ben^{HR}Dic^{MR}$ (phenotype II; 24%) and wild-type (phenotype W; 18%). The multiple resistant phenotype II was detected in plants treated at least once after transplanting with either fenhexamid, boscalid + pyraclostrobin or fludioxonil + cyprodinil (Table 2). This phenotype was not detected in plants treated only with chlorothalonil. On the other hand, phenotype I prevailed by 60% in the isolations made from the infected plants in the controls (Table 2). Phenotype-I strains were also most frequently detected in treatments where the QoI fungicide was included. No diseased plants were observed from the spray programmes with the mixture of fludioxonil + cyprodinil in the first trial (Tables 1 and 2).

Pathogenicity of fungicide resistant isolates in situ

Fenhexamid, pyraclostrobin, boscalid and cyprodinil failed to inhibit the development of *B. cinerea* lesions *in vitro* when the isolate used in inoculations was characterized as resistant to the respective fungicide. No differences were observed in lesion size compared to controls (Table 3). In contrast, fludioxonil was more effective, even against the isolate CR-32 which was characterized as moderately resistant to this fungicide. Chlorothalonil gave variable effects against the isolates with multiple resistances to other fungicides (Table 3). The mean lesion sizes ranged from 2.6 to 12.2 mm, although these strains had previously been characterized as sensitive *in vitro* to chlorothalonil.

DISCUSSION

Selection of resistant individuals in fungal populations subjected to selective pressure due to fungicides is an evolutionary mechanism that promotes advantageous genotypes (Walker *et al.*, 2013). In the present study, during a 2-year monitoring schedule in a glasshouse, multiple resistant isolates were detected after two to four fungicide applications per year. In addition, the phenotypic characterization of the isolates obtained in this

Table 3. *In situ* lesion development on lettuce leaves treated with fungicides and inoculated with multiple resistant isolates of *Botrytis cinerea*.

Isolate[a]	Size of lesion (mm)						
	Fenhexamid (1.5 g)[b]	Pyraclostrobin (0.4 g)	Boscalid (0.8 g)	Cyprodinil (0.375 g)	Fludioxonil (0.5 g)	Chlorothalonil (2.5 mL)	Control
C-01 (Type I)	0.0 **A**a (S)[d]	18.0 **BC**b (R)	27.0 **C**b (R)	15.4 **B**b (R)	4.6 **A**a (S)	3.4 **A**a (S)	20.0 **BC**b
CR-32 (Type II)	21.0 **BC**b[c] (R)	19.8 **BC**b (R)	24.2 **C**b (R)	18.4 **BC**b (R)	10.9 **A**b (MR)	12.2 **B**b (S)	23.8 **C**b
A-56 (Wild type)	3.0 **A**a (S)	4.6 **A**a (S)	0.0 **A**a (S)	5.4 **A**a (S)	2.6 **A**a (S)	2.6 **A**a (S)	15.4 **B**a

[a] Phenotype: **C-01**=QoI^{R}Bos^{R}Ani^{R}Ben^{HR}Dic^{MR}; **CR-32**=Hyd^{R}QoI^{R}Bos^{R}Ani^{R}Phen^{MR}Ben^{HR}Dic^{MR}; **A-56**=wild-type.
[b] Fungicide treatments (concentration per L). The fungicide formulations used were: fenhexamid as Teldor 50 WG (Bayer CropScience); pyraclostrobin as F500 25 EC (Syngenta Ltd); boscalid as 510F 50 WG (BASF SE); cyprodinil as Chorus 50 WG (Syngenta Crop Protection AG); fludioxonil as Geoxe 50 WG (Syngenta Crop Protection AG); chlorothalonil as Daconil 500 SC (Syngenta Ltd).
[c] Numbers followed by the same bold upper-case letters in rows and low-case letters in columns do not differ significantly according to Tukey's HSD post hoc test; $P = 0.05$.
[d] Sensitivity group: R=resistant, MR=moderately resistant, S=sensitive.

study, and previous genetic analyses, showed that several resistance alleles to different fungicide molecules were selected, due to fungicide pressure (Chatzidimopoulos *et al*., 2013; Chatzidimopoulos *et al*., 2014a; Chatzidimopoulos *et al*., 2014b). Furthermore, next generation sequencing data revealed that an underlying MDR mechanism was also present in these strains (Chatzidimopoulos *et al*., 2016). The presence of such strains in an isolated area after repeated use of fungicides suggests that a stepwise accumulation of resistances occurred over time, and that no pathogen migration took place from neighbouring crops, as has occurred in other cases (Rupp *et al*., 2016).

Different strategies have been adopted to avoid or reduce the risks of production losses due to fungicide resistance. These have included applications only in pre-formulated or tank fungicide mixes, or in rotations with effective non-cross-resistant fungicides, preferably multi-site inhibitors with low risk and limited numbers of treatments (Brent, 2012). In the present study, despite the existence of multi-resistant airborne inoculum, all the application programs improved disease control and left fungicide residues below the defined European Community MRLs. Few multi-site inhibitors with activity against *B. cinerea* are now available. Use of chlorothalonil, a multi-site inhibitor that is still available, is restricted to certain crops due to deposition of undesirable residues. However, when applied in this study on lettuce in the nursery, satisfactory disease control was achieved without detectable fungicide residues at harvest. Applications with chlorothalonil-based fungicides after transplanting should be avoided, however, since there is then risk of the remaining residues on lettuce being above the accepted LoQ limits. Due to recent decision of the European Standing Committee (SCoPAFF) against the renewal of chlorothalonil registration in EU countries, growers are likely to have (from spring 2020) one fewer vital tool to combat fungicide resistance.

Recent surveys made on lettuce revealed infections by *B. cinerea* strains that are multi-resistant to most available botryticides (Chatzidimopoulos *et al*., 2013; Weber and Wichura, 2013). By using an air sampler with a selective medium, as proposed by Edwards and Seddon (2001), modified and enriched with appropriate doses of fungicides (Chatzidimopoulos *et al*., 2014b)2014b, we revealed the prevalence of *B. cinerea* resistant strains in the air of the glasshouse throughout two experimental periods. Most trapped isolates exhibited multiple resistances to specific fungicides. High degrees of resistance to carbendazim (benzimidazole class) and moderate resistance to iprodione (dicarboximide class) were always present, even when neither of these fungicides was included in the spray programmes. Two dominant resistant phenotypes (QoI^{R}Bos^{R}Ani^{R}Ben^{HR}Dic^{mR} and Hyd^{R}QoI^{R}Bos^{R}Ani^{R}Phen^{MR}Ben^{HR}Dic^{MR}) were detected in the air of the experimental glasshouse and isolated from infected plants. These phenotypes constituted 84% of the total *Botrytis* population in the glasshouse, and had also been isolated from diseased plants originating from the same site in previous years (Chatzidimopoulos *et al*., 2013).

Recent studies have shown that in the absence of fungicide selection pressure, resistance to fenhexamid (Billard *et al*., 2012), cyprodinil (Bardas *et al*., 2008) or boscalid (Veloukas *et al*., 2014) may entail a fitness costs in *B. cinerea*. However, Rupp *et al*. (2016) con-

cluded that multiple resistant strains are likely to possess high fitness in the field, and that they are essentially immune to sprays with any of the current botryticides. In the present study, significant decreases of the airborne resistant populations were observed at the beginning of each growing period. Moreover, no strains highly resistant to fludioxonil have been detected in the field, although this phenylpyrrole fungicide has been used for over two decades (Baroffio *et al*., 2003; Chatzidimopoulos *et al*., 2013). Similarly, Fernández-Ortuño *et al.* (2012) reported the high efficacy of the mixture boscalid + pyraclostrobin against *B. cinerea* in strawberry, even though a resistant population to SDHIs and QoIs was present at high frequency. This information may explain the good efficacy of the fungicides in our field trials. The high frequency of resistant strains within the pathogen population in the atmosphere of the glasshouse may be the consequence of good efficacy of the fungicides against the wild-type *B. cinerea* strains. The present assays have shown that under strong disease pressure, (as for the mycelial plugs used in the *in situ* assays) fungicides lose their efficacy against resistant strains. Under conditions of low disease pressure, however, as for airborne spores in the second trial, the fungicides may retain their efficacy. When conidia of the selected strains were used to check the *in situ* efficacy of the fungicides, lesions were not formed in most cases (unpublished data). The phytoalexin lettucenin A in young plants may act preventively on infections triggered by spores (Bennett *et al*., 1994).

All fungicide applications only at the host nursery stage reduced disease incidence and severity, and increased the number of healthy plants at harvest in a trial with high disease pressure, and to a lesser extent (not significantly) under lower disease pressure. According to Sowley *et al.* (2010), the infections could be initiated at nursery stages and spread systemically throughout the plants without the development of visible symptoms. This disease progress can be arrested by fungicide protection of the young seedlings at the nursery or at early transplanting stages. The pre-transplanting applications provided improved disease control when these were combined with one or two post-transplanting applications. However, the additional applications did not significantly improve the number of healthy plants at harvest. In the second trial, under low disease pressure, the number of healthy plants in control plots was similar with the number of the healthy plants in the treated plots. The initial symptoms of infection were observed at 3 weeks after transplanting, but the disease progressed very slowly because of unfavourable climatic conditions in the glass house. Furthermore, recent studies have shown that lettuce crops become less susceptible to infections as they age (develop thick bases and resistant leaves), and some cultivars, such as iceberg and romaine types, are more prone to infections than others (Shim *et al*., 2014).

The mixture of fludioxonil + cyprodinil was the most effective of the different fungicides tested here against lettuce bottom rot at both rates of application. The high efficacy of this mixture in lettuce crops was also reported by Matheron and Porchas (2008). Kilani and Fillinger (2016) also reported that high resistance to fludioxonil does not exist among *B. cinerea* populations worldwide, and this status is not expected to change in the future. In addition, our results have shown that fludioxonil provided better control of *B. cinerea* resistant isolates *in situ*, in comparison with other fungicides. Similar results were observed by Rupp *et al.* (2016) in *in planta* tomato assays. The inclusion of this compound in the fungicide programmes could explain the effective disease control in field trials.

In conclusion, it was shown that appropriate selection and timing of fungicide sprays is fundamental for control of bottom rot of lettuce grown in hydroponic systems. In a trial with high disease pressure, two fungicide applications during plant growth in the nursery combined with one further application at transplanting, provided the best disease control without leaving detectable fungicide residues at harvest. However, under less disease pressure, the two applications at the nursery stage were enough to protect the plants from bottom rot caused by *B. cinerea*. Fungicide selection was made according to the risk for resistance development. Fludioxonil was the most effective compound against the multiple resistant strains of *B. cinerea*. Primary applications were evaluated, with a multi-site inhibitor (chlorothalonil) or mixtures of non-cross resistant compounds (e.g. fludioxonil + cyprodinil) in alternation with other effective compounds with different modes of action (such as fenhexamid or boscalid). These treatments gave satisfactory disease control, despite the predominance of multi-fungicide resistant populations of *B. cinerea*.

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Research Paper

Molecular diversity of *Alternaria* spp. from leafy vegetable crops, and their sensitivity to azoxystrobin and boscalid

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Summary. Severe outbreaks of Alternaria leaf spot have occurred in Northern Italy on leafy vegetable and ornamental hosts. This disease is mainly controlled by two classes of respiration inhibitor fungicides, QoIs (including azoxystrobin) and SDHIs (including boscalid). Thirty-six *Alternaria* strains were isolated from five leafy vegetable crops, and subjected to molecular characterization. Multilocus phylogenetic analyses assigned most of the strains (86%) to *A. alternata*, while the rest were *A. arborescens* and other *Alternaria* spp. *In vitro* sensitivity assays showed that 3% of the strains were of intermediate resistance, and 11% of reduced sensitivity to azoxystrobin, while 8% of the strains were resistant to boscalid. Sequencing of cytochrome b in an intermediately resistant strain of *Alternaria* revealed the G143A mutation. This strain was also resistant to boscalid. None of the tested *Alternaria* strains had amino acid mutations associated with boscalid resistance coded by the *SdhB* and *SdhC* genes. This is the first report of azoxystrobin resistance in *A. alternata* in Italy, as well as the first record of resistance of *Alternaria* spp. found on leafy vegetables. As boscalid resistance was not associated with any frequently reported mutations, further investigations of the additional resistance mechanisms are necessary. These results demonstrate the need for well-organized chemical control of emerging Alternaria diseases, to prevent the increase of resistance to QoI and SDHI fungicide classes, and the possibility of double fungicide resistance in these pathogens.

Keywords. Alternaria leaf spot, fungicide resistance, QoIs, SDHIs, molecular characterization.

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INTRODUCTION

Alternaria species are becoming emerging threats in vegetable growing areas in Europe, as the consequence of the globalization of the trade of seeds and plants, climate changes and intensification of cultivation (Gilardi

et al., 2018). Alternaria leaf spot on leafy vegetables is caused by *Alternaria alternata* (Fr.: Fr.) Keissl. and other *Alternaria* spp., including *A. japonica* and *A. arborescens* (Gullino *et al*., 2014; Subbarao *et al*., 2017; Gilardi *et al*., 2018). Symptoms first appear as small leaf spots which expand to brown-black lesions encircled by yellow haloes on aging leaves. Progressive plant defoliation occurs at later stages with the occasional death of the plants. These pathogens overwinter on infected crop residues, seeds and weeds. Conidia are airborne and can be dispersed over great distances throughout the growing season. Transmission by seeds facilitates wide pathogen dissemination (Simmons *et al*., 2007), and this distribution occurs with different leafy vegetables (Gullino *et al*., 2014).

The presence of *Alternaria* spp. on new vegetable hosts has recently been reported in different countries, including Italy, Greece, Poland, South Africa, Algeria, Pakistan, China, and the United States of America (Farr and Rossman, 2019). Outbreaks of Alternaria leaf spot have increased in Italy, and they are mainly caused by small-conidium *Alternaria* spp. including *A. alternata* and *A. arborescens*. These species have been recorded for the first time on a few vegetable crops in Italy; *A. alternata* has been reported on sweet basil, cultivated and wild rocket, pepper, chili pepper, cabbage and spinach, and *A. arborescens* on sweet basil (Garibaldi *et al*., 2011; Gullino *et al.,* 2014; Woudenberg *et al*., 2015; Garibaldi *et al*., 2019a; Gilardi *et al*., 2019). In addition, *A. alternata* has been reported on ornamental hosts, including purple coneflower, pineapple sage, fruit-scented sage, peppermint, *Digitalis purpurea* and *Ceratostigma willmottianum* (Garibaldi *et al*., 2018a, 2018b, 2018c, 2019b, 2019c, 2019d).

Alternaria spp. are mostly controlled using fungicides. The fungicides registered in the European Union against *Alternaria* spp. are: 1) copper-based fungicides, 2) dithiocarbamates, 3) dicarboximides, 4) phenylpyrroles, 5) quinone outside inhibitors (QoIs), 6) succinate dehydrogenase inhibitors (SDHIs), 7) methyl benzimidazole carbamates, and 8) demethylation inhibitors (http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=activesubstance.selection&language=EN). The main fungicide groups used for management of Alternaria leaf spot are respiration inhibitors; QoIs (including azoxystrobin, pyraclostrobin and fluoxastrobin) and SDHIs (boscalid and fluopyram). Azoxystrobin and boscalid are widely used on leafy vegetables against a number of soil-borne and foliar pathogens, due to their broad activity spectra (Margot *et al*., 1998; Matheron and Porchas, 2004). Careful monitoring is important to determine change in sensitivity of *Alternaria* spp. to these fungicides. In Italy and other countries, the mixture pyraclostrobin + boscalid is frequently applied against different fungal diseases (e.g. *Botrytis cinerea* and *Sclerotinia sclerotiorum*).

The QoIs, which have a common single-site mode of action, inhibit mitochondrial respiration at the outer, quinone oxidizing pocket (Qo site) within the cytochrome bc1 enzyme complex. This causes impairment of the electron transfer chain, resulting in energy deficit and insufficient ATP production (Becker *et al*., 1981). The cytochrome b (*cyt b*) gene, one of the coding genes of the enzyme complex, is related to QoI resistance, which appeared soon after QoIs were introduced into plant protection markets. QoI resistance was first reported in *Blumeria graminis* f. sp. *tritici* and then on many plant pathogenic oomycetes and fungi, including *A. alternata* (Sierotzki *et al*., 2000; Ma *et al*., 2003). The cyt b amino acid substitution from glycine to alanine at position 143 (G143A) was mainly reported in QoI-resistant *A. alternata* (Ma *et al*., 2003), while *A. solani* showed other cyt b mutations (phenylalanine with leucine at position 129; F129L mutation) (Pasche *et al*., 2005).

SDHIs are another group of respiration inhibitors that act on the mitochondrial succinate dehydrogenase (Sdh) complex. The Sdh complex contains four subunits: flavoprotein (FP or SdhA), ironesulfur protein (IP or SdhB) and two integral membrane-anchor proteins (SdhC and SdhD) (Hägerhäll, 1997). Reduced sensitivity to SDHI fungicides has been related to several point mutations in four subunits of the Sdh complex. Boscalid resistance was reported for the first time in *A. alternata* in pistachio under field and laboratory conditions, followed by *A. solani* in potato and *A. alternata* in tomato (Avenot and Michailides, 2007; Wharton *et al*., 2012; Malandrakis *et al*., 2018). SdhB amino acid substitutions from histidine (H) to tyrosine (Y) or to arginine (R) at position 277 aa (H277Y), (H277R) in *A. alternata*, and the same substitution at amino acid position 278 (H278Y), (H278R) in *A. solani,* have been found in strains showing SDHI resistance (Avenot *et al*., 2008a; Mallik *et al*., 2014). SDHI resistance has also been associated with mutations in two other SDH subunits; H134R in SdhC, and H133R and D123E in SdhD (Avenot *et al*., 2009; Mallik *et al*., 2014).

The objective of the present study was to characterize *Alternaria* isolates from leaf spot-affected vegetable crops using molecular techniques, and to investigate their sensitivity to azoxystrobin and boscalid. For this purpose, 36 *Alternaria* isolates, obtained from symptomatic plants, were subjected to molecular characterization by four-loci phylogenetic analyses. The sensitivity of the identified *Alternaria* strains to azoxystrobin and

boscalid was also assessed using *in vitro* tests, and presence of the fungicide-associated mutations was investigated by characterizing the *cyt b* gene (related to azoxystrobin resistance) and the *SdhB* and S*dhC* genes (associated with boscalid resistance).

MATERIALS AND METHODS

Isolate collection

Thirty-six isolates were collected during 2013–2017 from leaf spot-affected tissues of cabbage, cauliflower, cultivated rocket, wild rocket and basil plants, grown in soil-less or conventional systems in Northern Italy (Table 1). On the basis of conidium observations, all the isolates belonged to the small-conidium *Alternaria* spp. Four additional strains, EGS 34015 (CBS 918.96), EGS 34016 (CBS 916.96) (E.G. Simmons, Mycological Services), CBS 124274, and CBS 124278 (CBS-KNAW Collection), were used as reference strains for *A. alternata* or *A. arborescens* (Table 1).

DNA extraction and PCR

The DNA of the 36 isolates was isolated using an E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek), following the manufacturer's instructions, from 100 mg of mycelium grown on potato dextrose agar (PDA, Merck®) plates. Molecular identification was performed through amplification of the internal transcribed spacer (ITS; White *et al*., 1990) using primer ITS1/ITS4, endopolygalacturonase (*endoPG*; Andrew *et al*., 2009) using primer PG3/PG2b, β-tubulin (*tub2*; O' Donnell and Cigelnik 1997; Peever *et al*., 2004) using primer T2/β-tub2, and histone 3 (*H3*; Glass and Donaldson 1995) using primer H31a/H31b. The PCR products were purified using a QIAquick PCR purification kit (Qiagen), according to the manufacturer's instructions, and sequenced in both directions at the BMR Genomics Centre (Padua, Italy). Only the sequences of the studied and reference isolates with good quality scores (Phred scores greater than 30) were selected (Ewing *et al.,* 1998). These sequences were used for a successive contig assembly and sequence analyses. The sequences were deposited in GenBank under Accession Numbers: ITS (MH936379-MH936414), *endoPG* (MK140907-MK140935), *tub2* (MK044808-MK044820) and *H3* (MK239196-MK239231) (Table 1), with the exception of some isolates which had previously been sequenced in the *tub2* and *endoPG* region (Siciliano *et al*., 2017; 2018). The accession numbers of the reference isolates are also included in Table 1.

Sequence analyses

A sequence comparison with reference isolates of *Alternaria* spp. (Woudenberg *et al*., 2015; Siciliano *et al.,* 2018) available in the GenBank database was performed using the BLAST software package (www.ncbi.nlm.nih.gov). Phylogenetic analyses were based on Maximum Likelihood (ML) and Bayesian inference (BI). MEGA 7 software was used (Kumar *et al*., 2016) for the Maximum Likelihood analysis. A total of 1831 bp concatenated data sets were obtained with the ITS, *tub2*, *endoPG* and *H3* sequences. Findmodel was used to select the best-fit nucleotide model of each region (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) as follows: K80: Kimura 2-parameter for ITS, TrN: Tamura-Nei for *tub2*; GTR: General Time Reversible for *endoPG*, and TrN plus Gamma for *H3* and the concatenated tree. Maximum-likelihood trees were then constructed with bootstrap values obtained from 1,000 replications. The best-fit model of each dataset was determined for a Bayesian analysis (Huelsenbeck and Ronquist, 2001) using TOPALI v.2.5 (Milne *et al*., 2004): JC: Jukes and Cantor (ITS and *endoPG*), TrN plus Gamma (*tub2*), HKY85+I; Hasegawa, Kishino, and Yano, 1985 plus invariable sites (*H3*), and K81+I+G: Kimura, 1981 plus invariable sites plus Gamma (concatenated tree). The Bayesian analysis was performed discarding the first 25% of the sampled trees as burn-in phases, and the successive probabilities were estimated from the remaining trees (Ronquist *et al*., 2009).

In vitro *sensitivity testing of* Alternaria *spp. to azoxystrobin or boscalid*

The fungicides azoxystrobin (Ortiva®, 250 g L^{-1} a.i., Syngenta Italia S.p.A.) and boscalid (Cantus, 50% a.i., BASF Italia S.p.A.), each at concentrations of 0.1, 0.3, 1, 3, 10, 30, 100 or 300 mg L^{-1} of active ingredient, were used in Petri plate sensitivity assays. Salicylhydroxamic acid (SHAM, Sigma-Aldrich) was added to the medium at a final concentration of 100 mgL^{-1}, to prevent fungi from starting an alternative respiration process and to suppress resistance due to alternative oxidase (Kim *et al*., 2003; Pasce *et al*., 2004).

The effects of azoxystrobin and boscalid on the spore germination of different *Alternaria* isolates were evaluated on selective medium of corn meal agar (CMA, Sigma-Aldrich, 17 g L^{-1}) amended with streptomycin sulphate at 0.025 mg L^{-1} (AppliChem). Petri dishes containing CMA amended with antibiotic without fungicide, with or without SHAM, were used as experimental controls. Assay inoculum consisted of conidia gently scraped from a culture of each isolate grown on V8 medium

Table 1. *Alternaria* spp. isolates characterized by means of four molecular loci.

No.	Isolate	Host	Source	Location	ITS	*tub2*	*H3*	*endoPG*	Species
1	Cav 2/10	Cauliflower	Leaf	Italy	MH936379	*KT920427*	MK239196	MK140907	*A. alternata*
2	Cav 3/10	Cabbage	Leaf	Italy	MH936380	*KT920426*	MK239197	MK140908	*A. alternata*
3	Cav 4/10	Cauliflower	Leaf	Italy	MH936381	MK044808	MK239198	MK140909	*A. alternata*
4	Cav 5/10	Cabbage	Leaf	Italy	MH936382	*KT920423*	MK239199	MK140910	*A. alternata*
5	Cav 6/10	Cabbage	Leaf	Italy	MH936383	MK044809	MK239200	MK140911	*A. alternata*
6	Cav 7/10	Cabbage	Leaf	Italy	MH936384	*KT920425*	MK239201	MK140912	*A. alternata*
7	Cav 9/10	Cauliflower	Leaf	Italy	MH936385	MK044810	MK239202	MK140913	*A. alternata*
8	Cav 12/10	Cauliflower	Leaf	Italy	MH936386	*KT920424*	MK239203	MK140914	*A. alternata*
9	Cav 15/10	Cabbage	Leaf	Italy	MH936387	*KT920428*	MK239204	MK140915	*A. alternata*
10	Ruc 1/10	Cultivated rocket	Leaf	Italy	MH936388	*KJ909926*	MK239205	MK140916	*A. alternata*
11	Ruc 3/10	Wild Rocket	Leaf	Italy	MH936389	MK044811	MK239206	MK140917	*A. alternata*
12	Ruc 4/10	Wild Rocket	Leaf	Italy	MH936390	*KT920413*	MK239207	MK140918	*A. alternata*
13	Ruc 5/10	Wild Rocket	Leaf	Italy	MH936391	*KT920412*	MK239208	MK140919	*A. alternata*
14	Ruc 7/10	Wild Rocket	Leaf	Italy	MH936392	MK044812	MK239209	MK140920	*Alternaria* sp.
15	Ruc 8/10	Cultivated rocket	Leaf	Italy	MH936393	MK044813	MK239210	MK140921	*A. alternata*
16	Ruc 9/10	Cultivated rocket	Leaf	Italy	MH936394	*KT920411*	MK239211	MK140922	*A. alternata*
17	Ruc 10/10	Cultivated rocket	Leaf	Italy	MH936395	MK044814	MK239212	MK140923	*A. alternata*
18	Ruc 12/10	Cultivated rocket	Leaf	Italy	MH936396	*KT920417*	MK239213	MK140924	*A. alternata*
19	Ruc 13/10	Cultivated rocket	Leaf	Italy	MH936397	*KT920416*	MK239214	MK140925	*A. alternata*
20	Ruc PMP 4	Cultivated rocket	Seed	Italy	MH936399	KT920419	MK239216	MK140927	*Alternaria* sp.
21	Ruc PMP 8	Cultivated rocket	Seed	Italy	MH936398	KT920420	MK239215	MK140926	A. alternata
22	Ruc PMP 9	Cultivated rocket	Seed	Italy	MH936400	KT920418	MK239217	MK140928	*A. alternata*
23	Ruc PMP 12	Cultivated rocket	Seed	Italy	MH936401	KT920422	MK239218	MK140929	*A. alternata*
24	Ruc PMP 19	Cultivated rocket	Seed	Italy	MH936402	*KT920421*	MK239219	MK140930	*A. alternata*
25	Bas 1/10	Basil	Leaf	Italy	MH936403	*MF070269*	MK239220	*MF070304*	*A. alternata*
26	Bas 2/10	Basil	Leaf	Italy	MH936404	*MF070270*	MK239221	*MF070305*	*A. alternata*
27	Bas 4/10	Basil	Leaf	Italy	MH936405	MK044815	MK239222	MK140931	*A. alternata*
28	Bas 5/10	Basil	Leaf	Italy	MH936406	MK044816	MK239223	MK140932	*A. alternata*
29	Bas 6/10	Basil	Leaf	Italy	MH936407	*MF070271*	MK239224	*MF070306*	*A. alternata*
30	Bas G1	Basil	Seed	Italy	MH936408	*MF070272*	MK239225	*MF070307*	*A. arborescens*
31	Bas BIO 10	Basil	Seed	Italy	MH936409	MK044817	MK239226	MK140933	*Alternaria* sp.
32	Bas BIO 11	Basil	Seed	Italy	MH936410	MK044818	MK239227	MK140934	*Alternaria* sp.
33	Bas 4-1BA	Basil	Seed	Italy	MH936411	MK044819	MK239228	*MF070295*	*A. alternata*
34	Bas 18-1BA	Basil	Seed	Italy	MH936412	MK044820	MK239229	MK140935	*A. alternata*
35	Bas 23-1BA	Basil	Seed	Italy	MH936413	*MF070261*	MK239230	*MF070294*	*A. alternata*
36	Bas 27-1BA	Basil	Seed	Italy	MH936414	*MF070259*	MK239231	*MF070292*	*A. alternata*
37	EGS 34015	*Dianthus* sp.	-	The UK	*AF347032*	*MF070252*	-	*KP124026*	*A. alternata*
38	EGS 34016	Peanut	-	India	*AF347031*	*MF070244*	-	*JQ811978*	*A. alternata*
39	CBS124274	Cherry	Fruit	Denmark	*KP124413*	*MF070253*	-	*MF070287*	*A. arborescens*
40	CBS124278	Cherry	Fruit	Denmark	*KP124374*	*MF070256*	-	*MF070290*	*A. alternata*

GenBank accession numbers obtained from Woudenberg *et al.* (2015) and Siciliano *et al.* (2018) are shown in italics.

(100 mL Campbell's V8 juice, 1.5 g $CaCO_3$, 15 g of agar, 900 mL distilled water) using a sterile scalpel. Conidia were mixed into 4 mL of sterile distilled water containing 0.1% Tween-20 (VWR International), and adjusted to 10^4 conidia mL^{-1}. The conidium suspension of each isolate (100 µL) was spread on each fungicide-amended plate. The plates were then placed in the dark at 22±1°C for 4 to 6 h, and the germination of 100 conidia in each plate was assessed under a microscope. Germination of each conidium was defined as the presence of a germ tube at least half the length of the conidium. Plates were arranged in a completely randomized design with three treatment replicates per trial. The experiments were performed three times per each isolate.

Conidium germination for each fungicide concentration (Gf) was compared with the germination for the controls (Gc). The percent of germination inhibition (GI) was calculated as: % GI = (Gc – Gf / Gc) × 100. EC_{50} values (concentrations giving 50% inhibition) were calculated using the log/logit dose response relation of the GraphPadPrism® software (version 7.02; La Jolla, CA, USA). A log fungicide concentration versus normalized response-variable method was calculated as: Y = Bottom + (Top-Bottom) / {1 + 10 [($LogEC_{50}$-X) × HillSlope]}, where Y refers to the response (GI) and X indicates the fungicide concentration.

The *A. alternata* isolates were divided into four groups according to their sensitivity to azoxystrobin or boscalid. An isolate was considered sensitive (S) if EC_{50} was between 0 and 1 μg mL^{-1}, with reduced sensitivity (RS) if EC_{50} was between 1 and 15 μg mL^{-1}, intermediate-resistant (IR) for EC_{50} of between 15 and 100 μg mL^{-1}, and resistant (R) for EC_{50} > 100 μg mL^{-1} (Avenot *et al*., 2008b).

Cross-resistance relationships between the two classes of respiration inhibitor fungicides were assessed by regression analysis (regression coefficient r^2), where log EC_{50} values of the individual isolates were compared for boscalid × azoxystrobin pairs.

Molecular characterization of the cytochrome b gene

The portion of the *cytb* gene of eighteen *Alternaria* strains was amplified with the cytb2f (5'-CTA TGG ATC TTA CAG AGC AC-3') and DTRcytb2-INTr (5'-GTA TGT AAC CGT CTC CGT C-3') primers (Vega *et al*., 2012). The PCR cycling conditions included an initial denaturing step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR products were purified and sequenced as described above. Using the distance-based matrix of the *cytb* gene, a Principal Coordinate Analysis (PCoA) was carried out by GenAlEx 6.502 software (Peakall and Smouse, 2012) to analyze the genetic structure among *Alternaria* subpopulations (sensitive or resistant to azoxystrobin).

Molecular characterization of the SdhB *and* SdhC *genes*

Amplification of the *SdhB* gene was performed with the SdhBF6 (5'-AAGGAAGATCGCAAGAAGCTC-3') and SdhBR6 (5'-AAT GGC TAG CGC AGG GTT CA-3') (Avenot *et al*., 2008a) primers, and the *SdhC* gene was amplified with the SdhC-(A-G)F1 (5'-CAC CTG GCC ATC TAC AAG C-3') and SdhC-(A-G)R1 (5'-TGG TTC TTG AAA CCA ATA CCG-3') primers (Avenot *et al*., 2009). The PCR conditions for both genes were as follows: an initial denaturing step at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 51°C for 50 s, an extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR products were purified and sequenced as described above. A Principal Coordinate Analysis (PCoA) based on the concatenated *SdhB* and *SdhC* genes was performed to genetically distinguish between *Alternaria* subpopulations (sensitive or resistant to boscalid).

RESULTS

Molecular identification and phylogenetic analyses

During the 2013–2017 period, different leafy vegetable plants (2–5 months old) showed severe leaf spot symptoms in different areas of northern Italy. On the basis of morphological observations, the isolates obtained from infected plant tissues mainly belonged to small-conidium *Alternaria* species. Thirty-six *Alternaria* isolates were collected and subjected to molecular characterization. The ITS, *tub2, endoPG* and *H3* sequences of these isolates were compared with those available at NCBI, and all gave the greatest similarity with *A. alternata* and *A. arborescens* (98–100%). The only exception was the ruc PMP 4 isolate from cultivated rocket, which showed greatest similarity to *A. brassicicola* in ITS and the *H3* gene (respectively, 96 and 99%). Since the single gene sequence analyses were not conclusive in identifying *Alternaria* sp., four loci-phylogenetic analyses were carried out.

Phylogenetic analyses were performed on gene portions of 400–500 bp for ITS, *H3* and *endoPG,* and 700 bp for *tub2*. A concatenated tree, based on these genes, was used to study the genetic diversity of 36 tested *Alternaria* strains isolated from five vegetable hosts, together with four reference strains (Table 1). One main cluster, divided into two sub-clusters, was observed (Figure 1); the first sub-cluster grouped 31 strains together with two reference *A. alternata* strains (EGS 34015 and EGS 34016); the second sub-cluster grouped three strains, which gave the greatest similarity (99–100%) with *A. arborescens* in *tub2* and *endoPG* genes (ruc 7/10, bas BIO 10, and bas G1 strains). The reference *A. arborescens* strain CBS124274 was outside the main cluster. One minor cluster contained two *Alternaria* strains (ruc PMP 4 and bas BIO 11) that were not grouped together with the *A. alternata* or *A. arborescens* strains. No specific grouping of the strains was observed that was related to a plant host or source of

the isolation. Moreover, moderate intraspecies molecular diversity was observed for *A. alternata*, to which belonged a major number of identified strains with several phylogenetic subgroups from various hosts. The Bayesian consensus tree for four loci agreed with the tree topologies obtained from the ML analyses (Supplementary data, Figure 1). Furthermore, the phylogenetic analysis based only on the *endoPG* gene, as suggested by Woudenberg *et al.* (2015) for better separation of *A. alternata* and *A. arborescens*, again clustered the ruc 7/10, bas BIO 10 and bas G1 strains together with three reference *A. arborescens* and a few additional *A. alternata* strains (Figure 2).

Figure 1. Phylogenetic relationships of *Alternaria* spp. based on ITS, *tub2*, *endoPG* and *H3* sequences. The concatenated phylogenetic tree was obtained from a Maximum Likelihood analysis using a Tamura Nei model. The name and host affiliation are indicated for each strain. Reference isolates of *A. alternata* and *A. arborescens* (Woudenberg *et al.*, 2015) are shown in bold.

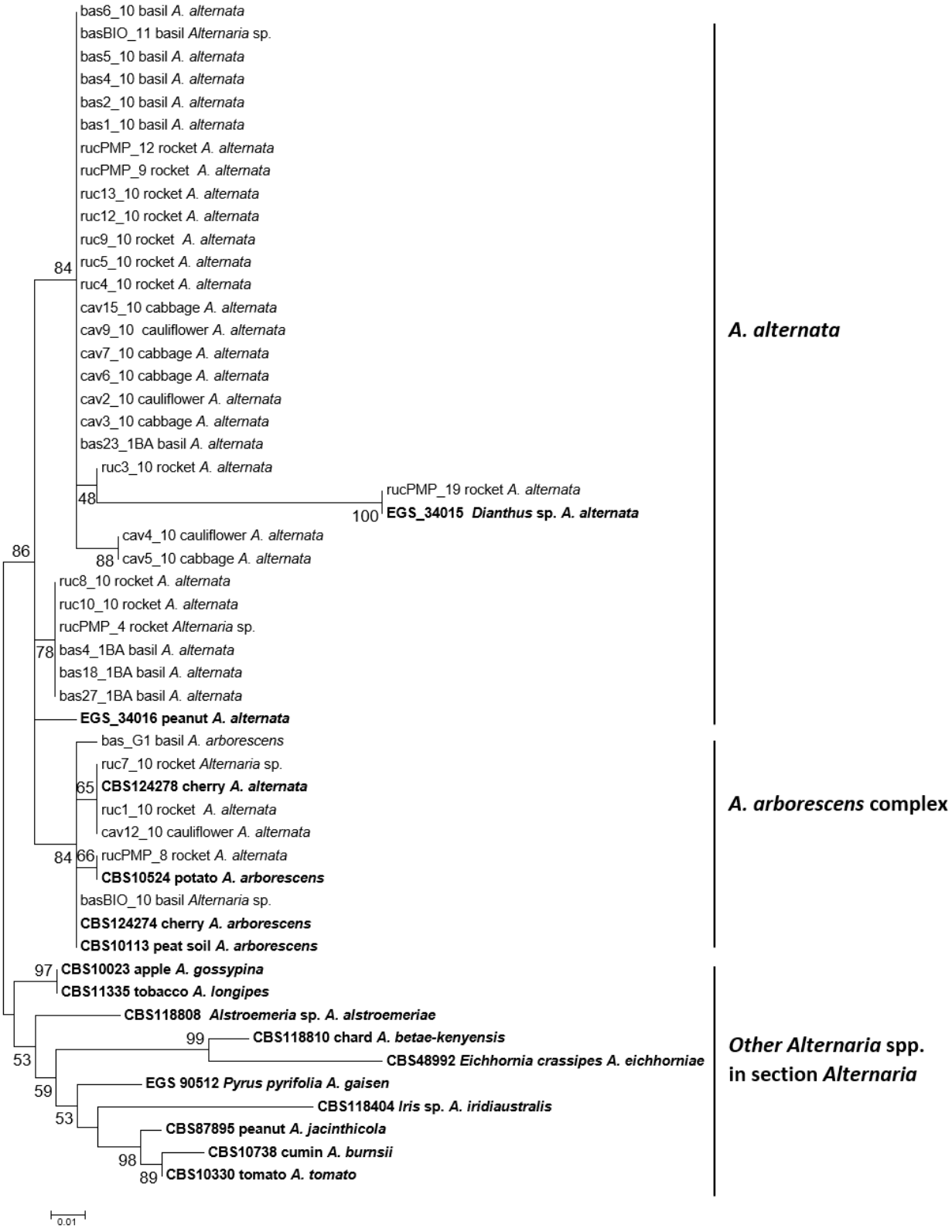

Figure 2. Phylogenetic relationships of the *Alternaria* species and the *A. arborescens* species complex within an *Alternaria* section based on *endoPG* sequences. The phylogenetic tree was obtained from a Maximum Likelihood analysis. The name, fungal species and host affiliation are indicated for each strain. Reference isolates (Woudenberg *et al*., 2015) are shown in bold.

Table 2. Sensitivity to azoxystrobin and boscalid of *Alternaria* spp. obtained from different hosts.

Isolate	Host	Species	Azoxystrobin EC_{50} (mg/L)			Boscalid EC_{50} (mg/L)			
			S	RS	IR	S	RS	IR	R
Cav 2/10	Cauliflower	*A. alternata*	0.01			0.06			
Cav 3/10	Cabbage	*A. alternata*	0.12				3.91		
Cav 4/10	Cauliflower	*A. alternata*	0.12					29.79	
Cav 5/10	Cabbage	*A. alternata*	0.04			0.54			
Cav 6/10	Cabbage	*A. alternata*	0.86				1.74		
Cav 7/10	Cabbage	*A. alternata*	0.06			0.34			
Cav 9/10	Cauliflower	*A. alternata*	0.86			0.14			
Cav12/10	Cauliflower	*A. alternata*	0.19			0.24			
Cav15/10	Cabbage	*A. alternata*	0.19				8.69		
Ruc 1/10	Cultivated rocket	*A. alternata*			30.42				134.4
Ruc 3/10	Wild Rocket	*A. alternata*	0.59				3.02		
Ruc 4/10	Wild Rocket	*A. alternata*	0.003				2.74		
Ruc 5/10	Wild Rocket	*A. alternata*	0.40			0.04			
Ruc 7/10	Wild Rocket	*Alternaria* sp.	0.13			0.04			
Ruc 8/10	Cultivated rocket	*A. alternata*	0.19			0.04			
Ruc 9/10	Cultivated rocket	*A. alternata*		2.42			6.48		
Ruc 10/10	Cultivated rocket	*A. alternata*		9.76			8.69		
Ruc 12/10	Cultivated rocket	*A. alternata*	0.20						153.2
Ruc 13/10	Cultivated rocket	*A. alternata*	0.43				1.04		
Ruc PMP 4	Cultivated rocket	*Alternaria* sp.		1.07			4.81		
Ruc PMP 8	Cultivated rocket	*A. alternata*	0.01			0.05			
Ruc PMP 9	Cultivated rocket	*A. alternata*	0.16			0.27			
Ruc PMP 12	Cultivated rocket	*A. alternata*	0.38				5.93		
Ruc PMP 19	Cultivated rocket	*A. alternata*	0.21				1.67		
Bas 1/10	Basil	*A. alternata*	0.11					38.85	
Bas 2/10	Basil	*A. alternata*	0.46				10.71		
Bas 4/10	Basil	*A. alternata*	0.61			0.46			
Bas 5/10	Basil	*A. alternata*	0.08				6.58		
Bas 6/10	Basil	*A. alternata*	0.02						102.8
Bas G1	Basil	*A. arborescens*	0.06				2.79		
Bas BIO 10	Basil	*Alternaria* sp.	0.02			0.20			
Bas BIO 11	Basil	*Alternaria* sp.		1.31			1.86		
Bas 4-1BA	Basil	*A. alternata*	0.007			0.11			
Bas 18-1BA	Basil	*A. alternata*	0.03			0.41			
Bas 23-1BA	Basil	*A. alternata*	0.10				1.44		
Bas 27-1BA	Basil	*A. alternata*	0.05			0.08			
Mean EC_{50}			0.22±0.02	3.64±0.44	30.42±2.93	0.23±0.02	4.51±0.38	34.32±2.31	130.13±15.67
EGS 34015*	*Dianthus* sp.	*A. alternata*	0.02				4.59		
EGS 34016*	Peanut	*A. alternata*	0.15			0.65			
CBS124274*	Cherry	*A. arborescens*	0.04			0.27			
CBS124278*	Cherry	*A. alternata*	0.05			0.11			

*Reference strains CBS.
S = sensitive isolates; RS= isolate with reduced sensitivity; IR = intermediate resistant isolates; R = resistant isolates.

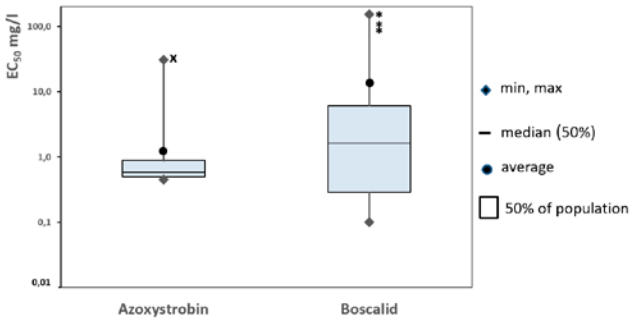

Figure 3. Sensitivity distribution (EC_{50}) of *Alternaria* spp. populations to azoxystrobin and boscalid. Reference isolates are included in the analyses. *Alternaria alternata* strain ruc 1/10 with intermediate resistance to azoxystrobin is indicated by a cross, and *A. alternata* strains (ruc 1/10, ruc 12/10, and bas 6/10) resistant to boscalid are shown by stars. The sensitivity distribution is plotted on a log EC_{50} scale.

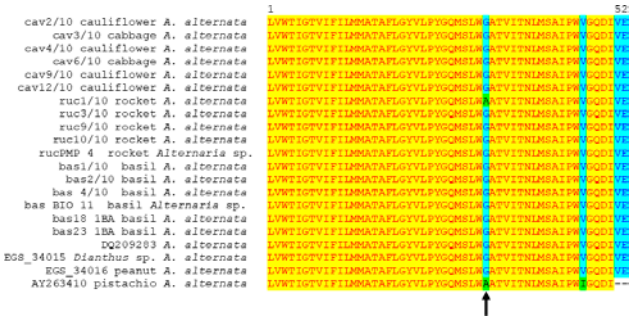

Figure 4. Amino acid alignment of the partial cytochrome b coding region. The eighteen studied and four reference *A. alternata* strains are shown (Ma *et al.*, 2003; Grasso *et al.*, 2006; Vega *et al.*, 2012). Amino acid position 143, where glycine (GGT) was substituted with alanine (GCT) in the ruc 1/10 strain, is indicated by the arrow.

Sensitivity of Alternaria *spp. to azoxystrobin or boscalid*

Thirty-six *Alternaria* isolates, originating from five vegetable hosts (and the four reference strains of *A. alternata* and *A. arborescens*), were evaluated in spore germination assays to establish their sensitivity to azoxystrobin (Table 2). Most of the isolates were sensitive to azoxystrobin, but four *Alternaria* isolates showed reduced sensitivity (mean EC_{50} = 3.64), and one isolate showed intermediate resistance (EC_{50} = 30.42). The four reduced sensitivity isolates had resistance factors of 17, and that for the intermediate resistance isolate was 138, compared to the sensitive isolates. The sensitivity range (between the most and the least sensitive isolate) was 122-fold, with a non-continuous sensitivity distribution of the isolates.

The *Alternaria* isolates were also tested for their sensitivity to boscalid (Table 2). Fifteen isolates (42%) were sensitive with a mean EC_{50} of 0.23. Sixteen isolates (44%) showed reduced sensitivity to boscalid (mean EC_{50} = 4.51), two isolates (6%) showed intermediate resistance (mean EC_{50} = 34.32), and three isolates (8%) were resistant (mean EC_{50} = 130.13). The sensitivity range was 14-fold, and this was narrower than the range for azoxystrobin, showing a more continuous sensitivity distribution of the isolates.

The box and whiskers plots showed high sensitivity variations in the 50% box of the population for sensitivity to azoxystrobin, and high maximum whiskers values for boscalid (Figure 3). The median line of the *Alternaria* spp. populations was less (EC_{50} < 1 mg L^{-1}) for azoxystrobin compared to that of boscalid. The sensitivity of the *A. alternata* isolate ruc 1/10 was outside the 50% box for azoxystrobin, and of the *A. alternata* isolates ruc 1/10, ruc 12/10, and bas 6/10 was outside the 50% box for boscalid, as well.

No cross-resistance was observed between azoxystrobin and boscalid (Supplementary data, Figure 2). A weak correlation (r^2 = 0.27) indicated that the isolates showing reduced sensitivity to both fungicides possessed a double resistance mechanism.

Molecular characterization of the cytb *gene*

Eighteen *Alternaria* strains (including the strain ruc 1/10 with intermediate resistance to azoxystrobin) were amplified in the *cyt b* region, in which the azoxystrobin resistance-associated mutation (G143A) was reported in *A. alternata* (Ma *et al.*, 2003). One intron was found, starting at position 164 aa (S164), after the T of the codon encoding for serine (TCA) in all of the sequenced strains (data not shown), as reported for other *A. alternata* strains (Vega *et al.*, 2012). Out of 18 strains, only the ruc 1/10 strain from cultivated rocket showed the cytb mutation at position 143 aa (glycine to alanine, G143A) (Figure 4). The rest of the cytb sequence was identical in all of the strains (either sensitive or with reduced sensitivity), with the exception of two nt polymorphisms in the exon region in the rocket ruc PMP 4 strain. The intron region was very similar to that of the group of *A. alternata* citrus strains reported by Vega *et al.* (2012), while it was different from those reported by Grasso *et al.* (2006).

It was not possible to distinguish between the sensitive *Alternaria* spp. subpopulation and the subpopulation with reduced sensitivity, based on the observed *cytb* nt polymorphisms and PCoA analyses (Supplementary data, Figure 4a). The only exceptions were the ruc 1/10 strain with intermediate resistance to azoxystrobin and

the cytb G143A mutation, and the ruc PMP 4 strain with reduced sensitivity to azoxystrobin. Both of these strains were distant from the rest of the analyzed strains.

Molecular characterization of the SdhB *and* SdhC *genes*

Eighteen strains (including boscalid-resistant strains and strains with reduced sensitivity) were sequenced in the portions of the *SdhB* and *SdhC* genes known to be related to boscalid resistance (Avenot *et al*., 2008a). Two synonymous mutations were found in the cauliflower cav 2/10 and rocket ruc 1/10 strains, while the basil bas BIO 10 strain showed only a few nt polymorphisms in both the *SdhB* and *SdhC* genes. However, no amino acid mutations, including the mutations related to boscalid resistance in *SdhB* (H277Y, H277R) and *SdhC* (H134R), were observed in either of the proteins (Supplementary data, Figure 3).

Identified *SdhB* and *SdhC* nt polymorphisms were not able to differentiate the subpopulations (sensitive, with reduced sensitivity, with intermediate resistance, or resistant) by PCoA analyses, and the resistant subpopulation was grouped together with sensitive subpopulations (Supplementary data, Figure 4b).

DISCUSSION

Alternaria sect. *Alternaria* comprises approx. 60 host-specific and small-conidium *Alternaria* species, which affect plants, animals and humans (Woudenberg *et al*., 2013). Recent genome and transcriptome studies of different *Alternaria* morphospecies have indicated that *Alternaria* sect. *Alternaria* contained only 11 phylogenetic species and one species complex (*A. arborescens* species complex), which are genetically very similar (97–98 % of the full-genome similarity; Woudenberg *et al*., 2015). Thirty-five morphospecies, which are indistinguishable according to multi-gene phylogeny, have also been synonymized as *A. alternata* (Woudenberg *et al*., 2015). Owing to the high genetic similarity, molecular characterization of the *Alternaria* sect. based on one locus is inconclusive, and is not sufficient to differentiate the small-conidium *Alternaria* species within this section. Thus, *A. alternata* cannot be differentiated from *A. arborescens* on the basis of only single markers, such as ITS, *tub2*, SSU, LSU or *gapdh* (Lawrence *et al*., 2013; Woudenberg *et al*., 2015). Multi-locus phylogenetic studies are widely used for the molecular characterization and better separation of *Alternaria* spp. (Woudenberg *et al*., 2015; Siciliano *et al*., 2018; Nishikawa and Nakashima, 2019). However, expanded multi-gene phylogenetics, in which the most diverse genes selected from the comparison of the whole-genome *Alternaria* sequences are considered, is not always sufficient to differentiate all of the *Alternaria* phylogenetic species in *Alternaria* sect. *Alternaria*. The markers such as *rpb2*, *tef1*, OPA10-2, *Alt a 1*, *endoPG*, KOG1058 and KOG1077 suggested by Woudenberg *et al*. (2015), and ATPase and cmdA used by Zhu and Xiao (2015), should permit differentiation of *A. alternata* and *A. arborescens*. This could be combined with morphological characteristics of conidium formation and culture, and a *TaqI* restriction site in the *endoPG* gene, as a specific marker for differentiation of *A. alternata* and *A. arborescens* (Andrew *et al*., 2009; Woudenberg *et al*., 2015; Ozkilinc and Sevinc, 2018).

Thirty-six *Alternaria* strains, isolated from leaf spot diseased plants of cauliflower, cabbage, cultivated rocket, wild rocket and basil, were characterized on a molecular basis in this study. Four commonly used markers for *Alternaria* sp. differentiation (ITS, *tub2*, *endoPG* and *H3)* were used for a multi-locus phylogenetic analysis. Some of these isolates had been characterized in a previous study, but not on the basis of all of these four loci (Siciliano *et al*., 2017; 2018). The present analysis showed that the majority of the strains were *A. alternata*. The ruc 7/10 and bas BIO 10 strains were grouped together with the bas G1 strain, which was identified by five other genes as *A. arborescens* in a study by Siciliano *et al*. (2018). These three strains also showed the TCGA sequence (*TaqI* restriction site), specific for *A. arborescens* (Ozkilinc and Sevinc, 2018). Based on morphological characteristics, the ruc 7/10, bas BIO 10 and bas G1 strains were also similar to each other, exhibiting ovate conidia (10.8 to 34.2 × 6.1 to 14.9 µm) and dark green-gray colonies (data not shown). In order to confirm the identification of the ruc 7/10 and bas BIO 10 strains as *A. arborescens*, it will be useful to include more molecular markers in future studies.

Two strains (ruc PMP 4 and bas BIO 11) were outside the main cluster that included the *A. alternata* and *A. arborescens* strains. These two strains need more profound molecular analyses in which other *Alternaria* sections should be included, since they were found to be more phylogenetically distant from all of the rest of the studied strains. Compared to the work of Siciliano *et al*. (2017), which was only based on the *tub2* gene, it was found that the strain ruc 1/10 from cultivated rocket was *A. alternata* instead of *A. japonica*. With respect to the work of Siciliano *et al*. (2018), which was based on seven genes, the basil strains were all confirmed as the same *Alternaria* spp.

Moderate molecular diversity with subgroup structuring to different plant hosts and isolation sources (seeds and leaves) was observed among the strains of *A. alternata*, which suggests non-recent introduction of the

pathogen into new areas and subsequent emergence of leaf spot diseases. More probably, a new disease outbreak is associated with the seed transmission of *A. alternata*, with globalization of the seed market and introduction of new agricultural practices (Rotem, 1994; Gullino *et al*., 2014; Mangwende *et al*., 2018). High percentage of seed contamination has been found for basil and rocket (respectively, 7% and 0.4% of non-disinfected seeds) (Gilardi *et al*., 2013a; 2015a). The pathogen can also be spread by airborne conidia (Simmons, 2007), that could explain the appearance of *A. alternata* on new ornamental hosts in Northern Italian areas close to the cultivation zones of leafy vegetables (Garibaldi *et al*., 2018a, 2018b, 2018c). Recent outbreaks of *A. alternata* on ornamental hosts should be investigated to determine if airborne inoculum came from leafy vegetable crops.

Emerging Alternaria leaf spot disease in Italy is predominantly controlled by two respiration inhibitor fungicide classes; QoIs using azoxystrobin, and SDHIs using boscalid. Based on the genetic diversity data obtained in the present study, indicating that introduction of the causative pathogen into Northern Italy has probably been non-recent, and its presence on other hosts previously treated with QoI and SDHI fungicides, the further objective of this study was to evaluate the sensitivity to azoxystrobin and boscalid of *Alternaria* strains isolated from vegetable hosts affected with this emerging disease. Soon after the first description of QoI resistance in the plant pathogen *B. graminis* f. sp. *tritici* (Sierotzki *et al*., 2000), QoI resistance also occurred in *A. alternata* on several vegetable and cereal crops, including pistachio, apple, citrus, potato and tomato, in different countries (Ma *et al*., 2003; FRAC, 2016; Duba *et al*., 2018). An amino acid change from glycine to alanine at 143 aa (G143A) has been reported in the majority of *A. alternata* strains resistant to azoxystrobin (Ma *et al*., 2003). In the present study, a low proportion (3%) of the azoxystrobin resistant *Alternaria* strains was found in the conidium germination assays. Only one *A. alternata* strain originating from cultivated rocket (ruc1/10 strain) was resistant to azoxystrobin. This is the first report in Italy of azoxystrobin resistance in *A. alternata* originating from leafy vegetable hosts. The reason why the G143A mutation was only found in the rocket ruc1/10 strain with intermediate resistance, and not in those strains with reduced sensitivity, could be related to the recent appearance of azoxystrobin resistance in leafy vegetable *A. alternata* strains, or to other mechanisms for this kind of resistance. The azoxystrobin resistance could be related to a recompense mechanism of the energy deficit caused by the fungicide, upstream of the NADH dehydrogenase in the respiratory chain, as has already been proposed for *Venturia inaequalis*. This is through modification of the alternative oxidase gene, or through a reduced accumulation of the bc1 inhibitor (Avila-Adame and Köller, 2002; Esser *et al*., 2014).

Alternaria strains were also tested to establish their sensitivity to SDHIs, represented by boscalid. The first descriptions of SDHI resistance in *A. alternata* were for isolates from pistachio in California, a few years after this fungicide was registered in the USA (Avenot and Michallides 2007; Avenot *et al*. 2008a, 2008b). This was followed by a report of boscalid resistance in *A. solani* from potato and *A. alternata* from peach in other USA states (Wharton *et al*., 2012; Yang *et al*., 2015). Four years later, boscalid resistance was found in *A. alternata* and *A. solani* populations from potato fields in Belgium (Landschoot *et al*., 2017). All of these reports associated resistance to boscalid with Sdh complex mutations, mainly with those in the *SdhB* or *SdhC* genes, or occasionally with *SdhD* mutation. However, in the present study, boscalid resistant strains isolated from leafy vegetable hosts did not show any aa mutation in SdhB or SdhC. This could mean that the boscalid resistance may be related to the *SdhD* gene or to some uncommon mutations in non-sequenced portions of the *ShB* and *SdhC* genes, which means further molecular studies are needed to verify these possibilities. It is also possible that the resistance in the evaluated *A. alternata* strains was governed by some other mechanism. There have been reports of SDHI resistance in other plant pathogens, such as *B. cinerea* from grapevine (Leroux *et al*., 2010), *Monilia fructicola* from peach, (Chen *et al*., 2013), *Pyrenophora teres* from barley (Wieczorek, *et al*., 2016) and *Zymoseptoria tritici* from wheat (Yamashita and Fraaije, 2018), which did not show any *SdhB* and *SdhC* mutations, or had some Sdh mutations present in both sensitive and resistant strains. The fungicide efflux membrane proteins, the ATP-binding cassette (ABC), and major facilitator superfamily (MFS) transporters (in *B. cinerea*), and nucleobase transporters (in *Aspergillus nidulans*), have been related to boscalid resistance and also to multi fungicide resistance (boscalid and other fungicides; Kretschmer *et al*., 2009; Leroux *et al*., 2010; Kalampokis *et al*., 2018).

In the present study, the ruc 1/10 strain with intermediate resistance to azoxystrobin was also resistant to boscalid, as has already reported for *A. alternata* (Avenot and Michailides, 2007; Avenot *et al*., 2008b; Landschoot *et al*., 2017). However, there was no cross-resistance between QoIs and SDHI in this isolate, suggesting the double resistance mechanism, and confirming the data of the previous study of *A. alternata* with double resistance (Malandrakis *et al.,* 2018).

Particular attention should be paid because QoI and SDHI fungicides can be formulated in combination products, and since multiple fungicide resistance is not associated with target gene alteration and provokes a wide spectrum of resistance (Leroux *et al*., 2010). Furthermore, the selection pressure exerted by both fungicide classes, used against other pathogens, such as *Peronospora belbahrii, Plectosphaerella cucumerina, B. cinerea* and *S. sclerotiorum* (Gilardi *et al*., 2013b; 2015b; Homa *et al*., 2014) should be considered for its effect on changes in sensitivity in *Alternaria* spp. originating from leafy vegetable hosts where pathogen populations already showed reduced sensitivity to both classes.

In conclusion, a small proportion of *Alternaria* strains identified from five vegetable crops were sensitive to azoxystrobin, while more than half of the strains showed reduced sensitivity or resistance to boscalid. These results are similar to recently reported resistance to pyraclostrobin and boscalid in *A. alternata* from tomato in Greece (Malandrakis *et al*., 2018). Resistance in the strains evaluated in the present study was not associated with commonly reported mutations, with the exception of one strain that was resistant to azoxystrobin. This aspect requires further investigation of the additional resistance mechanisms, with particular attention to fungicide efflux transporters. Adequate fungicide mixtures and rotations with chemicals with different modes of actions (particularly multi-site and eco-sustainable fungicides) may delay the development of single and double fungicide resistance in pathogen populations. This should be supported by improved management of Alternaria leaf spot disease on vegetable crops. This will include information on the sanitary status of seeds, host resistance of cultivars to these diseases, and appropriate choice of best agricultural practices.

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Research Paper

Characterization of *Phytophthora capsici* isolates from lima bean grown in Delaware, United States of America

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Summary. Pod rot of lima bean (*Phaseolus lunatus* L.), caused by the broad host range oomycete *Phytophthora capsici,* is an emerging threat to lima bean production in the mid-Atlantic region of the United States of America (USA). There is little known about survival and spread of this pathogen in the State of Delaware, an area of major lima bean production. Irrigation water was sampled in 2014 and 2015 for the presence of *P. capsici* using baiting methods. Over three seasons, isolations from water sources, weeds, and soil samples did not yield *P. capsici*. However, field samples from symptomatic lima bean, watermelon, muskmelon, pepper, pickling cucumber, and pumpkin yielded 64 *P. capsici* isolates. Characterization of the isolates showed that 42 were of the A2 mating type, 31 were sensitive to mefenoxam, 18 were intermediately sensitive, and four were insensitive to this fungicide. All isolates were pathogenic on the eight lima bean and two snap bean cultivars tested. Three EST-SSR markers, PCSSR19, PCN3, and PCN7, used in combinations of PCSSR19/PCN3 or PCSSR19/PCN7 were significantly associated with mefenoxam sensitivity. This study is the first of its kind in Delaware, providing key information as a basis for effective management of *P. capsici*, including mating type, mefenoxam insensitivity, host range, and survival.

Keywords. Fungicide resistance, oomycete, plant pathogen, mefenoxam.

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INTRODUCTION

The causal agent of pod rot of lima bean, *Phytophthora capsici* (Leonian 1922), infects members of at least 27 plant families, including vegetable crops in the Cucurbitaceae, Solanaceae, and Fabaceae, as well as conifers, weeds,

and tropical crops around the world (Erwin and Ribeiro, 1996; Davidson *et al.* 2002; Gevens *et al*., 2008; Roberts *et al*., 2008; Quesada-Ocampo *et al*., 2009; Granke *et al*., 2012). This heterothallic, hemi-biotrophic oomycete, belonging to the Peronosporales and Pythiaceae, causes root, stem, fruit and crown rot, foliar blight, and stunting on various hosts (Gevens *et al*., 2008; Quesada-Ocampo *et al*., 2016). The pathogen has a broad host range and can cause 50% crop losses in agro-ecosystems (reviewed in Sanogo and Ji, 2012). Lima bean (*Phaseolus lunatus* L.) and snap bean (*Phaseolus vulgaris* L.), however, are the only reported legumes affected by *P. capsici* (Davidson *et al*., 2002; Tian and Babadoost, 2003; Gevens *et al*., 2004). Lima bean is the cornerstone of the Delaware vegetable processing industry and a greater area is grown in the mid-Atlantic region (MAR) than elsewhere in the United States of America (USA). Approximately 5,600 ha of lima bean crops are planted in Delaware annually (https: //www.nass.usda.gov/Statistics_by_State/Delaware/index.php). Lima bean fields are often planted after an early season vegetable crop, such as peas or cucumbers. Planting susceptible vegetable crops in rotation or as a double crop ahead of lima bean in fields with previous histories of *P. capsici* may increase the risk of lima bean pod rot (Hausbeck and Lamour, 2004). Along with lima bean, snap bean crops are also grown in and around Delaware, often as warm season vegetable crops for autumn harvest. In 2017, 890 ha of snap bean crops were grown in Delaware (https: //www.nass.usda.gov/Quick_Stats/Ag_Overview/stateOverview.php?state=DELAWARE). Though there are reports of *P. capsici* affecting snap beans in other states of the USA (Gevens *et al*., 2008; McGrath and Dillard, 2011), occurrence of snap bean pod rot in Delaware has not yet been reported.

The asexual sporangia of *P. capsici* produce motile zoospores that may spread in irrigation water or rain (Ristaino *et al*., 1992; Granke *et al*., 2012). Zoospores may remain viable for hours or days in water (Roberts *et al*., 2005). Dispersal of *P. capsici* sporangia by wind is not frequent and the dispersal to other fields solely by wind is unlikely (Granke *et al*., 2009). Sexual oospores are produced in the presence of A1 and A2 mating types (MT), and oospores survive in soil for variable times (Babadoost and Pavon, 2013). oospores may survive in infested soil and plant debris for more than 5 years, resulting in infective propagules after crop rotations with non-host crops (Lamour and Hausbeck, 2001). This could result in significant genetic variation of outcrossing populations in the field (Lamour *et al*., 2012). Weeds may be alternative hosts in the absence of host crops, and host weeds found and reported in the eastern USA for *P. capsici* include black nightshade (*Solanum americanum*, *S. nigrum*), common purslane (*Portulaca oleracea)*, velvet leaf (*Abutilon theophrasti)*, and Carolina geranium (*Geranium carolinianum)* (Tian and Babadoost, 2003).

Studies on *Phytophthora capsici* have demonstrated broad genetic diversity, demonstrated with genetic fingerprinting and molecular markers (Hu *et al*., 2013; Lamour *et al*., 2012), with genetic clustering (Granke *et al*., 2012), and with physiological race testing in pepper in New Mexico (Glosier *et al*., 2008; Monroy-Barbosa and Bosland, 2011). These results indicate that physiological races exist in *P. capsici*. A physiological race is defined as "a subdivision of a pathogen species, particularly fungi, distinguished from other members of the species by specialization for pathogenicity in different host cultivars" (Kirk *et al*., 2001). Cultivar differentials are generally used to identify physiological races.

Management strategies for *P. capsici* include applications of the fungicide mefenoxam, which has been used widely for *Pythium* and *Phytophthora*. However, prolonged use of this compound has contributed to the emergence of fungicide insensitivity in *P. capsici* populations and some *Pythium* species (Brent and Hollomond 1998; Parra and Ristaino 1998; Weiland *et al*., 2014). Fungicide insensitivity in *P. capsici* to mefenoxam, hymexazol, cyazofamid, pyrimorph, and flumorph has been reported (Jackson *et al*., 2012; Pang *et al*., 2013, 2016; Jones *et al*., 2014). In the Mid-Atlantic region of the USA, mefenoxam insensitive isolates of *P. capsici* from lima bean were first reported in 2008 (Davey *et al*., 2008). Traditionally, mefenoxam sensitivity of an isolate is tested using *in vitro* assays with fungicide-amended media (Parra and Ristaino, 2001; Hausbeck and Lamour, 2004; Keinath, 2007). While these assays are still performed with *P. capsici* isolates (Qi *et al*., 2012; Ma *et al*., 2018), we wished to develop a molecular marker-based method to rapidly identify mefenoxam sensitivity.

In the last two decades, molecular markers have been used to identify specific traits or changing populations in *Phytophthora* species. Lamour and Hausbeck (2001) used amplified fragment length polymorphism (AFLP) markers to resolve population dynamics of a recombinant field population of *P. capsici*. Additionally, Pei-Qing *et al*., (2013) identified four expressed sequence tag, simple sequence repeat (EST-SSR) markers to unravel diversity in *P. capsici* populations in China. While Hu *et al*., (2014) reported a sequence characterized amplified region (SCAR) marker that can distinguish mefenoxam insensitive populations and sensitive populations of *P. nicotianae*, to date there are no reported markers capable of distinguishing between insensitive and sensitive *P. capsici* isolates.

Surface water sources used for irrigation in Michigan, Georgia, New York, and other states in the USA have been shown to carry *P. capsici* (Bush *et al*., 2003; Roberts *et al*., 2005; Wang *et al*., 2009; Gevens *et al*., 2007; Jones *et al*., 2014). Though the pathogen is not known to overwinter in irrigation water sources, water may aid in its spread. The objectives of the present study were to characterize *P. capsici* isolates collected from irrigation water sources, weed and crop hosts and soil samples from the states of Delaware and Maryland (USA) to (1) determine *P. capsici* mating types, mefenoxam insensitivity, host range, and dispersal; (2) develop molecular markers to distinguish mefenoxam sensitive isolates from insensitive isolates; and (3) detect the presence of other *Phytophthora* species. This information would aid development of effective disease management strategies. We undertook these studies in order to better understand this economically important, broad host range pathogen on an important crop in our region; if lima bean cannot be produced profitably in the MAR, other processing vegetables such as peas, snap beans, sweet corn and spinach, would not be produced, resulting in severe economic losses to the region (Evans *et al*., 2007).

MATERIALS AND METHODS

Sampling: baiting, infected field samples, water, weeds, and soil samples

Thirteen surface water sources including lakes, streams, and naturally-fed ponds were sampled in Kent and Sussex counties in Delaware during the summers of 2014, and 2015. Soil proximal to water sources, known potential *P. capsici* weed hosts, and infected fruits from grower fields were also sampled in the summers of 2014, 2015 and 2016. Baiting traps were each constructed by attaching a polyethylene foam cylinder (5.7 cm in diameter) to a mesh laundry bag (30 cm × 30 cm) with a zipper. Two unripe pears, one whole eggplant, and two cucumbers were placed in each trap as bait along with two rhododendron leaves. Fruits and leaves were surface-sterilized using 0.825% sodium hypochlorite (NaOCl) with two ≅10 µL drops of Tween 20 (Agdia Inc.) for 100 mL of the solution, for 2 min, and washed with sterile distilled water before adding to the bait bag. Bait bags were kept in the water for 4–6 d.

Numerous infected plant samples from 18 crop fields were collected or obtained from the University of Delaware Plant Diagnostic Clinic, from locations in Delaware and Maryland over the 3 year study. Extensive field sampling was carried out in one location in Bridgeville, Delaware (field 17, Table S1) in the summer of 2016. This field was planted with pickling cucumber (*Cucumis sativus*) early in the growing season, then double-cropped with lima beans later in the same growing season. At least 20-25 samples from different locations of this field were sampled to avoid sampling bias that could lead to non-recovery of isolates belonging to both mating types.

Twenty-six water samples (two 1 L bottles per source) were collected from 13 water sources. Two rhododendron leaves were added to one water bottle (1 L) and incubated in the dark. After 3 d, water was removed, and the leaves were washed with sterile distilled water and kept under moist conditions at 25°C for 3 d until lesions developed. The remaining water was vacuum filtered in 100 mL batches using 3.5 cm diam. (P5 Fisher Brand, 3 µm) filter papers. Filter papers were then placed face down on PARP-V8 selective medium (Ferguson and Jeffers, 1999) and incubated at 25°C in the dark for 3–5 d for colony development.

Two 15 g soil samples were collected near the edge of each surface water source. For each 15 g of soil, 200 mL of distilled water was added, mixed to uniformity, and then filtered through several layers of cheesecloth to remove soil and other debris. Filtered water samples were then processed using the vacuum filtration technique described above.

Roots of weeds near water banks were collected if the plants were previously reported as hosts for *P. capsici*. Carolina geranium (*Geranium carolinianum*) and common purslane (*Portulaca oleracea*) were the only weeds known to be hosts for *P. capsici* that were present near the water sources sampled. Roots were washed thoroughly with running tap water and surface sterilized as described previously.

All samples collected (except the water and soil samples) were rinsed in sterile distilled water and dried under ambient laboratory conditions in a biosafety hood. Tissues from margins of lesions from each of these samples were placed on PARP-V8 medium and incubated at 25°C in the dark for 3–5 d.

Morphological identification and generation of single zoospore cultures

Hyphal tips of potential *P. capsici* isolates growing on PARP-V8 medium were transferred to 60% strength potato dextrose agar (PDA; BD Difco) and unclarified V8 medium (600 mL distilled water, 163 mL unfiltered V8 juice, 12 g agar, 1.7 g $CaCO_3$) to examine isolate growth patterns. Sporangium formation was induced in 7–10 d-old cultures grown on unclarified V8 medium using Chen-Zentmeyer salt solution (Chen and Zent-

meyer, 1970), and the isolates were identified based on morphometric characteristics (Erwin and Ribeiro 1996; Gallegly and Hong 2008). Single zoospore cultures (SZC) were obtained from field isolates positively identified as *P. capsici* (morphologically and molecularly). Sporangium formation was induced on 10 d-old *P. capsici* isolates with Chen-Zentmeyer salt solution. After 18–20 h, the solution was decanted and 10 mL of sterile distilled water added. Plates were maintained at 4°C for 1 h followed by 15–30 min at room temperature, to induce release of zoospores. Twenty to 50 µL of each zoospore suspension was spread on a water agar plate, incubated at 25°C for 2–3 d, and then examined under a dissecting microscope. Three germinating zoospores per each hyphal-tipped field isolate were each transferred to a low strength PDA plate, and then to a V8 agar plate. Single zoospore cultures were stored long term in sterile distilled water within sterile screw-capped tubes each containing two hemp seeds and three cucumber seeds, and were maintained at 20°C.

DNA extraction

For each isolate tested, a 7 mm diam. plug of actively growing 7-d-old culture was added to 25 mL of lima bean broth (Calvert *et al.*, 1960) and incubated at 25°C for 3 d on an orbital shaker. Mycelial mats growing on broth were harvested using vacuum filtration through Whatman #1 filter paper. After removing the original colonized agar plugs, the mycelial mats were washed with sterile distilled water, lyophilized, and then kept in -80°C until processed. DNA was extracted with a Wizard Genomic DNA purification kit (Promega). DNA was dissolved in 100 µL of DNA rehydration solution and diluted to a final concentration of 25 ng μL^{-1}.

Molecular identification and primers

DNA samples were amplified using two sets of *P. capsici* specific primers (Zhang *et al.*, 2006, Lan *et al.*, 2013) to confirm identification. PCR reactions were carried out with a nested PCR protocol with universal ITS1/ITS4 for the first PCR round, and the primers PC-1 (5'-GTCTTGTACCCTATCATGGCG-3') and PC-2 (5'-CGCCACAGCAGGAAAAGCATT-3') for the second PCR round, as described by Zhang *et al.* (2006). The expected amplified product size was 560 bp. Isolates were also characterized using the *P. capsici* specific primers Pc1F (5'-GTATAGCAGAGGTTTAGTGAA-3') and Pc1R (5'-ACTGAAGTTCTGCGTGCGTT-3'), as described in Lan *et al.* (2013). The expected product size was 364 bp. A 25 µL PCR reaction mixture containing 2 µL of template DNA (25 ng μL^{-1}), 5 µL 5× LongAmp *Taq* Master Mix (New England Biolabs), 300 µM dNTPs, 0.4 µM primer, and 2.5 U LongAmp *Taq* DNA Polymerase (New England Biolabs) was used for marker amplification. The PCR amplification protocol used was as follows: initial denaturation at 94 °C for 10 min, 35 cycles of 94°C for 30 s, 56–60 °C for 30 s, 72°C for 30 s, followed by a final extension at 72°C for 5 min. PCR products were separated on a 2% agarose gel containing ethidium bromide in 10× Tris-Borate-EDTA buffer. Electrophoresis was carried out at a constant 58 V for 2 h and DNA bands were visualized under a UV trans-illuminator. A 100 bp DNA ladder (New England Biolabs) was used to determine amplicon sizes. DNA of isolates confirmed as *P. capsici* were then tested with one Inter-Simple Sequence Repeats (ISSR) marker and five SSR markers, selected from published *P. capsici* population markers, to identify polymorphic molecular markers using a subset of five isolates (Wang *et al.*, 2009, Pei-Qing *et al.*, 2013). One polymorphic marker reported in Pei-Qing *et al.* (2013), PCSSR19 (F-5'-GTCTTCGCTAAAGCCTCCG- 3', R- 5'-AGATGGCCAACAGCGGTTA-3'), showed co-segregation with mefenoxam sensitivity in our population, so this was utilized for further study. Polymorphism of a marker was initially tested with five isolates with varying mefenoxam sensitivity (two mefenoxam sensitive and three mefenoxam insensitive isolates). Markers showing possible co-segregation with mefenoxam sensitivity were further tested with a small subset of 18 isolates before screening all 64 isolates. PCSSR19 primers were searched using BLAST in Fungidb (http: // fungidb.org/fungidb/), and matched one predicated gene in *P. capsici* (PHYCA_548602T0), which when searched using BLAST in NCBI, was shown to be most similar (84% similarity at nucleotide level) to an RNA helicase from *P. infestans*. This coding sequence was then utilized to design additional primers pairs (designated PCN3 and PCN7), using the Primer3 program (Koressaar *et al.*, 2007; Untergasser *et al.*, 2012). The three primer pair sets PCSSR19, PCN3, and PCN7 were then tested on a small set of 18 isolates, and the amplification products were found to be polymorphic between sensitive and insensitive isolates. These three primer sets, PCN3 (F-5'-CGTGGCTTAACCAGTGTTCT-3', R-5'- GACGGTCATAACCACCGTAG-3'), PCN7 (F-5'-CGTTTTCCTACCGATTCCAA-3', R-5'-GACGCGGTACGTATGCAGAT-3'), and PCSSR19, were therefore used to screen all isolates. Conditions for PCR reactions were as follows: initial denaturation at 94°C for 10 min, 35 cycles of 94°C for 30 s, 56–60°C for 30 s, 72°C for 30 s, followed by a final extension at 72°C for 5 min. Products

of PCR were separated on a 4% agarose gel (low range ultra agarose; Biorad) containing ethidium bromide in 10× Tris-Borate-EDTA buffer (Fisher Scientific). Electrophoresis was conducted at a constant 58 V for 5 h, and DNA bands were visualized under a UV trans-illuminator. Isolates were screened with markers two additional times to confirm the correct banding pattern.

Mating type determination

Mating type of each *P. capsici* isolate was determined by pairing single-zoospore cultures with known isolates of *P. capsici*, [A1 (10193) or A2 (SP98) MT], as described by Gevens *et al.* (2007). A 7 mm plug of 7-d-old SZC of *P. capsici*, grown on a V-8 plate at 25°C, was placed 3 cm from a 7 mm plug of either the A1 or A2 MT tester isolate on a V8 plate, and incubated at 25°C in the dark. Plates were examined under a compound microscope for the presence of oospores after 14 to 21 d.

Mefenoxam sensitivity

Mefenoxam sensitivity was determined as outlined in Gevens *et al.* (2007). A 7 mm plug from an actively growing plate of SZC of *P. capsici* was placed on three, 100 mm diam. plates of V8 agar amended with 100 ppm of mefenoxam (Technical grade, or Ridomil Gold SL, 45.3%, Syngenta) and two V8 non-amended controls. The active ingredient in Ridomil Gold SL mefenoxam at 45.3%, with the remainder of the formulation being proprietary additives. Technical grade mefenoxam was dissolved in acetone and Ridomil Gold SL was dissolved in sterile distilled water prior to adding to the media cooled to the touch (approx. 55°C). Three SZC from each of the 64 *P. capsici* field isolates were tested with Ridomil Gold SL. After incubating plates at 23°C in the dark for 3 d, perpendicular colony diameters were measured, and percent average growth of an isolate was obtained by comparing growth on V8 plates with growth on V8 amended with mefenoxam. Isolates were rated as follows: sensitive isolates had <30% growth compared to the controls, intermediately insensitive isolates had 30-90% growth compared to the controls, and insensitive isolates had >90% growth compared to the controls, as described in Gevens *et al.* (2007). The experiment was repeated. All three of the SZC's from each isolate were observed and recorded for mefenoxam phenotype, because in several instances, one SZC differed from the other two. To account for these discrepancies, homogeneity of variance among SZC's from the two experiments were assessed with Bartlett tests. Statistical significance of the mefenoxam reactions between SZC was also tested using a Student's t test at $\alpha = 0.05$, using JMP Pro 13 (JMP®, Version *13*. SAS Institute Inc., 1989-2019). The correlations between mefenoxam sensitivity and MT were tested with the Pearson coefficient at $\alpha = 0.05$ also using JMP Pro 13.

Pathogenicity of Phytophthora capsici *to lima bean and snap bean*

Plants of the commercial lima bean cultivars 'Cypress', 'C- Elite Select', 'Fordhook (FH) 242', 'Bridgeton', 'Eastland', 'Maffei (M) 15', '8-78', and '184-85' were each inoculated with isolates of *P. capsici* collected during 2014-2016. A lima bean plant introduction PI477041 from Arizona, determined to have some resistance to *P. capsici* in field and greenhouse inoculations (N. Gregory, personal communication) was also included. Plants were grown under greenhouse conditions at 25°C with a photoperiod of 10 h and light intensity of 2500-3500 $\mu Em^{-2} sec^{-1}$ until pod set. Accession PI477041 was grown with a photoperiod of 12 h to induce flowers and pods. Young, flat pods were collected and challenged, as described by McGrath (2009) for snap bean pods, with slight modifications. Pods were surface sterilized using a 0.825% NaOCl solution (as above) and completely dried under a laminar flow hood. Two pods were placed on each Petri plate (100 × 15 mm Fisherbrand™) lined with a filter paper moistened with sterile distilled water. A 7 mm diam. plug of an actively growing *P. capsici* culture, grown on V8 plates for 7 d at 25°C, was placed on each pod. Plugs of V8 culture medium without the pathogen were placed on pods as experimental controls. Plates were sealed with Parafilm and incubated at 25°C and a 10 h photoperiod. Pods were assessed as susceptible or resistant 3 to 5 d post inoculation. *Phytophthora capsici* was re-isolated from symptomatic tissue placed on PARP-V8 selective media. The experiment was repeated twice. Two isolates identified as a *Pythium*/*Phytopythium*-like spp., isolated from water baits, were also tested on the differential group of lima bean cultivars, using the method described above, to determine whether they were pathogenic on lima bean.

Snap bean cultivars 'Provider' and 'Caprice' were inoculated with isolates of *P. capsici* under laboratory and greenhouse conditions. Thirteen *P. capsici* isolates out of the 64 total were tested on young snap bean pods in the laboratory, using the protocol adopted from McGrath (2009). Six pods were tested per isolate and the experiment was replicated once. Greenhouse testing was done for three of the 13 isolates used in the laboratory testing to observe *in planta* reactions. Lima bean culti-

vars 'M15' and '8-78' were used as positive controls, and greenhouse assays consisted of four plants of each cultivar per isolate. Isolates were grown on V8 agar plates for 14 d, which were subsequently flooded with sterile distilled water, with sporangia being dislodged with a glass rod. Pods were spray-inoculated with a spore suspensions (3×10^4 mL^{-1}) until run off and kept under moist conditions (plastic chamber on a greenhouse bench at 24°C with misting for 1 min every hour during daylight hours from 7 am to 7 pm) until symptom development. Control plants were sprayed with sterile distilled water. Symptoms were evaluated 4 an 12 d post-inoculation. Pods from a total of four replicated plants were examined, and rated as susceptible if they showed typical symptoms of *P. capsici* infections. This experiment was repeated once. Infections were confirmed by re-isolating *P. capsici* from inoculated and symptomatic tissues.

RESULTS

Isolate collection and identification

More than 200 microbial isolates were collected from irrigation water, soil samples, plant hosts, and weed hosts. However, none of the isolates recovered from direct sampling of irrigation water, baited fruits, leaf baits, soil samples, or roots of weed hosts were *P. capsici*. *Pythium*/*Phytopythium*-like isolates were mainly recovered from surface water sources in late August and early September. *Phytophthora capsici* isolates were recovered only from the infected plant material from crop fields. The numbers of *P. capsici* isolates collected from infected plant material from these fields were 22 in 2014, 16 in 2015, and 26 in 2016, for a total of 64 isolates. All the isolates were positively identified morphologically as *P. capsici*, and yielded the expected 560 and 364 bp bands, when amplified, respectively, with *P. capsici* specific primers PC1/PC2 (used in the nested PCR with ITS1/ITS4 primers) and Pc1F/Pc1R primers. Isolate information is presented in Table 1.

Mating type (MT) determination testing

Isolates belonging to both MT of *P. capsici* were recovered in all three years. Of the 64 *P. capsici* isolates recovered, 42 out of 63 field isolates were A2 MT, with 17 A2 MT isolates recovered from lima bean (Tables 1 and S1). Mating types A1 and A2 were recovered from four field locations in Delaware (fields 8, 12, 17, and 18), including the intensively sampled field in Bridgeville Delaware in the summer of 2016 (Table S1; field 17, 20 samples).

Pathogenicity on commercial lima bean and snap bean cultivars

Pathogenicity testing of *P. capsici* isolates showed all 64 isolates were virulent on all the commercial lima bean cultivars tested ('Cypress', 'C- Elite Select', 'Ford-

Table 1. Numbers of *Phytophthora capsici* isolates, their host sources, mating types, mefenoxam sensitivities, and locations, for isolates collected in the 2014, 2015 and 2016 field seasons.

Host	Number of isolates	Mating types		Mefenoxam sensitivity[a]					Location	
		A1	A2	S[b]	S-IS[c]	IS[d]	IS-I[e]	I[f]	DE[g]	MD[h]
Lima bean	27	10	17	12	2	9	2	2	20	7
Pumpkin	5	2	3	3	1	1	0	0	4	1
Pepper	4	0	4	2	1	1	0	0	3	1
Muskmelon	1	1	0	0	0	0	0	1	1	0
Watermelon	15	2	13	7	2	5	0	1	15	0
Pickling cucumber	12	7	5	7	3	2	0	0	12	0
Totals	64	22	42	31	9	18	2	4	55	9

[a] Mefenoxam sensitivity of field isolates are categorized based on the reaction of the three single zoospore cultures tested for each field isolate.
[b] Sensitive.
[c] Sensitive to Intermediately sensitive.
[d] Intermediately sensitive.
[e] Intermediately sensitive to Insensitive.
[f] Insensitive.
[g] Delaware.
[h] Maryland.

hook (FH) 242', 'Bridgeton', 'Eastland', 'Maffei (M) 15', '8-78', and '184-85'), evidenced by clear signs of white sporulation. Isolate PC67 is an accurate representation of susceptible interactions on these cultivars (Figure. 1). Controls showed no signs of infection or discolouration (Figure 1). Most isolates were pathogenic on PI477041, with the exceptions of isolate PC33 (no sporulation), PC61 and PC62 (reduced sporulation and browning), and PC51 and PC37 (reduced sporulation) (Figure S1). Two of the *Pythium/Phytopythium*-like isolates identified in this study were tested on pods of the lima bean cultivars, and were non-pathogenic, with the exception of isolate Phy4 on 'Eastland' (Figure S2). The two snap bean cultivars, 'Caprice' and 'Provider', were susceptible to the 13 isolates tested in the laboratory, and to a subset of three representative isolates tested in the greenhouse, showing similar signs of sporulation. Control pods inoculated with sterile distilled water showed no symptoms. *Phytophthora capsici* was re-isolated from the infected pods.

Mefenoxam sensitivity

Since the replicates were homogeneous between experiments (Bartlett test - F ratio = 0.0046, $P = 0.9954$), the results from both experiments were averaged to obtain the mefenoxam reaction for the isolates. Of the 64 field isolates tested, 31 were sensitive (S), 18 intermediately sensitive (IS), and four insensitive (I) to Ridomil Gold SL (Table 1). Single zoospore cultures derived from eleven isolates showed variability in their reaction to mefenoxam (Tables 1 and S1). Nine isolates showed S-IS phenotype (of these, six were statistically significant at $\alpha = 0.05$) while two isolates showed IS-I phenotype (neither were statistically significant at $\alpha = 0.05$) (Table S1). There was no significant correlation between the mefenoxam sensitivity and the mating type at $\alpha = 0.05$ (Pearson coefficient = -0.23 $P = 0.063$, Table S1). We wished to determine whether SZCs showed different responses to Ridomil Gold SL (45.3% mefenoxam) versus technical grade mefenoxam (97% ai). This assay showed that SZCs from 14 field isolates exhibited the same reactions while SZCs from several other field isolates gave variable reactions to both mefenoxam formulations (Table S2). For example, all three SZCs derived from two field isolates (PC33 and PC43) gave different reactions, while two out of three SZCs from one field isolate (PC34), and one of three SZCs from four field isolates (PC36, 44, 46, and 55) showed different reactions to both formulations (Table S2). However, only the reactions observed for SZCs of PC33 and PC34 were statistically significant at $\alpha = 0.05$, suggesting that reaction of SZCs

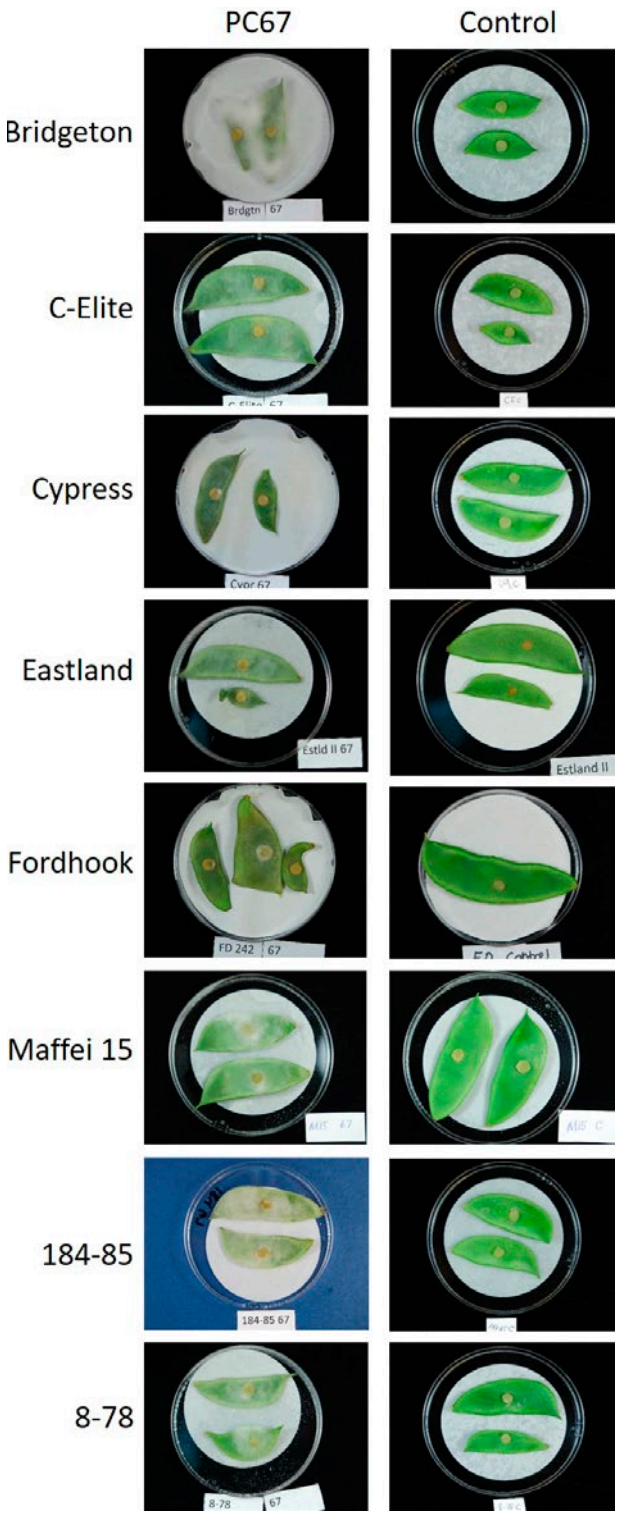

Figure 1. Lima bean pod assay. Pathogenicity testing of *Phytophthora capsici* isolate 67 on lima bean pods from eight different cultivars. Isolate 67 inoculations resulted in sporulation on all eight cultivars, representative of all of other isolate reactions with the lima bean cultivars. Pods were photographed 5 d post inoculation. The experiment was repeated once, with similar results.

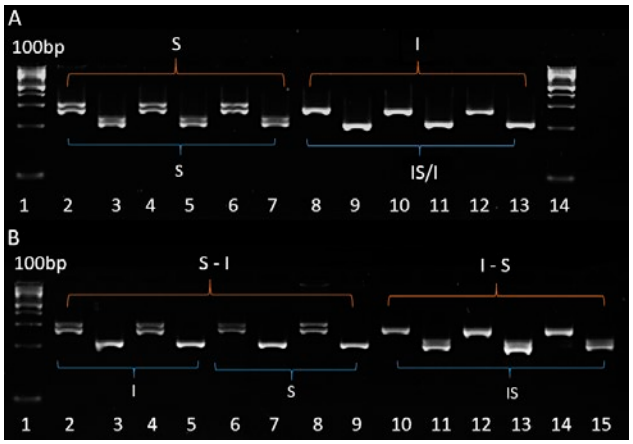

Figure 2. Segregation of mefenoxam sensitive and intermediately sensitive/insensitive *Phytophthora capsici* isolates, based on the SSR markers PCSSR19 and PCN3. S = sensitive, IS = intermediately sensitive, I = insensitive. Predictions based on the markers are shown above the fragments, and mefenoxam sensitivities of the isolates are shown below the fragments. Fragment sizes for each primer pair are as follows: PCSSR19 (S, 263 bp, 281 bp and 325 bp; IS/I, 252 bp and 270 bp) , PCN3 (S, 240-250 bp and 205-215 bp; IS/I, 205-215 bp). (A) Isolates showing the same segregation pattern for both PCSSR19 and PCN3. Lanes 1 and 14 are the 100 bp ladder. Even numbered lanes 2 to 12 are marker PCSSR19, odd numbered lanes 3 to 13 are PCN3. (B) Isolates exhibiting different segregation patterns with the markers PCSSR19 and PCN3. Lane 1, 100 bp ladder, even numbered lanes 2 to 14, PCSSR19, odd numbered lanes 3 to 15, PCN3.

to the technical grade mefenoxam and to Ridomil Gold SL were very similar.

Three EST-SSR markers tested, PCSSR19, PCN3, and PCN7 (which co-segregates with PCN3), were significantly correlated with mefenoxam sensitivity at $\alpha = 0.05$ (Pearson coefficients 0.507 (P = <0.001) for PCSSR 19 and 0.402 (P = 0.001) , for PCSSR 19 and for PCN3 and PCN7). Mefenoxam sensitive isolates yielded three bands of 263, 281, and 325 bp, while the isolates assessed as IS or I to mefenoxam yielded two bands of 252 and 270 bp with PCSSR19. Mefenoxam sensitive isolates produced two bands with PCN3 and PCN7 (240-250 and 205-215 bp), while mefenoxam insensitive isolates showed only the 205-215 bp band with PCN3 and PCN7. Mefenoxam sensitivity was predicted more accurately when both markers yielded the same banding pattern (Figure 2A, Table S3). Accuracy of sensitivity prediction when both markers predicted sensitivity was 96%, or for insensitivity was 85%. Twenty-five out of the 26 field isolates predicted to be mefenoxam sensitive by both markers were sensitive to Ridomil Gold SL in plate-based assays, and 11 out of the 13 field isolates predicted to be insensitive to mefenoxam exhibited insensitivity to Ridomil Gold SL. When PCSSR19 predicts insensitivity and PCN3 predicts sensitivity to mefenoxam, the plate-based reaction was IS (Figure 2B, Table S3). Isolates IS to mefenoxam could at times yield the bands for sensitivity with the PCN3 marker. Prediction of the actual sensitivity reaction was less accurate when PCSSR19 predicted sensitivity and PCN3 predicted insensitivity (Figure 2B). Based on these there was a 56% probability for the actual reaction to be IS (Table S3). Correlations between the marker combinations PCSSR19/PCN3 or PCSSR19/PCN7 with mefenoxam reaction, not considering instances where PCSSR19 predicted sensitivity and PCN3 predicted insensitivity, were significantly greater (Person coefficient 0.745 (P < 0.001).

DISCUSSION

Phytophthora capsici is an emerging threat to lima bean production in the main USA growing regions of Delaware, Maryland and Virginia. The goal of the present study was to isolate, identify and characterize many lima bean-infecting pathogen isolates in Delaware, and to determine their sensitivity to the widely-applied fungicide mefenoxam. In the course of 3 years, we recovered 64 *P. capsici* field isolates out of 200 samples collected. To identify a potential source of this important pathogen in grower fields, samples were collected from irrigation water sources, weed hosts, soils, and infected plant material. Of all these sources, *P. capsici* isolates were only obtained from infected plant material in crop fields. We were unable to identify any *P. capsici* isolates from irrigation sources, although they have been identified from irrigation sources in Florida, Michigan, and Georgia (Roberts *et al.*, 2005; Gevens *et al.*, 2007; Wang *et al.*, 2009). Our baiting method was similar to those of Wang *et al.*, (2009), although they used infected bait fruits to then inoculate pepper stems, which resulted in increased numbers of *P. capsici* isolates recovered. They observed that plating directly from bait fruits can favour organisms which outgrow *P. capsici*. Thirty seven isolates representative of the isolates recovered from irrigation sources were assessed for molecular identification. Details of this identification are listed in Table S4. None of these species were known pathogens of lima bean, though some where known to cause diseases on other hosts. Many of the isolates recovered from irrigation sources were morphologically similar to *P. capsici*. Based on the sequence data for the maternally inherited Cox1 gene and the ITS, however, several of the isolates recovered from water sources in Delaware were identified as the putative maternal parents of *Phytophthora* x *stag-*

num (Yang *et al*., 2014; Table S4). The hybrid species *P.* x *stagnum* was recently described (Yang *et al*., 2014). The paternal parent of *P.* x *stagnum* is reported as *P. chlamydospora*, previously known as *P.* taxon Pgchlamydo, (Hansen *et al*., 2015), and the maternal parent is thought to be an unknown species close to *P. mississippiae*. The present study is the first report of the identification of the putative maternal parent of *P.* x *stagnum* (C. Hong, personal communication). Of the other species recovered from the water sources, isolates of *P. irrigata* have been reported from water sources in other studies (Kong *et al*., 2003; Hong *et al*., 2008). *Phytopythium* is a novel genus in the Pythiaceae, exhibiting morphological characteristics in between *Pythium* and *Phytophthora* (de Cock *et al*., 2015). However, both isolates of the *Pythium*/*Phytopythium*-like species tested on the lima bean cultivars in the present study did not cause disease on pods except for isolate 4 on 'Eastland'. This indicates that these isolates were non-pathogenic on lima bean, but further greenhouse testing is needed, in particular, further testing on the 'Eastland' cultivar.

The presence of both MTs of *P. capsici* in the same field has been reported previously in New Jersey and North Carolina (Papavizas *et al*., 1981; Ristaino, 1990; Dunn *et al*., 2010). Mating type A2 appears to be dominant in Delaware, as approx. 66% of the field isolates tested were of this MT. Our results also demonstrate that both MTs can be present in the same field. This has significant ramifications for management of *P. capsici*, as new strains could quickly emerge due to sexual reproduction. Oospores can be viable in fields for more than 5 years, even after rotations with non-host crops (Lamour and Hausbeck, 2001). Hence, rotations of lima bean with non-host crops could become a less effective management strategy in grower fields in Delaware, if both MTs were present in the same field. *Phytophthora capsici* isolates belonging to both MTs were recovered from a field in Bridgeville, Delaware (field 17), which was planted with pickling cucumber early in the 2016 field season and then with lima bean later in the same growing season. Field 12 also tested positive for *P. capsici* in both 2015 and 2016, with both MTs found in 2016 (Table S1). This indicates that the pathogen survived over time, which could have been due to the presence of compatible MTs leading to formation of oospores are able to survive adverse conditions. Only one isolate was collected in 2015 from this field, and this could possibly be the reason for not detecting both MTs in this field in 2015.

Sampling a field multiple times could affect the accuracy of a study. While large numbers of samples could increase chances of detecting isolates of both MTs and different mefenoxam sensitivities within a field, these could also result in the possibility of sampling clones of individual isolates multiple times. This could affect the ratios for MTs and the accuracy of markers used to predict mefenoxam sensitivity. We sampled fields in multiple locations to offset this issue. Assuming the isolates of one MT and the same mefenoxam sensitivity to be clones of a single isolate within a field (disregarding the year collected and the source), we found 34 isolates of which 23 (68%) were of A2 MT. Though slightly greater, this proportion was is not significantly different from the 66% reported for the 64 isolates ($\chi^2_{df=1} = 0.184$, $P = 0.668$). At the same time, despite having the same MT and mefenoxam sensitivity, isolates collected from different years or different sources from the same field could also be different. Therefore, further analyses using sequence data are required to clearly identify the clones of individual isolates.

Out of 64 isolates collected, 31 were sensitive to mefenoxam, four were insensitive, and 18 were of intermediate sensitivity to the compound. There was variability among the SZCs of the remaining field isolates, indicating that each field isolate was a population of individuals. For example, SZCs of 11 field isolates had S to IS phenotypes, and two SZCs had IS to I phenotypes. Six out of these 11 reactions were significantly different between the individual zoospore isolates. These results indicate that *P. capsici* isolates insensitive to mefenoxam have increased in field populations since 2008, when mefenoxam insensitive isolates were first reported from MAR (Davey *et al*., 2008). This emphasizes the need for alternative fungicides for management of *P. capsici* diseases. We tested a subset of 21 field isolates for reaction to technical grade mefenoxam to determine whether this gave the same result as the commercial formulation Ridomil Gold SL, which contains only 45.3% mefenoxam. Enough technical grade fungicide was available to test three SZCs each for 21 field isolates with both forms of mefenoxam. Results indicated that 91% of the isolates give the same, or very similar, reaction (reactions not statistically different), regardless of whether they were exposed on technical grade mefenoxam or Ridomil Gold SL (Table S2). The two field isolates (PC33 and PC34) that gave significantly different phenotypes ($\alpha = 0.05$) could have resulted from effects of proprietary additives in the commercial Ridomil Gold SL formulation. However, this also suggests that each *P. capsici* field isolate is likely to be a population of individuals; each SZC cultured from the original field isolate, is not necessarily a clone. This assay provided the same phenotypes in two separate replicates. Further experiments should include at least 25 SZC from a single field isolate in plate-based and molecular-based mefenoxam sensitivity assays, to

provide insights into the nature of each field isolate.

Molecular markers were identified as associated with mefenoxam sensitivity, which could be important tools for initial screening of isolates. Mefenoxam sensitivity prediction was more accurate when both markers, PCSSR19 and PCN3, predicted the same reaction to mefenoxam. In a rare case, the two markers were inconsistent with each other, when PCSSR19 predicted sensitivity and PCN3 predicted insensitivity. In these instances, a plate-based assay should be used to confirm the reactions. Isolates PC34 and PC36, which showed S-IS reaction to Ridomil Gold SL, were S to technical grade mefenoxam (Table S2). Both PCSSR19 and PCN3 predicted sensitivity to mefenoxam for these two isolates. These markers are potentially robust tools for rapidly identifying fungicide-insensitive isolates. Future studies will include testing on a larger sample of *P. capsici* isolates than reported here, as well as generation and testing of a segregating population of the pathogen.

Results of the pod assays indicated that all commercial lima bean cultivars used in this study were susceptible to all *P. capsici* isolates tested, including isolates recovered from cucurbits and crops other than lima bean. Susceptibility of all commercial lima bean cultivars highlights one of the biggest disease management challenges. Accession PI477041 exhibited limited sporulation to some *P. capsici* isolates. It is hoped that this line and other resistant landraces can be used in breeding for resistance to *P. capsici* in the future. The detached pod assay could be used as an initial screening system for identifying resistance to this pathogen in future breeding efforts. Extensive greenhouse and field-testing could then be carried out on selected germplasm lines, with reduced cost and resources. Snap bean inoculations in this study indicated that *P. capsici* isolates from Delaware can cause disease on snap beans under optimal conditions for infection. Snap beans are widely grown in Delaware with no reports of *P. capsici* infections in field sites. However, there are reports of *P. capsici* infecting snap bean in other states (Gevens *et al.*, 2008; McGrath and Dillard, 2011). Examining the host range of *P. capsici* in Delaware is important information for farmers, as well as for developing effective management strategies to control *P. capsici* in the MAR.

As a pathogen, *P. capsici* has a broad host range and can attack different host tissues (Hausbeck and Lamours 2004). The presence in a field of both MTs of this pathogen, and isolates which are insensitive to mefenoxam, could result in oospore production and long-term survival of the pathogen, and also in reduced fungicide efficacy against the diseases it causes, posing severe management implications for lima bean production.

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Research Paper

Biological control of clementine branch canker, caused by *Phytophthora citrophthora*

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Summary. Significant losses of clementine trees (*Citrus* × *clementina*) due to *Phytophthora* branch canker were observed in the Cap Bon Peninsula in northeastern Tunisia. This disease is caused by *Phytophthora citrophthora*. The low efficiency of available cultural and host resistance disease management methods, and potential harmful impacts of pesticide strategies, lead to a search of new control alternatives. This study investigated potential biocontrol agents. The halophilic bacterial strains *Bacillus pumilus* M3-16 and *Halomonas elongata* L80, previously selected for their antifungal activity, were assessed in a greenhouse trial. In addition, 69 endophytic bacteria were isolated from citrus roots and assessed for their antagonistic activities. Five isolates were selected because they showed strong growth inhibition of *P. citrophthora* in dual culture tests. Based on phenotypic characteristics, biochemical tests and sequence analyses of the 16S region of rDNA, the bacteria were identified as *Bacillus pumilus* (S19), *Bacillus amyloliquefaciens* (S24), *Bacillus siamensis* (S54) *Paenibacillus polymyxa* (S31), and *Pseudomonas veronii* (S40). These metabolized different carbon sources, and possessed antibiotic genes, produced siderophores and phytohormones, and solubilized phosphates. In greenhouse trials, two endophytic strains (S24, S31) and two extremophile strains (M3-16, L80) were also assessed, these bacteria significantly reduced ($P<$ 0.0001) necrotic lesions on host plants, by 35% for strain S24 of *Bacillus amyloliquefaciens* to 51% for strain L80 of *Halomonas elongata*. This is the first report of *Halomonas* sp. Controlling a *Phytophthora* sp. The strains described in this study offer a foundation for developing an efficient biofungicide.

Keywords. Branch canker, biocontrol, *Bacillus*, *Halomonas elongata*.

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INTRODUCTION

Citrus fruits are an economically important crop in Tunisia, which produces approx. 350,000 tons of citrus fruits annually (Mahjbi *et al*., 2016) on over 21,000 ha (FAO, 2013). However, intensification of cropping techniques, exchange of plant material, and competition between citrus producing countries have contributed to the emergence of disease epidemics such as Citrus

greening, Citrus canker and tristeza (Cambra*et al*., 2000; Gottwald *et al*.,2002; Bove, 2006). Among the pathogenic fungi infecting citrus, *Phytophthora* is one of the most virulent, causing damping off, root rot, brown rot, and gummosis (Graham *et al*., 2003). Although ten species have been reported to be pathogenic toward citrus trees around the world, three cause the most serious disease, stem gummosis, as well as root and fruit rot. These are *Phytophthora citrophthora*, *P. nicotianae* (syn. *P. parasitica*), and *P. palmivora* (Erwin *et al*., 1996). These three *Phytophthora* species have distinct temporal and climatic requirements, so their relative distributions and importance vary in different production areas (Matheron *et al*., 1997). *Phytophthora nicotianae* and *P. palmivora* are major causes of citrus diseases in the United States of America, whereas *P. citrophthora* predominantly causes disease in the Mediterranean Basin (Graham *et al*., 1998; Cacciola*et al*., 2008; Khanchouch *et al.,* 2017).

Clementine production has expanded in the last few years to reach second in citrus production in Tunisia, after the 'Maltaise` cultivar, and third in citrus exports (GIFruits, 2018). This progress comes after the introduction of new varieties such as 'Marisol' and 'Hernandina' via the FAO program in 1996 from Corsica, France.

Since 2015, in the Cap-Bon Peninsula in northeastern Tunisia, a region which represents 70% of the country's citrus fruit production, a new serious disease has appeared, which was found to be caused by *Phytophthora citrophthora* (Zouaoui *et al*., 2016). This syndrome was reported in Spain in 2008 (Alvarez *et al*., 2008a) and in South Africa in 2010 (Schutte *et al*., 2010), and is characterized by host gum exudation, browning of the crust, cracking, and cankers. Reasons for emergence of this disease are still unclear, but could include several hypotheses including climate change, the introduction of susceptible varieties, the appearance of a virulent strain of *P. citrophthora* (Cohen *et al*., 2003) and the roles of snails and ants as dispersal vectors of *P. citrophthora* (Alvarez *et al*., 2009).

The use of chemical pesticides is decreasing, due to potential negative side effects, including contamination of soil and ground water, health risks to humans, and development of pesticide resistance in pathogens (Conacher and Mes, 1993; Jepson *et al*., 2014). Biocontrol, particularly the suppression of plant pathogens by antagonistic bacteria, is a potential alternative.

Endophytes are plant-associated microorganisms that live in plant tissues without causing any detrimental effects to their hosts (Kloepper *et al*., 2006; Ryan *et al*., 2008). In many studies, endophytic bacteria such as *Pseudomonas* and *Bacillus* have provided effective ways to manage plant diseases, and improve plant growth (Lee *et al*., 2008; Khabbaz *et al*., 2015). These bacteria commonly produce modified or unmodified peptides, simple heterocyclic (phenazine) and aliphatic compounds, hydrogen cyanide (HCN), siderophores, volatile compounds, proteolytic enzymes such as cellulase and β-1, 3-glucanase (Susi *et al*., 2011; Radhakrishnan *et al*., 2017; Biessy and Filion, 2018). *Bacillus* spp. have several advantages such as their ubiquity, spore production, heat resistance, and antibiotic production. *Pseudomonas* spp. are excellent competitors towards soil fungal and bacterial microflora. *Pseudomonas* bacteria have the ability to use plant exudates as nutrients (Lugtenberg *et al*., 2002; Espinosa-Urgel 2004), and to produce chelating ferric ions and a wide variety of secondary metabolites, and induce systemic resistance (ISR) in plants. All these characteristics are essential for effective biocontrol.

Previous studies have shown the efficiency of halotolerant and moderately halophilic bacteria isolated from different Tunisian Sebkhas (shallow salt lakes) to control several phytopathogenic fungi, including *Fusarium sambucinum* causing dry rot of potato and gray mold in strawberry and tomato fruits (Sadfi *et al*., 2001). The halophilic bacteria are known to produce a wide range of extracellular antibiotics and volatile compounds, as well as antifungal enzymes such as protease, chitinase and glucanase (Sadfi-Zouaoui *et al*., 2008a; Essghaier *et al*., 2009b).

The objectives of the present study were to: (i) isolate and evaluate endophytic bacteria from citrus roots for control of Citrus branch canker, (ii) identify plant growth-promoting bacteria (PGPB) traits of antagonism and antibiotic and phytohormone production, and the degradation of different carbon sources, and (iii) assess the abilities of selected halolerant and endophytic bacteria to reduce development of host necrosis caused by *P. citrophthora* under greenhouse conditions.

MATERIALS AND METHODS

Isolation of citrus root endophytic bacteria

Endophytic bacteria were isolated from citrus roots. Ten different citrus cultivars, all grafted on sour orange, were randomly collected from Cap Bon Peninsula. Sampled roots were surface disinfected with 1% sodium hypochlorite solution for 10 min. The external portion of each root (approx. 5 mm from the margin) was removed with a sterile scalpel, and the root tissue was triturated in a sterile porcelain mortar in 10 mM phosphate buffer (pH 7.2). The root extracts were spread on tryptic soy agar, (TSA, Biolife) and then incubated at 28°C for 48

h. Resulting bacterial isolates were initially screened for antagonistic properties, and grouped by morphological characteristics (Haque *et al*., 2016).

Antagonistic bacteria

Two halophilic antifungal bacterial strains from Tunisian Sebkhas, *Bacillus pumilus* isolate number M3-16, and *Halomonas elongata* L80, were used in this study. The morphological, physiological, and molecular characteristics of these strains were previously described and their 16S rDNA sequences have been deposited in the GenBank database under the accession numbers EU435355 for M3-16 and EU435356 for L80 (Essghaier *et al*., 2009a).

Pathogen inoculum

The strain *Phytophthora citrophthora* (E1P1) was previously reported as the causal agent of clementine trunk and branch canker in Tunisia (Zouaoui *et al*., 2016). The ITS1/ITS4 rDNA sequences of this strain have been deposited in the GenBank database under the accession number KX269827.

Dual culture tests for antagonism

Antagonistic bacteria were selected by the co-culture test. For each test, a mycelium plug (5 mm diam.) of *P. citrophthora* was transplanted at 2.5 cm from each side of the bacterial strip located in the middle of each Petri dish. These tests were carried out on potato dextrose agar, and the incubation was for 7 d at 28°C. The percentage inhibition of *P. citrophthora* was calculated according to the formula developed by Whipps (1987): I = (R 1 -R 2) / R1 * 100. R1 was the radial distance of the pathogen colony measured from the centre to the side of the Petri plate, and R2 was the radial distance of the colony from the centre to the bacterial strip (Sadfi-Zouaoui *et al*., 2008a).

Morphological characterization of bacterium strains

The effective endophytic bacteria were phenotypically characterized according to morphological, physiological, and chemical analyses, based on the following tests: colony and cell morphology, motility, Gram staining, pigmentation, and catalase and oxidase tests (Trotel-Aziz *et al*., 2008). In addition, biochemical features of the highly antagonistic bacteria were investigated using API 20NE and ZYM strips (bioMérieux), following the manufacturer's instructions.

Molecular characterization of strains

To extract the DNA, bacteria from 24 h tryptic soy broth (TSB, Biolife) cultures were processed by centrifugation at 8000 rpm for 10 min. The bacterial pellets were then suspended in 500 µL of TE solution (10 mM Tris-HCl, 1 mM EDTA, pH 8) and treated with 17 µL of lysozyme (30 mg mL^{-1}) for 30 min at 37°C. Six µL of proteinase K (20 mg mL^{-1}) and 40 µL of 10% SDS were then added to this suspension which was incubated at 37°C. The solution was homogenized thoroughly after the addition of 100 µL of 5M NaCl and 80 µL of CTAB / NaCl (10% 0.7M) and incubated at 65°C for 10 min. An equal volume of chloroform/isoamyl alcohol (24/1) solution was then added. The aqueous phase obtained after centrifugation for 20 min at 12000 rpm was transferred to a new tube, to which 2 volumes of isopropanol was added. After incubation at -20°C for 1 h, the preparation was centrifuged for 5 min at 13000 rpm. The precipitated DNA was washed with 70% ethanol, dried and then resuspended in 50 µL of TE solution (Sambrook *et al*., 1989). The DNA obtained was stored at -20°C.

Amplification of the 16 rDNA was carried out using PCR, with the universal primers 27f (5′- AGA GTT TGA TYM TGG CTC AG-3′) and 1492r (5′-TAC CTT GTT A**Y**G ACT T-3′) (Reysenbach *et al*., 1992). The PCR profile was initial denaturation at 96°C for 3 min followed by 30 annealing cycles at 57°C for 30 s, extension at 72°C for 2 min, and denaturation at 96°C for 30 s, and an extension cycle of 72°C for 7 min. The PCR products were cleaned using the PCR purification kit (Promega) and sent for sequencing. The sequences were compared using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) for identification of the isolates. A neighbour-joining phylogenetic tree was produced by MEGA v7.0 (Kumar *et al*., 2016).

Modes of action of antagonistic bacteria

Cellulase production

Endoglucanase activity of the strains was determined as described by Miller (1959), based on the amount of sugar released during the hydrolysis of cellulose. This is determined colorimetrically, using detection by dinitrosalicylic acid 3 (DNS). The protocol for each sample was as follows: 200 µL of supernatant was added to 200 µL of the substrate 1% carboxymethyl cellulose (CMC)

and 200 µL of sodium citrate buffer (pH 4.8), then the reaction mixture was incubated at 50°C for 30 min. The reaction was stopped by the addition of 800µL of DNS. The mixture was then heated to 100°C for 15 min and then cooled in an ice bath. The nil controls each consisted of 200µL of buffer plus 800µL of DNS. Absorbance was determined at 540 nm, and converted to the concentration of reducing sugars using a standard curve created by different concentrations of glucose as standard. One unit of enzyme activity was defined as the amount of enzyme that released 1 µM of reducing sugars per 30 min (Assareh *et al*., 2012).

Siderophore production

A medium containing chromium azurol S (CAS) was used to detect the secretion of siderophores (Schwyn and Neilands, 1987). The principle is that the culture medium is initially blue, due to the iron-hexadecyl-trimethylammonium complex (iron/CAS/HDTMA complex), which turns orange when the iron is displaced by siderophore produced by the microorganism. This competition for iron will favour the siderophore forming orange ferri-siderophores. After 14 d of culture incubation at 28°C, red to orange halos form around bacterial colonies. Visual examination ensured the ability of microorganisms to produce siderophores. The strain colonies were classified for growth as + (0 to 10 mm), ++ (10 to 20 mm), or +++ (20 to 30 mm).

Phosphate solubilization

Phosphate solubilization was detected with the method of Islama *et al.* (2007), using the National Botanical Research Institute's phosphate (NBRIP) growth medium. This contained (per litre) 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5g $MgCl_2{\cdot}6H_2O$, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, and 15 g agar. pH 7.0. Inoculated plates were incubated at 24±2°C. The solubilization zones around bacterial colonies were assessed 7 d after inoculation, by subtracting the diameters of bacterial colonies from the total zone diameters (dp). The strains colonies were classified as + (5 mm <dp> 15 mm), ++ (15 <dp> 20 mm), or +++ (dp>20 mm).

Indole acetic acid (IAA) production

For each sample, 100 µL of fresh culture was inoculated into 10 mL of TSB medium and incubated at 28°C for 5 d. with agitation. The cultures were each centrifuged at 10000 rpm for 5 min, 1 mL of the supernatant was then removed, 2 mL of Salkowskire agent and 100 µL of orthophosphoric acid (10 mM) were added, and the cultures were then incubated for 30 min at 25°C. Observation of pink color indicated production of IAA (Ahmad *et al*., 2008; Tarnawski *et al*., 2006). Quantity of IAA was determined by absorbance at 530 nm, converted to concentration of IAA (μgmL^{-1}) using a standard IAA curve.

PCR detection of antibiotic biosynthesis genes

The five selected bacterial strains were screened for production of lipopeptide antibiotics, using specific primers that amplify genes from fengycin (FENDF, GGCCCGTTCTCTAAATCCAT; FENDR, GTCATGCTGACGAGAGCAAA), bacillomycin (BMYBF, GAATCCCGTTGTTCTCCAAA; BMYBR, GCGGGTATTGAATGCTTGTT), bacilysin (BACF, CAGCTCATGGGAATGCTTTT; BACR, CTCGGTCCTGAAGGGACAAG), Surfactin (SRFAF, TCGGGACAGGAAGACATCAT; SRFAR, CCACTCAAACGGATAATCCTGA) and iturin (ITUD1F, GATGCGATCTCCTTGGATGT; ITUD1R, ATCGTCATGTGCTGCTTGAG). The PCR amplifications were performed in 15 µL reaction mixtures each containing 0.06 µL (5 U μL^{-1}) *Taq* DNA polymerase, 1 µL (20 ng μL^{-1}) of DNA template, 1.5 µL of 10× PCR buffer, 0.45 µL (50 mmol L^{-1}) of $MgCl_2$, 1 µL (10 mmol L^{-1}) of dNTPs, 0.3 µL (10 µmol L^{-1}) of each primer, 0.75 µL (10 mg L^{-1}) of bovine serum albumin (BSA), and 9.64 µLof filter-sterilised Milli-Q water (Mora *et al*., 2011; Khabbaz *et al*., 2015).

Biocontrol screening in planta

Pathogen inoculum

Phytophthora citrophthora (strain E1P1) was grown on V8 juice agar (JV8A) in Petri plates for sporangium production. The strain was incubated for 2 d in the dark at 24°C. The Petri plates were then filled with the Chen-Zentmyer's Salt Solution (containing, per L of distilled water: 1.64 g (Ca $(NO_3)_2$, 0.05 g KNO_3, 0.48 g $MgSO_4$, and 1 mL of chelate iron solution (13.05 g L^{-1}EDTA; 7.5 g L^{-1} KOH; 24.9 g $L^{-1}FeSO_4$; pH.7), and incubated under continuous fluorescent light at 24°C. Sporangia were produced within 1 to 2 days.

Bacterium strains

Four bacteria were selected for *in-planta* tests. Two halophilic strains (M3-16, L80) and two endophytic bacteria (S24 and S31) were used. They were grown for 48 h on TSA, supplemented with 5% NaCl for the two halo-

philic strains. After 48 h, colonies were scraped from the agar surfaces in Petri dishes, and were diluted in sterilized saline solution (1% NaCl). Bacterium concentrations were determined by dilution plating on TSA, and adjusted to 10^8 colony forming units (CFU) mL^{-1} for inoculations.

Experimental design

One hundred 2-year-old clementine plants ('Hernandina'), grafted on 'Carrizo' citrange rootstock, were grown in a partially controlled greenhouse at the Tunisian National Institute of Agricultural Research. The experiment was arranged in a completely randomized design. Five treatments were applied, each on 20 plants with two inoculation points for each plant. These were: untreated plant inoculated with *P. citrophthora* (E1P1), d, (ii) plant inoculated with antagonist *B. amyloliquefaciens* (S24) and *P. citrophthora*, (iii) plant inoculated with the antagonist *P. polymyxa* (S31) and *P. citrophthora*, (iv) plant inoculated with the halophilic antagonist *H. elongata* (L80) and *P. citrophthora*, and (v) plant inoculated with the halophilic antagonist *B. pumilus* (M3-16) and *P. citrophthora*.

Co-inoculations

For each plant, two cuts of length 5 to 10 mm were made with a scalpelon the bark of the scion, at 15 to 20 cm from each other. Each wound was immediately drop-inoculated with 100 µL of suspension of the bacterial strains (10^8 CFU mL^{-1}). The liquid was allowed to dry for 20–40 min at air temperature, and the incision was filled with a JV8A plug containing sporangia of *P. citrophthora* E1P1 placed in direct contact with the stem cambium. The wound was then wrapped with foil, moistened, and sealed with a strip of paraffin film to prevent desiccation.

Pathogenicity assessments and data analyses

The results were assessed 10 d after inoculation. Bark of the inoculated area on each plant was scraped with a scalpel, and margins of lesions were measured with a ruler. Statistical analyses for these data were performed using R statistical software version 3.4.0 (R Core Team, 2017). Normal distributions of the data were verified by the Shapiro-Wilk test before being subject to the non-parametric Kruskal-Wallis tests. Additionally, average reduction in lesion size [% reduction = 100 – (average lesion size for treatment × 100)/average lesion size for experimental control)] was calculated for the treatments that displayed statistically significant differences from the non-treated controls (Alvarez *et al.*, 2008b).

RESULTS

Isolation, characterization and identification of antagonistic bacteria

Among the 69 bacteria isolated from citrus roots, five isolates (S19, S24, S31, S40 and S54) were shown to be the most effective for inhibiting growth of *P. citrophthora* in the dual culture tests (Figure 1). Growth inhibition percentage (GI%) ranged from 67% for S31 to 74% for isolate S19. The GI% for both S24 and S54 was 69%, and for S40 was 62% (Figure 2).

Isolates S19, S24, S31, and S54 were identified as belonging to *Bacillus* and S40 to *Pseudomonas* on the basis of several phenotypic features (Table 1). The colony morphology of the *Bacillus* isolates was circular with cream pigmentation, the isolates had rod cell shape and were Gram positive, while the *Pseudomonas* isolate had pale yellow colonies, coccoid cell shape and was Gram negative (Table 1). According to API 20NE strip (bioMérieux) tests, positive reactions were observed among all the isolates for nitrate reduction, esculin and gelatin hydrolyses. Negative reactions were observed for L-tryptophane, D-glucose, L-arginine, urease, capric acid, adipic acid and phenylacetic acid. All other reactions were different among the isolates (Table 1). For

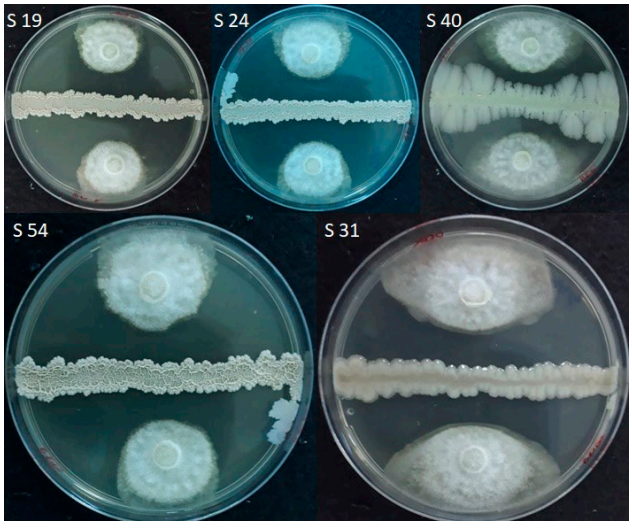

Figure 1. Dual culture showing antagonistic activities of the endophytic bacteria (strains S19, S24, S31, S40 and S54) toward *Phytophthora citrophthora*.

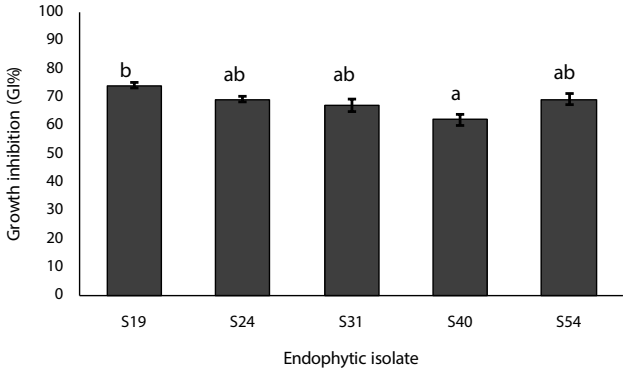

Figure 2. Growth inhibition rates of the endophytic bacteria toward *Phytophthora citrophthora*. Growth inhibition values followed by different letters are significantly different (Tukey Test, $P< 0.05$, n = 6, (± standard errors)).

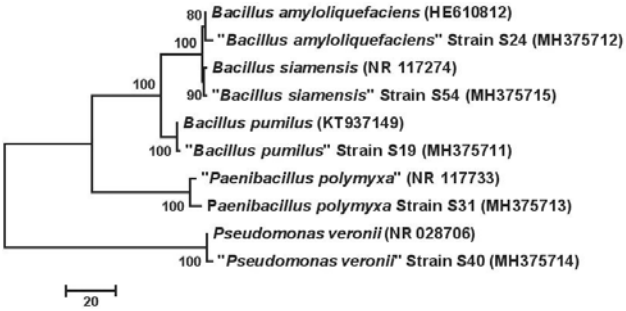

Figure 3. Neighbour-joining tree based on 16S rDNA sequences showing the phylogenetic relationship between strains S19, S24, S31, S40 and S54. Bootstrap values (expressed as percentages of 1000 replications) >70% are given at the nodes.

API ZYM strip tests, positive reactions were observed for alcaline phosphatase, esterase (C4), esterase lipase (C8), acid phosphatase and naphthol-AS-BI-phosphohydrolase. Positive reactions for α-chymotrypsin were only observed for the isolates S31 and S40, and positive reaction for α-galactosidase occurred only for the isolate S19. All other reactions were negative (Table 1).

BLAST analyses of the 16SrDNA sequences showed that there was 99% homology of isolate S19 with *Bacillus pumilus* L14 (KT937149), isolate S24 with *Bacillus amyloliquefaciens* BD18C2-B16 (HE610812), isolate S31 with *Paenibacillus polymyxa* DSM13, S40 with *Pseudomonas veronii* CIP104663 (NR_028706), and isolate S54 with *Bacillus siamensis*PD-A10 (NR_117274). The 16S rDNA sequences were deposited in the GenBank database, under the accession numbers MH375711 for S19, MH375712 for S24, MH375713 for S31, MH375714 for S40 and MH375715 for S54 (Figure 3).

Table 1. Phenotypic characterization of selected antagonistic bacteria[a].

Characteristic	S19	S24	S31	S40	S54
Colony morphology	Circular	Circular	Circular	Circular	Circular
Cellshape	Rod	Rod	Rod	coccoid	Rod
Mobility	+	+	+	+	+
Pigmentation	Cream	Cream	Cream	Cream	Cream
Gram strain	+	+	+	-	+
Catalase	+	+	+	+	+
Oxidase	+	-	-	+	+
Nitrate reduction	+	+	+	+	+
L-tryptophane	-	-	-	-	-
D-glucose	-	-	-	-	-
L-arginine	-	-	-	-	-
Urease	-	-	-	-	-
Esculinhydrolysis	+	+	+	+	+
Gelatinhydrolysis	+	+	+	+	+
4-nitrophenyl-βD-galactopyranoside	-	-	+	-	-
L-arabinose	+	+	+	+	-
D-mannose	+	+	-	+	+
D-mannitol	+	+	-	+	+
N-acetyl-glucosamine	+	+	-	-	+
D-maltose	+	+	-	+	+
Potassium gluconate	+	-	+	+	+
Capricacid	-	-	-	-	-
Adipicacid	-	-	-	-	-
Malicacid	+	+	-	+	+
Trisodium	+	-	-	+	+
Phenylacetic	-	-	-	-	-
Alcaline phosphatase	+	+	+	+	+
Esterase (C4)	+	+	+	+	+
Lipase (C8)	+	+	+	+	+
Lipase (C 14)	-	-	-	-	-
Leucine arylamidase	-	-	-	-	-
Valine arylamidase	-	-	-	-	-
Cystine arylamidase	-	-	-	-	-
Trypsin	-	-	-	-	-
α-chymotrypsin	-	-	+	+	-
Acid phosphatase	+	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+
α-galactosidase	+	-	-	-	-
ß-galactosidase	-	-	-	-	-
ß-glucuronidase	-	-	-	-	-
α-glucosidase	-	-	-	-	-
ß-glucosidase	-	-	-	-	-
N-acetyl-ß-glucosaminidase	-	-	-	-	-
α-mannosidase	-	-	-	-	-
α-fucosidase	-	-	-	-	-

[a]S19, S24, S31 and S54: Strains of *Bacillus* sp.; S40: Strain of *Pseudomonas* sp.
(+) Positive reaction ; (-) negative reaction.

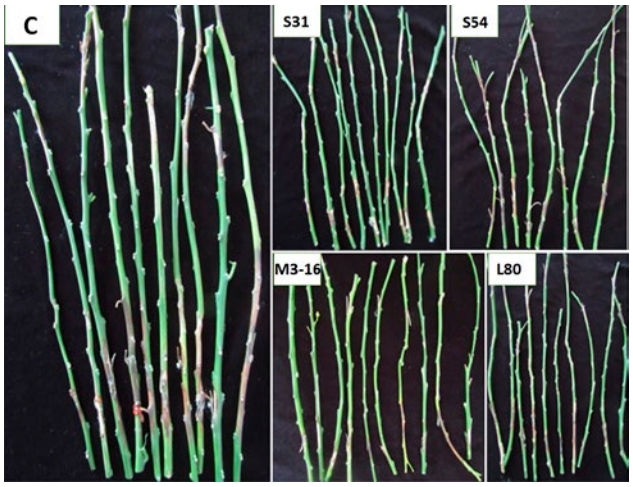

Figure 4. Suppression of branch canker on clementine mandarin for the trial conducted under partly controlled greenhouse conditions. Host branches were cut and leaves removed for improved view of the symptoms. C: Plants inoculated with *Phytophthora citrophthora* (Pc) without bacterial treatment. S31 and S24 indicate plants treated with endophytic bacteria, and M3-16 and L80 indicate those treated with halophilic bacteria.

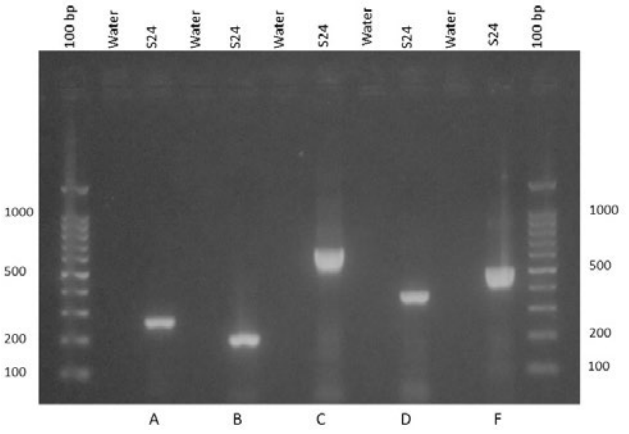

Figure 5. PCR amplification products of antibiotic biosynthetic genes in *Bacillus amyloliquefaciens* (S24), with primers (A) fengycin (FENDF/R) 269 bp; (B) surfactin (SRFAF/R) 201 bp; (C) iturin A (ITUD1F/R) 647 bp; (D) bacillomycin (BMYBF/R) 370 bp; and (E) bacilysin (BCAF/R) 498 bp

Modes of action of antagonistic bacteria

Production of antibiotic genes

The five lipopeptides genes Fengycin, Surfactin, Iturin A, Bacillomycin and Bacilysin were detected in all the *Bacillus* strains (*B. pumilus*S19 and S24, *B. amyloliquefaciens,* and *B. siamensis* (S54). However, the Bacillomycin gene was not detected in the *P. polymyxa* strain (S31) (Table 2; Figure 5).

Cellulase production

The *P. veronii* strain (S40) was the most efficient for production of cellulase (2.64 U mL^{-1}), followed by the three strains of *Bacillus* with S19 producing 1.62 U mL^{-1}, S24 producing 2.13 U $mL^{-1}$and S54 producing 2.51 U mL^{-1}. Cellulase production from *Paenibacillus polymyxa* strain S31 was 1.21 UmL^{-1} (Table 2).

Siderophore production

All the strains produced siderophores. Strain S24 produced the smallest halo (+) from 1 to 10 mm, and the strains S31 and S54 produced the largest halo zones (+++) of 20 to 30 mm (Table 2).

Table 2. Modes of action of selected antagonistic bacteria in dual culture assays with *Phytophthora citrophthora.*

Strain	Hydrolytic enzyme[a]					PGPB traits[b]			Antibiotic molecular screening[a]				
	Lip	Amy	Ure	Prot	Cell (UmL^{-1}) [c]	Sid	Phos	Aux	Fen	Bcin	Bsin	Surf	Itu A
S19	-	+	-	-	1.62±0.12ab	+	++	-	+	+	+	+	+
S24	+	+	-	+	2.13±0.05ab	+	++	-	+	+	+	+	+
S31	-	+	-	+	1.21±0.13a	+++	+	+	+	-	+	+	+
S40	-	+	-	-	2.64±0.06b	+	+++	-	-	-	-	-	-
S54	+	+	-	+	2.51±0.02ab	+++	++	-	+	+	+	+	+

[a] (+) Positive reaction (-) and negative reactions for: Lip , Lipase; Amy, Amylase; Ure, Urease; Prot, protease; Cell, cellulase; Sid, Siderophore production; Phos, phosphate solubilization; Aux, Auxin production; Fn, Fengycin; Bcin, Bacillomycin; Bsin, Bacilysin; Surf,: Surfactin; Itu A, Iturin A.

[b] PGPB: Plant growth-promoting bacteria.+, Growth ranging from 1 to 10 mm; ++, growth ranging from 10 to 20 mm; +++, growth ranging from 20 to 30 mm.

[c]Mean cellulase activity (U mL^{-1}) followed by different letters are significantly different ($P < 0.05$, n = 6; Tukey tests) (± standard errors).

Table 3. *In planta* antagonistic activity of four bioactive bacteria toward *Phytophthora citrophthora.*

Treatments with bioactive bacteria[a]	Mean lesion length(cm)[b]	Average reduction in lesion size (%)[c]
Control	4.9±0.47a[d]	
S24	3.2±0.42bc	35
S31	2.9±0.19b	41
L80	2.4±0.20b	51
M3-16	3.1±0.22c	37

[a] Treatments by bioactive bacteria applied at 10^8 UFC mL^{-1}.
[b] Mean lesion length 10 d after inoculation with *P. citrophthora*. Mean of 40 inoculation points.
[c] Average reduction in lesion size [% reduction = 100 – (Av. lesion size treatment × 100)/Av. lesion size control] was calculated for the treatments that displayed statistically significant differences from the non treated controls.
[d] Means followed by the same letter are not significantly different ($P < 0.05$, n = 40) according to Dunn tests.

Phosphate solubilization

The three *Bacillus* strains produced clear phosphate solubilized zones, ranging from 15 to 20 mm after 9 d. The *Ps. veronii* strain S40 produced the largest zone of more than 20 mm. *Paenibacillus polymyxa* (S31) was the weakest strain for this character, with zones less than 15 mm (Table 2).

Auxin production

Only the strain *Paenibacillus polymyxa* S31 produced IAA (18.5 µg mL^{-1},Table 2).

Suppression of branch canker on clementine mandarin plants

The endophytic and halophilic bacterium strains all reduced ($P<0.0001$; Kuskalwallis tests) the size of necrotic lesions caused by *P. citrophthora* on clementine plants 10 dafter inoculations, compared to the non-treated plants (Figure 4). The strain L80 of *Halomonas elongata* was the most effective for lesion reduction, giving 51% reduction of mean lesion size (Table 3). The other bacteria reduced lesion sizes, proportional reductions not exceeding 41%.

DISCUSSION

The Tunisian citrus industry has flourished in recent years, and this has been partly due to increased clementine production. However, trunk and branch canker caused by *P. citrophthora* represents a serious threat to productive clementine cultivation. Biological control of this new disease has not been previously investigated. In order to establish an eco-friendly strategy for management of *Phytophthor*a, 69 isolates of bacteria from citrus roots were identified and screened for antagonistic activity against *P. citrophthora*. Five strains had strong *in vitro* antagonistic effects against *P. citrophthora*. Molecular characterization allowed us to identify these strains, three of which were *Bacillus*, one was *Paenibacillus*, and the fifth was *Pseudomonas*. Köberl *et al.* (2011) found that *Bacillus* and *Paenibacillus* represent 96% of the antagonists towards phytopathogens in agriculture. Size of the inhibition zone in dual culture was the first criterion used to select the antagonist candidates. The use of PDA as a rich nutrient medium could exclude competition as a mode of action. No physical contact between the isolates and the pathogen in the dual culture tests could be related to the production of antifungal metabolites that inhibit mycelium growth (Montealegre *et al.*, 2003; Lee *et al.*, 2008). The growth inhibition proportion (GI%) of the selected bacteria varied from 74% for *B. pumilus* S19 to 62% for *Ps. veronii* S40. Among those most antagonistic bacteria, *B. amyloliquefaciens* and *P. polymyxa* were selected using a preliminary *in vivo* trial (results not shown) to study their ability to suppress branch canker under greenhouse conditions. In addition, two halophilic bacteria isolated from Tunisian sebkhas were selected. These were *Bacillus pumilus* M3-16 and *Halomonas elongata* L80. These halophilic bacteria produced extracellular antifungal enzymes such as chitinase, glucanase, and protease, and were characterized by tolerance and stability in the presence of extreme conditions (pH, temperature and salts) compared to other antifungal enzymes reported in the literature (Essghaier *et al.*, 2009b, 2010, 2012). Other reports have shown that halophilic bacteria with inability to produce inhibition zones in solid medium co-culture tests, have also demonstrated promising disease inhibition in *in vivo* tests. *Bacillus thuringiensis* strains unable to form inhibition zones in dual cultures were very effective *in vivo* on potato tubers (Sadfi *et al.*, 2001). *Bacillus subtilis* effectively inhibited grey mold on wounded tomatoes but was ineffective *in vitro* on PDA medium (Sadfi-Zouaoui *et al.*, 2008b).

The endophytic bacteria isolated from citrus roots demonstrated abilities to produce cell wall-degrading enzymes such as endoglucanase and protease, which are important for breaking through plant cell walls. Reinhold-Hurek*et al.* (2006) confirmed that endoglucanases are essential for *Azoarcus* sp. to colonize rice roots. Many other studies have confirmed that bacterial endophytes

are mainly recruited from soil via rhizosphere and root systems, to reach xylem and phloem vessels and colonize plants intra- and extra-cellularly (Liu *et al*., 2017). Little is known about endophyte colonization of citrus tissues. Lacava *et al*.(2007) studied the colonization of *Citrus sinensis* by endophytic bacteria, choosing the model of a *K. pneumoniae* strain labelled with GFP genes. They concluded that the endophytic bacterium strain colonized xylem vessels of *C. roseus* branches and roots.

The greenhouse trial of the present study revealed that the endophytic *B. amyloliquefaciens* and the halophilic *B. pumilus* produced similar results, reducing necroses in citrus plants by, respectively, 35 and 37%. Several other studies have demonstrated the ability of *B. amyloliquefaciens* to control *in planta* infections by *Phytophthora* spp. (Chung *et al*., 2005; Anandhakumar *et al*., 2008; Li *et al*., 2014; Zhang *et al*., 2016). Anandhakumar *et al*. (2008) showed that *B. amyloliquefaciens* reduced red core and crown rot diseases of strawberry in a greenhouse trial, exhibiting a similar level of disease control (up to 59%) as the chemical fungicide Aliette®. The second most effective bacterium in the present study was *P. polymyxa,* which reduced necrosis by 41%. Recent publications have also indicated the potential of *P. polymyxa* as a bio-pesticide (Grady *et al*., 2016; Weselowski*et al*., 2016; Luo *et al*., 2018). However, it is the ability of this bacterium to inhibit *Phytophthora* diseases of citrus has not been previously explored.

The greatest *in vitro* inhibition of *P. citrophthora* was obtained with *H. elongata*, with 51% reduction of host necrosis. This is the first record of potential for *H. elongata*as a biocontrol agent against *Phytophthora* sp. In order to study the PGPB traits of this bacterium, direct and indirect mechanisms to promote citrus growth were evaluated. Direct mechanisms include increased availability of plant nutrients (biofertilization), from phosphate solubilization and azote fixation. According to the biochemical test 'Gallerie API 20 NE', all the five strains were able to reduce atmospheric azote (N_2) to nitrite (NO_2^-) and then to nitrate (NO_3^-). In addition, the *P. polymyxa* S31 strain could synthesize IAA enhances plant cell growth and proliferation (Grady *et al*., 2016). The indirect mechanism was demonstrated by the abilities of all the candidate bioactive bacteria to produce siderophores, lytic enzymes such as lipase, protease and cellulase, and to possess most of the lipopeptide genes. Interactions between lipopeptides can become synergistic to enhance their respective activities (Romero *et al*., 2007; Malfanova *et al*., 2012; Li *et al*., 2014). Previous studies have highlighted the role of *Bacillus* lipopeptides in biological control of plant diseases (Ongena and Jaques, 2008; Mora *et al*., 2015; Paraszkiewicz *et al*., 2017). Beside their involvement in antifungal and antimicrobial activities, some bacteria also facilitate root colonization and modulate plant immunity (Ongena and Jaques, 2008).

CONCLUSIONS

This study has demonstrated potential to overcome the new serious disease in clementine orchards by adopting an ecological biocontrol approach. After screening endophytic bacteria from citrus roots, five bacteria displayed strong antagonistic traits as *in vitro* pathogen inhibition rates, metabolization of different carbon sources, PGPB traits, and antibiotic production. In the greenhouse trial, the strains L80 of *H. elongata* and S31 of *P. polymyxa* showed the greatest potential as candidates for control the disease. This is the first biological control study of clementine branch canker. The bacterial strains S31 and L80 offer a good basis for developing efficient biofungicides, and should be further investigated under field conditions.

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Research Paper

Fungicide suspensions combined with hot-water treatments affect endogenous *Neofusicoccum parvum* infections and endophytic fungi in dormant grapevine canes

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Summary. *Neofusicoccum parvum* is an important opportunistic fungus causing Botryosphaeria dieback on grapevines. Because of its opportunistic nature, this pathogen spreads to many grape growing areas in a latent phase, and causes serious economic losses. The aims of the study were to examine hot-water treatments combined with fungicides in order to cure latent infections of artificially inoculated *N. parvum* in dormant grapevine canes and to assess the effects of these treatments on endophytic fungi. Artificially *N. parvum*-inoculated canes were dipped into cyprodinil + fludioxonil, tebuconazole or thiophanate-methyl suspensions under the following temperature-duration combinations; 30°C for 12 h, 35°C for 6 h, 40°C for 2 h or 50°C for 30 min (temperature at 50°C was not combined with fungicides). Treated canes were cooled in tap water (18°C) for 1 h, and pathogen re-isolations were immediately attempted from inner wood tissues. Extent to which these applications affected the presence of endophytic fungi were also determined by calculating pre- and post-treatment isolation rates. Hot-water treatments (without fungicides) below 52°C did not reduce *N. parvum* re-isolation rates, and were ineffective. However, these treatments combined with fungicides decreased pathogen incidence at 50°C and below. Maximum reduction (34%) was obtained with heated tebuconazole suspensions at 40°C for 2 h and 50°C for 30 min, and eradicative ability was superior to that of cyprodinil + fludioxonil or thiophanate-methyl. The hot-water treatments reduced incidence of endophytic fungi but greater reduction was observed with the hot-water and fungicide combinations. Fungicide penetration into wood tissues of propagation material could be enhanced by increasing water temperatures in hydration or hot-water treatment tanks, and this approach could be useful method for production of healthy grapevine plants in nurseries.

Keywords. *Vitis vinifera*, tebuconazole, cyprodinil, fludioxonil, thiophanate-methyl.

INTRODUCTION

Neofusicoccum parvum (Pennycook & Samuels) Crous, Slippers & Phillips is one of the most important pathogens causing Botryosphaeria dieback of grapevines. This fungus causes a variety of symptoms in grapevines, includ-

ing brown wedge-shaped wood cankers, xylem necroses, failure of spring growth and black dead arm (Úrbez-Torres, 2011). Although *N. parvum* pycniospores are the main inoculum sources for short distance dissemination, the pathogen is more likely to spread to different geographical areas through infected propagation materials. When the pathogen infects vines through wounds, natural openings or direct penetration of bark, it can progress systemically in cane vascular and wood tissues, and can cause decline of vines (Amponsah *et al*., 2012). Isolation of botryosphaeriaceous fungi from asymptomatic canes suggest the importance of latent infections on pathogen dissemination in vineyard areas (Slippers and Wingfield, 2007).

Hot-water treatment (HWT) is a promising and plausible method to reduce endogenous infections of all grapevine trunk disease (GTD) and other pathogens, for production of healthy propagation material (Fourie and Halleen 2004; Halleen *et al.,* 2007). Though some studies have reported that standard HWT (at 50°C for 30 min) can eliminate grapevine pests, some bacterial pathogens (such as *Xylella fastidiosa* and *Rhizobium vitis*) and phytoplasmas (Flavescense dorée) are not completely eradicated (Ophel *et al.,* 1990; Caudwell *et al.,* 1997; Crous *et al.,* 2001). There is also evidence that GTD fungi are not sufficiently eliminated from dormant grapevine canes by these treatments (Rooney and Gubler, 2001; Waite and May, 2005; Gramaje *et al.,* 2010). *Neofusicoccum parvum* is among the GTD fungi which were subjected to HWT studies both in laboratory and controlled conditions. *Diplodia seriata*, *Neofusicoccum luteum*, *N. parvum* and *Spencermartinsia viticola* were found to be the most susceptible species, while *Lasiodiplodia theobromae* and *N. vitifusiforme* were very tolerant to HWT in *in vitro* conditions. Mycelium survival of *N. parvum* was about 15% from HWT at 50°C for 30 min in test tubes containing sterile water (Elena *et al*., 2015). In this study, when the curative ability of HWT was tested with *N. parvum* inoculated grapevine canes, HWT (at 51°C for 30 min) eradicated the pathogen from dormant canes of 110-Richter rootstock. However, Billones-Baaijens *et al*. (2015) found controversial findings from their study, showing that HWT (at 50°C for 30 min) was less effective or ineffective, respectively, for eradication of *N. luteum* and *N. parvum* from infested cuttings of 101-14 Mgt rootstock. While this treatment reduced the isolation frequency of *N. luteum* by 50%, it did not reduce isolation of *N. parvum*. The expected performance from HWT was obtained just with 53°C for 30 min, but this combination was harmful for bud vitality of the treated rootstock cuttings. Billones-Baaijens *et al*. (2015) suggested that research should be carried out to improve hot-water treatments practicable for grapevine nursery industries. In Turkey, most nurseries avoid HWT because of detrimental effects on plants, such as delayed callusing, bud death, and delayed development and rooting.

Dipping of grapevine cuttings in fungicide suspensions is another control measure to produce healthy grapevine plants in nurseries. Rego *et al*. (2009) determined the efficacy of applications of cyprodinil + fludioxonil, metiram + pyraclostrobin, cyprodinil or fludioxonil suspensions, by hydration (tap water) of the rootstock cuttings prior to grafting. They found that the cyprodinil + fludioxonil reduced the incidence of black foot (by 47%) and botryosphaeriaceous fungi (by 60%) in plants grown in a field that was naturally infested with these pathogens. Halleen and Fourie (2016) developed a combined control strategy to reduce GTD pathogen infections South Africa, showing that benomyl and Sporekill® were useful chemicals for increasing healthy plant rates (up to 100%) in the grapevine nurseries. These studies were conducted in naturally infested fields or mother plants that were known to be infected with some GTD pathogens. Billones-Baaijens *et al*. (2015) showed that tebuconazole and carbendazim soakings (at tap water temperature) were effective for curing superficial bark infections by *N. parvum* or inoculated canes.

Curative performance of fungicide treatments against latent inner tissue infections of canes is important, because outer bark is a natural barrier preventing entry of pesticides into internal host tissues. Fungicide penetration via diffusion is known to be very inefficient (Waite and May, 2005). Therefore, there is a need to increase fungicide penetration into grapevine wood tissues during the soaking stage before grafting or cold storage for production of healthy propagation material.

The combination of HWTs with fungicides may be an effective approach for curing latent infections by GTD fungi in grapevine nurseries. This has previously been studied by many researchers who work on postharvest diseases of some fruits (Wells and Harvey, 1970; Barkai-Golan and Apelbaum, 1991). These studies showed that heat treatment in combinations with fungicides could increase penetration of the pesticides into fruit tissues. Schirra *et al*. (1996) treated lemon fruits with imazalil suspensions (1500 $mg \cdot L^{-1}$ at 20°C or 250 $mg \cdot L^{-1}$ at 50°C for 3 min) to cure *Penicillium* infections. The second of these treatments was as effective as the first for disease control, and four or five-fold increases in fungicide residues were recorded in albedo tissues of fruits from the treatment of 250 $mg \cdot L^{-1}$ imazalil at 50°C for 3 min.

Endophytic fungi live within plants without causing disease symptoms (Freeman, 1904). These fungi can contribute to plant adaption and survival in unfa-

vourable conditions. They can also produce secondary metabolites, trigger biochemical pathways or interact with other microorganisms (Aly *et al*., 2010; Kusari *et al*., 2012). Gonzalez and Tello (2011) assessed endophytic fungi from different organs of seven grape cultivars in central Spain, and obtained more than 500 isolates belonging to 68 species. While the endophytic composition differed according to vine cultivars, *Acremonium, Alternaria, Aureobasidium, Cladosporium, Epicoccum, Fusarium, Penicillium, Phoma*, and *Trichoderma* were isolated most frequently. Some of these fungi were reported to have antagonistic ability towards plant pathogenic fungi. Eichmeier *et al*. (2018) investigated effects of hot-water treatments (at 50°C or 53°C for 30 min) on endophytic fungi from dormant canes of Garnacha Tintorera/110 Richter and Sauvignon Blanc/SO4 grapevines. Although hot-water treatments decreased the incidence of endophytic fungi depending on temperature, HWTs did not sterilize dormant canes, and these fungi were recovered from hot-water treated plants grown for 8 months in nurseries. Although several studies have investigated curative effects of hot-water treatments (conducted generally at 50-54°C for 30, 45 or 60 min) on GTD pathogens (Habib *et al.,* 2009; Serra *et al.,* 2009), limited results are available where effects have been assessed for HWTs on endophytic fungi in dormant grapevine canes.

In evaluating current literature, we hypothesized that penetration of fungicides into dormant grapevine canes could be enhanced by heating fungicide suspensions (at low temperatures combined with extended durations) at the pre-grafting host stage. The aims of the study were: (i) to determine the efficacy of heated fungicide suspensions on latent infections of *N. parvum* and their effects on endophytic fungi in dormant grapevine canes; and (ii) to assess antagonistic abilities of some endophytic isolates against *N. parvum* in *in vitro*.

MATERIALS AND METHODS

Three fungicides (Table 1) with different modes of action were used to prepare hot-water treatment suspensions at 50°C and lower temperatures (30, 35 and 40°C). Dormant grapevine canes (*Vitis vinifera* 'Prima') were used as plant material in all experiments.

Isolation and molecular identification of endophytic and pathogenic fungi

The dormant canes were taken (in December 2017) from randomly selected vineyards in three different geographic regions of Turkey (Manisa, Tokat and Tarsus Cities; in, respectively, the Aegean, Central Anatolia and Mediterranean Regions). Fifty, one-year-old dormant canes (each with three to five buds and 50 cm long) were randomly taken from each vineyards (two vineyards per region, 25 canes from each vineyard). These canes were washed under running tap water for 10 min., and 10 cm-long cuttings were used for endophyte isolations. The cuttings were dipped into ethyl-alcohol (70%) for 1 min, sodium hypochlorite solution (2.5% NaOCl) for 3 min, and ethyl-alcohol (70%) (Schultz *et al*., 1993). Canes were then rinsed twice with sterile distilled water and bark tissues were removed with a sterile knife. Wood tissues were cut into 3 mm^2 pieces, and seven each were plated onto potato dextrose agar (PDA, Becton Dickinson) amended with streptomycin sulfate (150 $mg{\cdot}L^{-1}$). Petri dishes were incubated for 10 d at 24°C in the dark, and growing fungal colonies were sub-cultured to fresh PDA (without antibiotic) for further microscope examinations and molecular identification. After morphological discrimination and microscope examinations, the incidence of endophytic species was calculated by counting colonies (at genus or species level) growing around tissues and average isolation rates were determined from 25 Petri dishes for each vineyard. For each geographic region, average incidence of fungi was calculated as percent.

For molecular identification of fungi, 10-d-old mycelium mats of the isolates (growth from hyphal tip or single spore cultures) were harvested (50–100 mg), and DNA was extracted according to the protocol of Nejat *et al*. (2009). PCR amplification of the ITS1, 5.8S and ITS2 regions of rDNA was performed using ITS4 and ITS5 primers (White *et al*. 1990), and thermocycler (SimpliAmp A24811™; Applied Biosystems) conditions were adjusted as follow; 95°C for 3 min (initial denatura-

Table 1. Fungicides and application rates used in this study.

Active ingredients	Fungicide group	Application dose (product in water·L^{-1})	Trade name and formulation	Manufacturer
Cyprodinil (37.5%) + fludioxonil (25%)	Anilinopyrimidine + phenyl-pyrrole	5 g	Switch® 62.5 WG	Syngenta
Tebuconazole (250g·L^{-1})	DMI-triazole	4 mL	Orius® 20 EW	Adama
Thiophanate-methyl (70%)	Benzimidazole	5 g	Sumitop 70 WP	Sumitomo

tion), followed by 35 cycles each of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were purified and sequenced (Sanger dideoxy sequencing) by MedSanTek Co. (Istanbul, Turkey), and nucleotide sequences (in forward and reverse directions) of the isolates were contiged using Chromas Lite free software (Technelysium™). Consensus nucleotide sequences were compared with those deposited in the NCBI GenBank database using the BLAST program, and identification of the isolates was decided according to maximum identity results (with 99% or 100% rates). Sequences were deposited (except the well-known species, *Aspergillus niger, Aureobasidium pullulans, Epicoccum nigrum, Nigrospora oryzae, Penicillum* sp. *Rhizopus stolonifer*) to the NCBI database and accession numbers were obtained.

Inoculation and incubation of Neofusicoccum parvum

An electric drill method (Pouzoulet *et al.*, 2013) was used to inoculate *N. parvum* in all HWT experiments. The *N. parvum* isolate (MBAi27AG, GenBank accession no: KF182330), formerly shown to be highly virulent, was selected from the fungal library of Cukurova University, Department of Plant Protection. This isolate was grown on PDA at 25°C for 10 d in the dark. Dormant grapevine cuttings (each measuring 45 cm) were taken from a Prima vineyard (grafted on 1103 Paulsen, 10 years-old) in Tarsus city (Southern Turkey), and were transferred to the laboratory in December 2017. To prevent dehydration, they were immersed in clean tap water at ambient temperature (20°C) overnight. The cuttings were then trimmed to 35 cm, and the superficially sterilized in 2.5% NaOCl solution for 3 min and rinsed twice with sterile distilled water. Cane internodes were laterally drilled with a 2 mm-diam. drill bit and 2 mm-mycelium plugs were inserted into the drill holes which were then sealed with Parafilm®. In control plants, sterile agar pieces were inoculated into the wounds and the holes were sealed with Parafilm®. The inoculated canes were planted into plastic bags containing growth mixture (fine sawdust, perlite, sand, soil and peat, in equal volumes), and were then watered and maintained in a growth room (28°C. 90% relative humidity, 12 h dark/12 h light) for 2 months. For further experiments, each of the cuttings was numbered to compare initial and final endophytic flora after all treatments. The endophytic fungi were isolated from these cuttings before inoculation with *N. parvum*, and their isolation frequencies were recorded for further comparisons.

Hot-water treatment effects on Neofusicoccum parvum *infections and endophytic fungi*

The curative effects of hot-water treatments were investigated with and without fungicides. All HWT experiments were conducted in an adjustable 10 L capacity water bath device (Memmert WB 10). Air was pumped through the device using an aquarium pump to provide homogenity of fungicide suspensions. In fungicide-HWT treatment combinations, the fungicide suspensions were prepared at the application rates specified in Table 1, and at temperature/time treatments of 30°C for 12 h, 35°C for 6 h, 40°C for 2 h or 50°C for 30 min. The plants that were previously inoculated with *N. parvum* and grown for 2 months were uprooted from pots, and their roots and shoots were removed before HWTs. The cuttings (30 cm) were firstly washed and immersed in clean tap water at ambient temperature for 1 h. Bundles of cuttings, each comprising 12 cuttings, were then dipped into heated fungicide suspensions at the temperatures and for the durations specified above. The treated cuttings were cooled in clean tap water (at 18°C) for 1 h. Pathogen isolations were then carried out from the wood tissues from around inoculation points (4 cm from both sides of the inoculation holes), to investigate *N. parvum* survival, and from 10 cm from inoculation points to assess presence of endophytic fungi. Inner wood tissues were cut into 3 mm pieces with sterile secateurs and placed onto PDA amended with streptomycin-sulfate in Petri plates. The plates were then incubated at 25°C for 7 d in the dark. Control canes (inoculated but not treated with HWT-fungicide combinations) were subjected to the same isolation process. All the wood chips from a cane were placed on one plate, so 12 plates were used for each treatment (completely randomized design, four replicates and three plates from each replicate). Fungal colonies were counted and incidence was calculated as percentages.

The inoculated cuttings were also subjected to hot-water treatments (without fungicides) at temperature-time combinations of: 30°C for 12 h, 35°C for 6 h, 40°C for 2 h, or 50°C, 51°C, 52°C, or 53°C, each for 30 min. After HWTs, the cooling, isolation and incidence calculation procedures described above were carried out. This experiment was repeated once and average incidences were calculated.

In vitro *antagonistic effects of endophytic fungi against* Neofusicoccum parvum

Frequently isolated endophytic species (one isolate each) were selected to check antagonistic ability against *N. parvum*. Mycelial agar discs from 10-day-old cul-

tures of endophytic isolates were placed on one side of PDA plates. They were allowed to grow at 24°C for 72 h (because of slow growth), and *N. parvum* discs (from 10-d-old cultures) were placed on the other side of plates. These dual culture plates were incubated at 24°C for 10 d, with *N. parvum* growth observed daily and colony diameters (length-width) were measured to determine average colony diameters. Experimental control plate contained sterile agar discs opposite the *N. parvum* discs. Inhibition rates (%) were calculated according to Reyes-Chilpa *et al*. (1997) as cited by Felber *et al*. (2016). Interactions between endophytic isolates and *N. parvum* were grouped using the scale of Badalyan *et al*. (2002); where A = deadlock with mycelial contact, B = deadlock at a distance, and C = replacement, overgrowth with and without initial deadlock. Dual culture tests were arranged in a completely randomized design with five replications, and were repeated once.

Statistical analyses

Variance analyses were performed on data to reveal differences between means of the treatments, and the statistically similar groups were determined by Fisher's Least Significant Difference (LSD) test $P \leq 0.05$) in all experiments (Gomez and Gomez, 1984). The data of isolation percentages and inhibition rates were converted by Arc Sin transformations before variance analyses.

RESULTS

Endophytic and pathogenic fungi from apparently healthy cuttings

Twenty-three operational taxonomic units (OTUs) of fungi belonging to three phyla were obtained from grapevine cuttings from three regions in Turkey (Table 2). Wood pieces plated on PDA yielded more than one colony (average two) after 7-8 days of incubation. While most of the fungi (19 OTUs) were Ascomycota, the others were Basidiomycota (*Cerrena unicolor, Quambalaria cyanescens* and *Schizophyllum commune*) and Zygomycota (*Rhizopus stolonifer*). All of the OTUs were not regularly isolated from all the regions, but *Acremonium* sp. *Aureobasidium pullulans, Alternaria alternata, Aspergillus niger, Cladosporium cladosporioides, Penicil-*

Table 2. Endophytic and pathogenic fungi isolated from dormant grapevine canes in the study.

Order	Genus	Species	Isolate	GenBank Accession Number
Ascomycota	*Acremonium*	sp.	CUZFVG42	MK120286
Ascomycota	*Alternaria*	*alternata*	CUZFVG12	MK120281
Ascomycota	*Alternaria*	*tenuissima*	CUZFVG276	MK120296
Ascomycota	*Aspergillus*	*niger*	N/A	N/A
Ascomycota	*Aureobasidium*	*pullulans*	N/A	N/A
Basidiomycota	*Cerrena*	*unicolor*	CUZFVG176	MK120292
Ascomycota	*Chaetomium*	*globosum*	CUZFVG10	MK120280
Ascomycota	*Cladosporium*	*cladosporioides*	CUZFVG38	MK120283
Ascomycota	*Diaporthe*	*foeniculina*	CUZFVG125	MK120290
Ascomycota	*Diplodia*	*seriata*	CUZFVG4	MK120279
Ascomycota	*Epicoccum*	*nigrum*	N/A	N/A
Ascomycota	*Fusarium*	*equiseti*	CUZFVG87	MK120289
Ascomycota	*Geosmithia*	sp.	CUZFVG40	MK120285
Ascomycota	*Gnomonia*	*idaeicola*	CUZFVG36	MK120282
Ascomycota	*Lasiodiplodia*	sp.	CUZFVG250	MK120294
Ascomycota	*Neofusicoccum*	*parvum*	CUZFVG2	MK120278
Ascomycota	*Nigrospora*	sp.	N/A	N/A
Ascomycota	*Penicillium*	sp.	N/A	N/A
Ascomycota	*Phoma*	*glomerata*	CUZFVG212	MK120298
Zygomycota	*Rhizopus*	*stolonifer*	N/A	N/A
Basidiomycota	*Quambalaria*	*cyanescens*	CUZFVG39	MK120284
Basidiomycota	*Schizophyllum*	*commune*	CUZFVG173	MK120291
Ascomycota	*Trichoderma*	*atroviride*	CUZFVG243	MK120297

Table 3. Mean incidences of frequently isolated endophytic fungi in grapevine cuttings from three regions of Turkey.

Endophytic fungi	Incidence (%)		
	Manisa	Tarsus	Tokat
Acremonium sp.	2.0	3.6	17.1[a]
Aureobasidium pullulans	22.3	1.2	88.0
Alternaria spp.	14.3	16.3	12.6
Aspergillus niger	0.9	1.2	0.6
Cladosporium sp.	51.4	32.8	16.6
Penicillium sp.	0.9	5.2	0.8
Quambalaria cyanescens	0.1	9.9	0.1

[a]Means were for two vineyards for each location, and 25 grapevine cuttings from each vineyard were used for the isolations.

lium sp. and *Quambalaria cyanescens* were endophytic fungi isolated from all the regions and vineyards. The isolation percentages of endophytic fungi showed differences by geographical region. When compared to mean incidence rates, *Cladosporium* sp. was predominant in Manisa (51% recovery) and Tarsus (33%), but *A. pullulans* was predominant in Tokat (88%) and Manisa (22%). *Alternaria* sp. was the second most frequently isolated species in Manisa (14%) and Tarsus (16%). *Quambalaria cyanescens* was the third most common species in Tarsus (10%), but incidence of this species was very low (0.1%) in the other regions (Table 3). In apparently healthy canes, some pathogenic species (Botryosphaeriaceae,) were also isolated at low incidence rates, and only one isolate each of *S. commune* and *D. foeniculina* were obtained (Table 2).

Curative effects of hot-water treatments on Neofusicoccum parvum *infections*

No fungicide was added in the dipping tank for temperatures above 50°C due to risks of chemical degradation. The hot-water treatments below 50°C with extended durations (without fungicide), and 30 min durations at 50 or 51°C), had no curative effects on *N. parvum* infections. The 2 month incubation period ensured good establishment of the pathogen in plants, and the pathogen was re-isolated (100%) from all inoculated plants (hot-water treated and untreated). When water temperature was 52°C, the pathogen re-isolation frequency was 97%, or at 53°C was 74 % (Table 4).

Some of the hot-water treatments combined with fungicides significantly decreased fungus re-isolation rates. When comparing all treatments, the most effective combinations were tebuconazole suspensions at 40°C for 2 h and 50°C for 30 min, giving pathogen re-isolation of 66% from the cutting inoculation points. The curative effects of these combinations was less (34% recovery) than for HWT alone (26% recovery) at 53°C for 30 min, though these recovery rates were not significantly different. The efficacy of other combinations ranged from 3 to 34% (Table 4). Tebuconazole and thiophanate-methyl suspensions at 40°C reduced the incidence of *N. parvum* to some extent, but the efficacy of cyprodinil + fludioxonil decreased above 30°C (Figure 1). The 50°C for 30 min treatment did not reduce curative performance of tebuconazole but reduced the activity of thiophanate-methyl (Figure 1).

Table 4. Mean incidences and eradication rates (%) of *Neofusicoccum parvum* after different hot-water treatments and fungicide combinations.

Hot-water treatments (with and without fungicides)	Incidence of *N. parvum* (%) ± SEs	Eradication of *N. parvum* (%) ± SEs
40°C for 2 h, tebuconazole	65.7 ± 3.5 a [a]	34.3 ± 3.8 a
50°C for 30 min, tebuconazole	65.7 ± 3.5 a	34.3 ± 3.2 a
53°C for 30 min, water	74.3 ± 5.4 ab	25.7 ± 4.8 ab
40°C for 2 h, thiophanate-methyl	88.6 ± 2.9 bc	11.4 ± 2.5 bc
35°C for 6 h, tebuconazole	88.6 ± 5.4 bc	11.4 ± 2.9 bc
30°C for 12 h, cyprodinil + fludioxonil	88.6 ± 5.4 bc	11.4 ± 4.1 bc
35°C for 6 h, thiophanate-methyl	91.4 ± 3.5 cd	8.6 ± 3.1 cd
50°C for 30 min, thiophanate-methyl	94.3 ± 3.5 cde	5.7 ± 2.9 cde
35°C for 6 h, cyprodinil,+,fludioxonil	94.3 ± 5.7 cde	5.7 ± 4.2 cde
30°C for 12 h, tebuconazole	97.1 ± 2.9 de	2.9 ± 3.1 de
40°C for 2 h, cyprodinil + fludioxonil	97.1 ± 3.5 de	2.9 ± 3.0 de
52°C for 30 min, water	97.1 ± 2.9 de	2.9 ± 2.7 de
30°C for 12 h, thiophanate-methyl	100 ± 0.0 e	0 ± 0.0 e
51°C for 30 min, water	100 ± 0.0 e	0 ± 0.0 e
50°C for 30 min, water	100 ± 0.0 e	0 ± 0.0 e
50°C for 30 min, cyprodinil + fludioxonil	100 ± 0.0 e	0 ± 0.0 e
40°C for 2 h, water	100 ± 0.0 e	0 ± 0.0 e
35°C for 6 h, water	100 ± 0.0 e	0 ± 0.0 e
30°C for 12 h, water	100 ± 0.0 e	0 ± 0.0 e
Untreated control	100 ± 0.0 e	0 ± 0.0 e

[a] Mean values within each column are significantly different ($P \leq 0.05$; LSD tests).
LSD (incidence) = 14.1, F Value: 4.53. LSD (eradication) = 10.7, F value: 6.39.
SEs: Standard error values.
The experiment was repeated once and the results were calculated by averaging incidence and eradication rates obtained from both experiments.

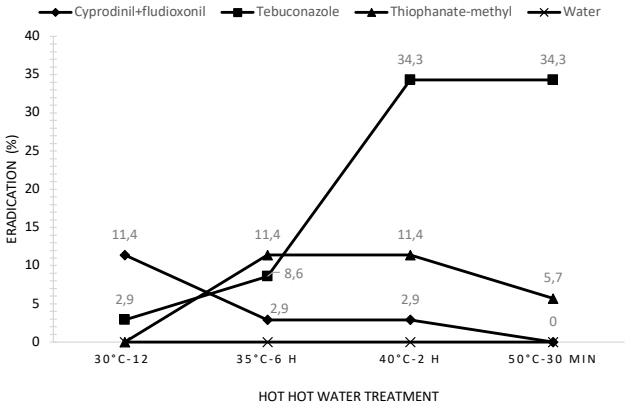

Figure 1. Mean eradication proportions (%) for *Neofusicoccum parvum* from grapevine cuttings by different fungicide suspensions and different hot-water treatments.

Effects of hot-water treatments on endophytic fungi

In the untreated control, most of the species had increased incidences during plant growth (Figure 2 a). However, with increased temperatures in the HWTs, there was progressive reductions in re-isolation rates of endophytic fungi in dormant canes (Figure 3 b-g). The 53°C for 30 min HWT almost eradicated all entophytic fungi except *Acremonium* sp. and *Alternaria alternata* (Figure 2h). *Acremonium* sp. was very tolerant to hot-water treatments, with increased re-isolation rates at from 30 to 52°C. In general, the HWTs decreased the other fungi. *Cladosporium* sp. also showed tolerance to HWTs. Compared with the initial isolation rates, final incidence of these fungi decreased for most of the treatments, but these fungi could not be fully eradicated, except at 53°C for 30 min. *Quambalaria cyanescens* was the most susceptible species, because this species was completely eradicated from dormant cuttings by temperatures of 50 to 53°C.

On the other hand, HWTs at 50°C and below, combined with fungicides gave greater eradication when compared to HWTs alone. Following these combined treatments, re-isolation rates of endophytic fungi (except *Acremonium* sp.) decreased, for almost in all of the combinations tested. Among the fungicides, tebuconazole was the most eradicative, at temperatures of 30°C to 50°C. At 30°C for 12 h dipping, most of the endophytic fungi were not be isolated after 10 d on PDA. All the tebuconazole suspension treatments completely suppressed endophytic fungi from wood pieces on isolation plates, however *Acremonium* sp. was observed at with low rates after 10 d (3 to 14%; Figure 3 b, e, h, k). For thiophanate-methyl, only *Penicillium* was eradicated at 40°C for 2h. Although incidences of *Alternaria* and

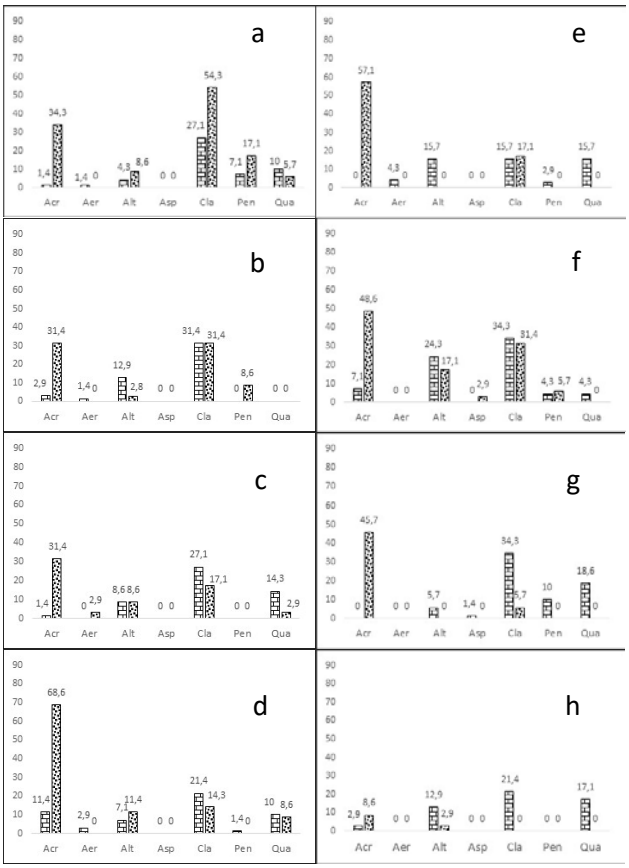

Figure 2. Mean incidence (%) of endophytic fungi in dormant grapevine cuttings after different hot-water treatments (without fungicides). The first and second columns in each histogram indicate, respectively, the re-isolation rates of endophytic fungi before and after the treatments. a) un-treated control, b) 30°C for 12 h, c) 35°C for 6 h, d) 40°C for 2 h, e) 50°C for 30 min, f) 51°C for 30 min, g) 52°C for 30 min, and h) 53°C for30 min.

Cladosporium were substantially decreased, they were not be eradicated with this treatment (Figure 3 i). The other combinations of thiophanate-methyl (30°C for 12h or 35°C for 6h) did not eradicate the other endophytic fungi (Figure 3 c, f). Cyprodinil + fludioxonil markedly suppressed occurrence of *Cladosporium* but did not affect *Acremonium* and *Alternaria* development (Figure 3 a, d, g, j).

Antagonistic effects of endophytes against Neofusicoccum parvum

The endophytic isolates gave inhibitive effects in the PDA dual culture experiment, and the inhibition interactions were of different types. *Trichoderma atroviride* gave the greatest inhibition of *N. parvum*.

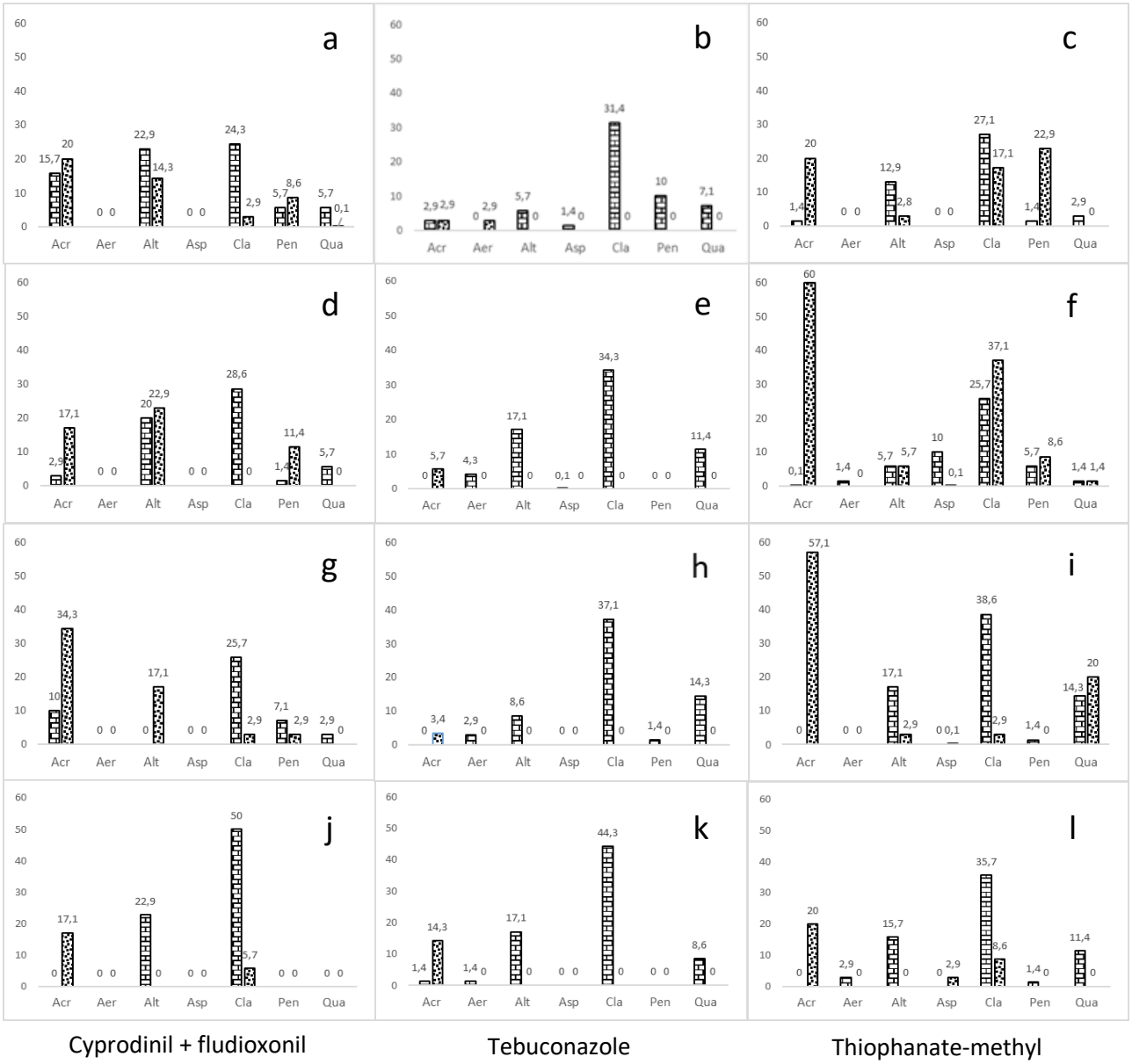

Figure 3. Mean incidence (%) of endophytic fungi in dormant grapevine cuttings after different hot-water treatments (combined with fungicides). The first and second columns in each histogram indicate, respectively, the re-isolation rates of endophytic species before or after the treatments were applied. a) b) c) 30°C for 12 h, d) e) f) 35°C for 6 h, g) h) i) 40°C for 2 h, j) k) l) 50°C for 30 min.

While *T. atroviride* (CUZFVG243) inhibited *N. parvum* growth by 69% (interaction class C), the inhibition percentages of *Acremonium* sp. (CUZFVG42), *E. nigrum* (CUZFVG88), *Geosmithia* sp. (CUZFVG40), and *Q. cyanescens* (CUZFVG39) were from 9 to 23% (class A). *Alternaria alternata* and *C. cladosporioides* moderately inhibited the pathogen, by, respectively, 31% and 30% (class B) (Table 5).

DISCUSSION

In this study, a variety of endophytic and pathogenic fungi were isolated from dormant and apparently healthy grapevine cuttings, taken from different geographical regions in Turkey. *Acremonium* sp., *Alternaria* spp., *Aureobasidium pullulans*, *Cladosporium* spp., and *Quambalaria cyanescens* were the most frequently isolated endophytic fungi, and *Diplodia seriata* was the

Table 5. Mean inhibition percentages, and competitive interactions with *N. parvum* in dual culture tests, for different isolates of grapevine endophytic fungi.

Endophytic isolates	Inhibition (%)	Interaction Class
Alternaria alternata (CUZFVG12)	31.1 ± 0.9 d [a]	B [b]
Acremonium sp. (CUZFVG42)	9.6 ± 1.0 a	A
Cladosporium cladosporioides (CUZFVG38)	29.5 ± 0.3 d	B
Epicoccum nigrum (CUZFVG88)	22.6 ± 1.1 c	A
Geosmithia sp. (CUZFVG40)	9.0 ± 1.4 a	A
Quambalaria cyanescens (CUZFVG39)	14.5 ± 1.0 b	A
Trichoderma atroviride (CUZFVG243)	69.0 ± 1.0 e	C

[a] Means values within the column are significantly different ($P \leq 0.05$), based on LSD tests.
LSD = 2.37, F value: 277.6, SEs: Standard error values
[b] Classification using the Badalyan scale (Badalyan *et al.*, 2002). A: deadlock with mycelial contact, B: deadlock at a distance, C: replacement, overgrowth with and without initial deadlock.

predominant pathogenic species from all of the regions. Halleen *et al.* (2003), screened fungi associated with healthy grapevine plants from nurseries in South Africa, and they obtained endophytic and pathogenic fungi from roots, and rootstock, grafting union and scion tissues. *Acremonium* spp. *Phoma* spp. *Alternaria* spp. *Aspergillus* spp. *Clonostachys* spp., and *Cladosporium* sp. were considered to be endophytic, but *Phaeomoniella chlamydospora, Phaeoacremonium* spp. *Botryosphaeria* spp. *Cylindrocarpon* spp. and *Phomopsis viticola* were found to be pathogenic. Pancher *et al.* (2012) investigated endophytic fungal communities from organic and Integrated Pest Management (IPM) vineyards at seven locations in Italy. Dormant canes of 'Cabernet Sauvignon' and 'Merlot' cultivars were examined for fungi using classical and molecular (ITS sequencing). Their results showed that organic vineyards had richer endophyte communities than IPM vineyards, and that *Alternaria* sp., *Epicoccum nigrum*, *Aureobasidium pullulans* and *Cladosporium* sp. were the most frequently isolated fungi from both types of vineyards. Kraus *et al.* (2019) investigated early development of endophytic fungi in healthy grapevines (from 2 months to 8 years old), and determined genera and species of fungi. Fast-growing fungi, such as *Alternaria* spp., *Aureobasidium pullulans*, *Cladosporium* spp. and *Epicoccum nigrum*, were isolated from vines that were less than 1-year-old, while many grapevine trunk diseases associated pathogens such as *D. seriata* or *Eutypa lata*, along with endophytic fungi, were obtained from perennial branches. *Alternaria* spp., *Aureobasidium pullulans*, *Cladosporium* spp. and *Epicoccum nigrum* made up 81% of the total fungal flora. Casieri *et al.* (2009) assessed vine wood samples taken from different regions and cultivars in Switzerland for endophytic and pathogenic fungi. They found that the composition of endophytes was different for each grape cultivar, and suggested that biochemical composition of the grape cultivars may have played an important role in fungal endophyte diversity. Endophytic and pathogenic fungi may vary according to plant age, geography, cultivar, type and age of tissues (Sieber *et al.*, 1991; Rodrigues, 1994). The results of these studies corroborate our isolation results, from 1-year-old dormant grapevine canes taken from three regions in Turkey.

The hot water treatments (below 50°C with extended duration) were not effective for eradication of *N. parvum* from inoculated dormant grapevine cuttings, but these treatments reduced re-isolation rates of the pathogen, when they were combined with fungicides. This indicates that increased water temperatures probably enhanced penetration of the fungicides into the inner wood tissues. We have been unable to find similar reported results for grapevine, but there are supportive studies conducted on postharvest disease management for fruits. Positive synergistic effects of hot-water and fungicide combinations have been reported Barkai-Golan and Appelbaum (1991), McGuire and Campbell (1993) and Smilanick *et al.* (1995). Cabras *et al.* (1999) have also demonstrated that the cuticle barrier of fruits was weakened with hot water, thus increasing the diffusion of fungicides into fruit rinds. Schirra *et al.* (1996) investigated curative effects of imazalil against blue mould of lemon (caused by *Penicillium italicum*) at the rates from 250 mg·L^{-1} to 1500 mg·L^{-1}, and with water temperatures of 20°C to 50°C for 3 min. Imazalil at 250 mg·L^{-1} and 50°C gave the same curative effect as 1500 mg·L^{-1} at 20°C, Imazalil residue in fruit rinds was 4-5 time greater for the low rate high temperature treatment than for high rate low temperature treatment. Waite *et al.* (2018) reported that dipping grapevine propagation material in hydration tanks for more than 30 min had caused softening of bark tissues which became prone to increased fungal infections. These results indicate that plant tissue softening caused by heated water may also enhance fungicide penetration. It is possible that fungicides may transport from the tips of grapevine cuttings to further parts, by passive diffusion, but this would only be possible if there was one-way flow of water during hydration. There would likely be very little water influx from the opposite ends of cuttings allowing passive infusion of fungicides in hydration tanks.

The heated suspensions of tebuconazole were more effective for eradication of *N. parvum* from dormant grapevine cuttings than those of cyprodinil + fludiox-

onil or thiophanate-methyl. Similar effects were also observed on endophytic fungi, so that most of the endophytes could not be isolated from the cuttings treated with hot-water and tebuconazole combinations. The fungicides assessed in the present study have been reported to be effective for preventing wound infections of vines in field conditions, or contamination of cuttings by many GTD pathogens in hydration tanks (Rolshausen *et al*., 2010; Amponsah *et al*., 2012; Pitt *et al*., 2012). However, in our experiments only tebuconazole showed high efficacy for reducing of colonization by *N. parvum*. We suggest that the differences in fungicide formulations may be responsible for these results. While tebuconazole was applied as an emulsion oil formulation in water, cyprodinil + fludioxonil and thiophanate-methyl were, respectively, wettable granule and wettable powder formulations. Active ingredients in powder or granule formulations may not have passed through grapevine barriers to reach inner tissues. Reports in PubChem indicate that these fungicides are stable in water, soil and air for long periods, so maximum duration in hot-water treatments (12 h) is unlikely to degrade the fungicides (Anonymous, 2019).

The present study investigated whether some endophytic fungi obtained from dormant grapevine cuttings had antagonistic effects against *N. parvum* in dual culture tests. All the endophyte isolates used in these tests were antagonistic to the pathogen. It has been previously shown that secondary metabolites secreted by endophytic fungi affect host plant physiology and other fungi (Schulz and Boyle, 2005). In an *in vitro* study by Wang *et al*. (2013), *Cladosporium cladosporioides* produced four different secondary metabolites (cladosporin, isocladosporin, 3,5'hydroxasperentin and cladosporin-8-methyl ether) in potato dextrose broth. Of these, cladosporin was the most inhibitory to mycelium growth of *Colletotrichum acutatum, Co. fragariae, Co. gloeosporioides* and *Phomopsis viticola* in a micro dilution broth assay reducing growth of these fungi by 80 to 93%. Springer *et al*. (1981), reported cladosporin to be a plant growth regulator, suppressing growth of etiolated coleoptiles of wheat. In our results, *Cladosporium* was a predominant endophytic genus isolated from vineyards in Manisa and Tarsus cities (located in warm climates), but antagonism by *Cladosporium* was moderate to *N. parvum* mycelium growth in dual culture tests. Musetti *et al*. (2007) extracted diketopiperazine compounds from the culture filtrates of *Alternaria alternata*, and tested inhibitory effects of these against downy mildew of grapes in greenhouse conditions. Spray applications of these secondary metabolites reduced (by up to 100%) disease severity, when vine leaves were treated with the compounds 2 or 24 h after inoculation with *Plasmopara viticola*. *Acremonium* has also been reported to produce biologically active metabolites, including β-lactam and cephalosporin antibiotics, and tremorgenic indole-diterpenoids. *Acremonium* also triggers some physiological pathways related to resistance of *Gramineae* plants (Gatenbay *et al*., 1999; Moussaif *et al*., 1997; Lindsey *et al*., 2002; Adinaryana *et al*., 2003). Assante *et al*. (2005) determined secondary metabolite profiles of endophytic *Acremonium* isolates (from grapevine leaves, cv. Reginabianca), and detected four different Acremines (A, B, C, D) in an *in vitro* study. These compounds have been found to inhibit sporangium germination of *P. viticola*, and maximum inhibition (99.8%) was obtained with "Acremin C" at 1 mM concentration. *Trichoderma atroviride* is a well-known endophytic and soil-borne fungus with antagonistic ability to many fungal pathogens, and some of the *Trichoderma* isolates are widely used in the control of GTD pathogens in Europe. Kotze *et al*. (2011) reported that *T. atroviride* was effective as a pruning wound protectant against GTD pathogens, including *N. parvum*, in South Africa. Their isolates provided satisfactory protection of grapevine pruning wounds against *Phompsis viticola*, *E. lata*, *P. chlamydospora*, *N. australe*, *N. parvum*, *D. seriata* and *L. theobromae*, reducing disease incidence by 69-92%. In Portugal, another strain of *T. atroviride* was reported to be highly suppressive to incidence and severity of disease caused by *P. chlamydospora* and *N. parvum* (Reis *et al*., 2017).

In the present study, the *T. atroviride* isolate (CUZ-FVG243) exhibited antagonistic performance (69% reduction) against *N. parvum* in PDA dual cultures competing for space and hyperparasitism. This isolate needs to be tested under field conditions for useful biocontrol effects. The endophytic *Q. cyanescens* was frequently isolated as an endophyte in Tarsus vineyards, but did not show strong antagonism to *N. parvum* (14.5%). This endophyte has been reported to affect grapevine physiology by producing secondary metabolites such as resveratrol (Srivastava, 2015).

CONCLUSIONS

Hot-water treatment is an effective strategy which should be used in grapevine nurseries to produce healthy plants. In the present study, hot-water treatments using fungicide suspensions, at 40°C and for 2 h durations, facilitated fungicide penetration into inner tissues of dormant grapevine canes. Although maximum reduction of *N. parvum* infections was 34%, this reduction could be practically significant because the propagation material

used was severely colonized by the pathogen. Reduction rates could be greater where infections were less severe. Hot-water treatment also eradicates endophytic fungi, and this effect could be negative for plant health. However, due to rapid proliferation and recovery by endophytes, these negative effect may be compensated by additional measures such as using antagonistic *Trichoderma* biocontrol products in nurseries. Additionally, fungicides need to be further evaluated for curative effects on latent infections by *N. parvum* in plant material, when applied in hydration and hot-water treatment systems.

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Research Paper

Activity of extracts from three tropical plants towards fungi pathogenic to tomato (*Solanum lycopersicum*)

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Summary. Antifungal properties were assessed of water and ethanol extracts from the pan-tropical plants *Oxalis barrelieri* L., *Stachytarpheta cayennensis* L., and *Euphorbia hirta* L. against *Fusarium oxysporum* f. sp. *vasinfectum*, *Alternaria solani* Sorauer, and *Rhizoctonia solani* Kuhn. The plant extracts inhibited fungal growth *in vitro* at 1.25-20 mg mL^{-1}, and the degrees of inhibition increased in a dose-dependent manner. Ethanol extracts from the plants inhibited fungal growth by 80-100%, while water extracts showed less antifungal activity, with maximum growth inhibition of 62%. Growth inhibition from ethanol extracts was two- to three-fold greater than for water extracts at equivalent concentrations. Antifungal activity of the extracts varied with their content and composition of phenolics, flavonoids, tannins, and alkaloids. In greenhouse experiments, spraying tomato plants (*Solanum lycopersicum* L.) with ethanol extract from *E. hirta* at 2.5 mg mL^{-1} did not cause phytotoxicity, and increased plant size, when compared to untreated plants. Spraying *E. hirta* ethanol extract on tomato plants infected by *R. solani* reduced disease severity up to 80%, when compared to non-sprayed plants. These results demonstrate potential of leaf extracts from *E. hirta*, *O. barrelieri*, and *S. cayennensis* as biofungicides for the control of *R. solani*, *A. solani*, and *F. oxysporum*, which are among the most important causal agents of tomato diseases.

Keywords. *Euphorbia hirta*, *Oxalis barrelieri*, *Stachytarpheta cayennensis*, antifungal activity, plant growth promotion.

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INTRODUCTION

In Cameroon, tomato is the most important vegetable crop, with production of over 1.18 million tons harvested from 92,626 hectares in 2016 (FAO, 2017). However, phytopathogenic fungi are responsible for the most prevalent

diseases, including: late blight, caused by *Phytophthora infestans* (Mont.) de Bary; early blight, caused by *Alternaria* spp.; damping-off and seedling blights, caused by *Pythium* spp.; *Rhizoctonia solani* Kuhn and *Verticillium albo-atrum* Reinke & Berthold; and Fusarium wilt and root rot, caused by *Fusarium oxysporum* f. spp. (Jones *et al*., 2014). Furthermore, bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al*., and bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davis *et al.* are also reported to cause severe crop losses in epidemic years (Fontem *et al*., 1999).

Most strategies for pest management rely on frequent application of synthetic chemical pesticides and copper compounds, to avoid yield and quality losses. These may have consequences for human health, the environment, and from development of resistant pathogen strains, due to continuous use of the same available chemicals (Ishii and Hollomon, 2015; Lucas *et al*., 2015). For these reasons, it is important to identify new effective strategies for disease management that pose reduced risks to human health and the environment.

Biofungicides have emerged as the main alternative to conventional fungicides, and the application of plant-derived products is a potential choice in disease management (Ribera and Zuñiga, 2012; Pusztahelyi *et al*., 2015). Tropical plants are rich sources of bioactive chemicals for the development of biopesticides as safe disease control agents, as reviewed by Suprapta (2016). Examples include papaya anthracnose (*Colletotrichum gloeosporioides*) that can be efficiently managed using extracts from tickberry (*Lantana camara*, *Verbenaceae*) (Ademe *et al*., 2013); and grey mold (*Botrytis cinerea*) of blackcurrant controlled by extracts from hyssop (*Hyssopus officinalis*, *Lamiaceae*) and summer savory (*Satureja hortensis*, *Lamiaceae*) (Sesan *et al*., 2015). Several plants are reported to produce diverse arrays of low molecular mass antimicrobial compounds, often called 'natural products'. These may also play important roles in plant physiology. Some of these compounds may provide antimicrobial potential and beneficial effects on plants, such as early seed germination, plant growth promotion, improved crop yield, and increased tolerance to abiotic and biotic stresses (Wink, 2010). The compounds can also enhance postharvest shelf-life of perishable products (Ji *et al*., 2005; Yang *et al*., 2011; Kharchoufi *et al*., 2018; Scavo *et al*., 2019).

Many local plants commonly present in several Cameroon provinces, including *Oxalis barrelieri* L., *Stachytarpheta cayennensis* L., and *Euphorbia hirta* L., have been gaining attention, based on their ethnobotanical uses, phytochemical and pharmacological properties, and their easiness to be cropped in tropical areas. *Euphorbia hirta* has been chemically studied and found to possess antifungal activity against *Fusarium moniliforme* Sheldon and *Phoma sorghina* Saccardo (Karanga *et al*., 2017). *Oxalis barrelieri* plant extracts showed inhibitory effects on mycelium growth and conidia germination of *F. oxysporum* and *P. infestans* (Dakole *et al*., 2016). Plant extract of *S. cayennensis* inhibited the growth of different bacteria (Okoye *et al*., 2010).

Effects of plant extracts on fungal pathogens of tomato have not been studied. The present study aimed to: i) examine the antifungal activity of water and hydro-ethanolic extracts of *E. hirta*, *O. barrelieri* and *S. cayennensis* against three major phytopathogenic fungi affecting tomato (*i.e. R. solani*, *A. solani*, and *F. oxysporum*); ii) determine the phytochemical composition of these extracts; and iii) assess the growth promoting and protective effects of the *E. hirta* ethanol extract on tomato. Once antimicrobial activity is confirmed, such extracts or their components may have potential to be developed as innovative agrochemicals, for implementing sustainable pest management in organic and integrated tomato production.

MATERIALS AND METHODS

Plant material

Three pan-tropical plant species, *Oxalis barrelieri* L. (*Oxalidaceae*), *Stachytarpheta cayennensis* L. (*Verbenaceae*) and *Euphorbia hirta* L. (*Euphorbiaceae*) were collected from a local area (Central Region, Yaoundé-Mbankomo, Cameroon) (Figure 1). These species were chosen based on their previously studied phytochemical properties (Senthikumar, 2018). They were grown until the flowering stage and harvested. After harvesting, the plant material was shade-dried for 2 weeks, and the dried leaves were milled into powder.

Tomato seeds 'Leader F1', obtained from ISI Sementi, Fiorenzuola d'Arda, Italy, were sown and transplanted in a greenhouse into trays containing a commercial soil mix (Dueemme Marketing srl.). The greenhouse was maintained at a constant temperature of 27°C, with a 16 h photoperiod each day. Tomato plants were used for experiments 3 weeks after sowing, at the five to six true leaf stage.

Fungus strains

Fusarium oxysporum f. sp. *vasinfectum*, strain FUSITS04 and *Alternaria solani* Sorauer, both originat-

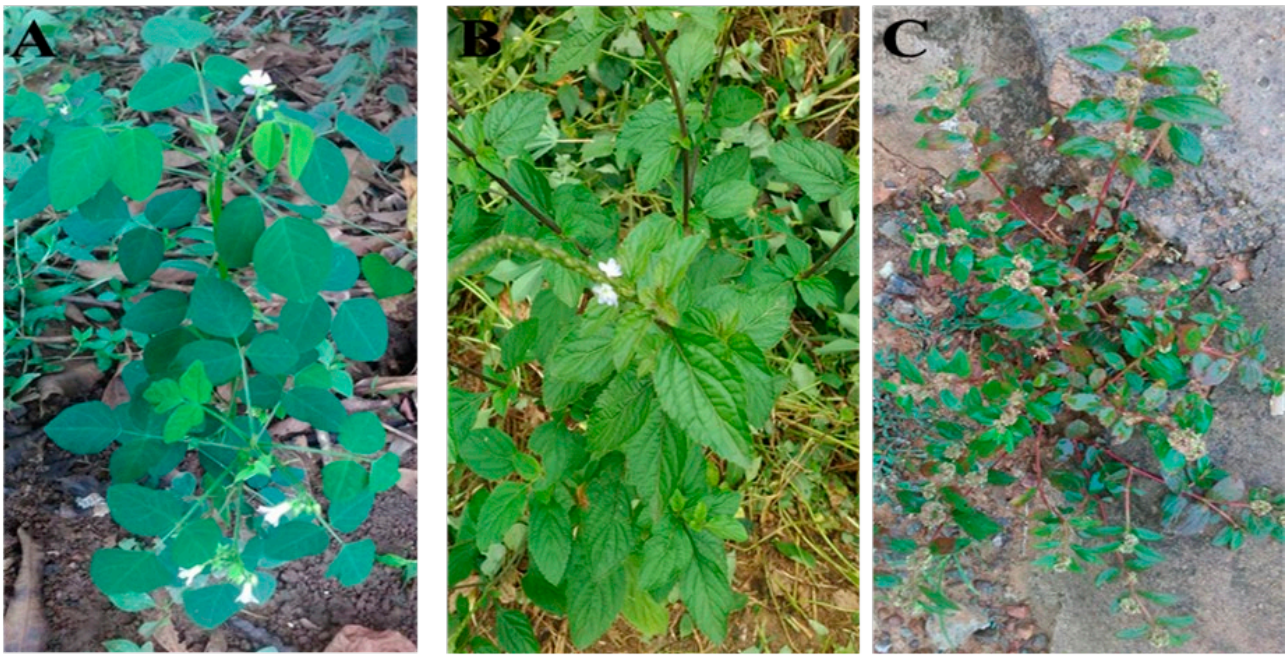

Figure 1. Pan-tropical plants examined in the present study: *Oxalis barrelieri* L. (A), *Stachytarpheta cayennensis* L. (B), and *Euphorbia hirta* L. (C). These plants are shown at the beginning of the flowering stage, during which they were harvested and dried.

ing from Cameroon, and *Rhizoctonia solani* Kuhn (courtesy of P. Nipoti, University of Bologna, Italy) were used during the experiments. These fungi were isolated from diseased tomato plants, and were maintained on 3.9% potato dextrose agar (PDA) at 4°C until used for experiments. *R. solani* was also used for greenhouse *in planta* experiments.

Preparation of crude plant extracts

Before extraction, leaf powders were defatted (1:6, w/v) by mixing in 600 mL of anhydrous n-hexane under continuous stirring at room temperature (21 ± 1°C) for 24 h. After filtration through fine cloth, each defatted plant residue was hexane-evaporated in a laminar flow cabinet. Extraction of the plant residues was achieved using either distilled water or 70% hydro-ethanol solution. Plant material was soaked (1:6, w/v) in 600 mL of distilled water or in 600 mL of ethanol solution under continuous stirring for 24 h, followed by filtration through Whatman No. 1 filter paper (11 µm pore size) and centrifugation at 5,200 × *g* for 10 min. The supernatants were then collected and the solvents evaporated overnight (12 h) in a ventilated oven at 50°C. The dried pellets obtained were designated as: WE*ox* (water extract of *O. barrelieri*), EE*ox* (ethanol extract of *O. barrelieri*), WE*st* (water extract of *S. cayennensis*, EE*st* (ethanol extract of *S. cayennensis*), WE*eu* (water extract of *E. hirta*), or EE*eu* (ethanol extract of *E. hirta*). The extracts were stored at 4°C until used.

Effects of plant extracts on fungus growth in vitro

Effects of different water and ethanol extracts on growth of fungi were assayed *in vitro* on PDA agar plates, amended with different concentrations of the plant extracts, using the supplemented agar method described by Rios *et al.* (1988).

Five increasing amounts of each plant extract, from 0.125 to 2 g, were added to flasks containing 100 mL of PDA medium before autoclaving (at 121°C for 15 min). This gave final concentrations of each extract in poured PDA plates of 1.25, 2.50, 5, 10, or 20 mg mL^{-1}. PDA plates without extract additions were used as experimental controls. Each agar plate was then inoculated with a 5 mm diam. mycelium plug taken from the margin of a 7-d-old culture, and kept in an incubator at 27°C. Growth was assessed after 7 d, by measuring two opposing diameters of the fungus colony. Growth inhibition relative to the controls was calculated according to the following equation:

$$\text{Growth inhibition (\%)} = \frac{D - d}{D} \times 100$$

where, D = colony diameter in the control PDA plate, and d = colony diameter in the amended PDA plate.

Each fungus-extract combination was replicated in five plates, and all experiments were independently repeated three times.

Greenhouse in planta *experiments*

Application of plant extract and fungus inoculation

To perform *in planta* experiments, the most active ethanol extract of *E. hirta* (EE*eu*) was chosen for assessment against *A. solani*. Prior to these experiments, a preliminary assay was carried out on a set of tomato plants to assess for possible phytotoxic effects of EE*eu*. Since no visible phytotoxic effects were observed in this assay after 1 week, an aqueous solution of EE*eu* was used as a spray onto the shoots canopy of tomato plants, at a concentration of 2.50 mg mL^{-1}.

Inoculum of *R. solani* was prepared from 7-d-old cultures grown on PDA by gently blending the mycelium with the agar to obtain an inoculum paste. Inoculation was carried out 48 h after EE*eu* applications, by transferring the tomato plants into new pots containing infested soil-perlite (3:1, v:v). The soil was infected by setting 2.5 g of *R. solani* inoculum paste at the bottom of a hole made at mid-depth in each pot (Logemann *et al*., 1992). The tomato plants were carefully uprooted from their previous pots, partially cut at their root ends and transferred into the inoculated pots, so that the inoculum paste became in contact with the wounded roots. The pots were then arranged in a completely randomized design in a greenhouse maintained at 27°C, under a 16 h photoperiod each day and at 70% relative humidity, and were watered appropriately. The experimental design considered four experimental treatments: i) plants sprayed with EE*eu* (TE); ii) plants inoculated with *R. solani* (TR); iii) plants sprayed with EE*eu*, followed by inoculation with *R. solani* (TE+R); or iv) untreated tomato plants, as the experimental control (TC). Ten replicate plants were used for each treatment, experiments were repeated three times independently.

Evaluation of tomato plant growth promotion and disease

For each treatment, the heights of the treated plants were measured at 7, 14, and 21 d after extract application, to evaluate effects of the extract on plant growth.

Disease severity was recorded for TR and TE+R treatments at 5, 10, 15, and 20 d after inoculation. Severity was scored using a 0-5 scale, were: 0 = healthy plant, 1 = 1-10% of leaves with initial wilts, 2 = 11- 25% of leaves with wilts, 3 = 26-49% of leaves showing wilting and chlorosis, 4 = 50-74% of leaves showing pronounced wilting and development of necrotic areas, and 5 = whole leaves wilting. Severity was calculated according to the following equation:

$$\text{Disease severity (\%)} = \frac{\Sigma dn}{DN} \times 100$$

where, d = severity score; n = number of disease plants with the same severity score; N = total number of the examined plants, and D = the greatest severity score.

Disease reduction was calculated by comparing disease severity observed on plants using the following equation:

$$\text{Disease reduction (\%)} = \frac{S - s}{S} \times 100$$

where, S = disease severity on tomato inoculated with *R. solani* (treatment TR), and s = disease severity on tomato treated with EE*eu* then inoculated with *R. solani* (treatment TE+R).

Analyses of phytochemical contents and antioxidant potential

Preliminary analyses of crude plant extracts were performed to assess their phytochemical compositions. Each extract was analyzed for: i) total phenolic content using the Folin-Ciocalteu reagent, using the method of Singleton *et al.* (1999); ii) total flavonoid content, using the method of Zhishen *et al.* (1999); iii) total tannin content, using the method described by Verzelloni *et al.* (2010); iv) total alkaloid content, using bromocresol green reagent and the method described by Tabasum *et al.* (2016); v) total polysaccharides content using phenol-sulfuric acid after mild acid hydrolysis, using the method of Dubois *et al.* (1956); and vi) total protein content using Bradford's reagent, and Bradford's method (Bradford, 1976).

Antioxidant activity of the plant extracts was measured using the free radical cation 2,2-azinobis-3-ethyl benzothiazoline-6-sulfonic acid, using the method described by Re *et al.* (1999).

Statistical analyses

All data were statistically analyzed using the MaxStat Lite software, version 3.60 (available at: https://maxstat-lite.soft112.com/). Data were subjected to analysis of

variance (ANOVA) and the means comparison carried out using Tukey's multiple range test at $P \leq 0.05$. Principal component analyses (PCA) were performed using the software package Solo, version 8.6.1, (Eigenvector Research, Inc. Manson, WA, USA), considering the analytical properties as variables.

RESULTS

Characteristics of plants extracts

The mean yields of the extracts, based on initial dry biomass of leaves, varied between 5 and 8% (w/w). More precisely, extraction productivity for the different plant extracts was: WE*ox*, 6.2%; EE*ox*, 5.1%; We*st*, 7.4%; EE*st*, 5.3%; WE*eu*, 8.2%; and EE*eu*, 5.6%.

Extracts differed in colour: water extracts were dark brown, and the ethanol extracts were grayish-green.

Antifungal activity of plant extracts

The addition of the plant extracts to PDA medium at all concentrations did not affect medium consistency, but only its colour. Growth of the three phytopathogenic fungi on PDA medium, without addition of plant extracts, reached the following mean colony diameters after 1 week: 8.50 cm for *R. solani*, 7.50 cm for *F. oxysporum* f. sp. *vasinfectum* and 7.38 cm for *A. solani*. Addition of the plant extracts to PDA medium inhibited fungus growth in all of the substrate/fungus combinations, and inhibition was related to extract concentration in the medium (Figure 2A). Increasing concentration of EE*eu* led to complete inhibition of *F. oxysporum*. The same concentration of WE*eu* also inhibited growth of *F. oxysporum*, but to a lesser extent than for EE*eu* (Figure 2B). The tested plant extracts consistently reduced mycelium growth of *R. solani*, *F. oxysporum*, and *A. solani*.

The ethanol extracts gave greater antifungal activity than the water extracts. As little as 1.25 mg mL^{-1} of ethanol extracts from the three plants were sufficient to inhibit fungus growth from 10 to 28% (depending on fungus). Between 10 to 20 mg mL^{-1} of ethanol extract, growth inhibition was 90–100% (Figures 3A, 4A, and 5A). Therefore, the ethanol extracts in sufficient concentration completely inhibited growth of all three fungi. At low doses, water extracts from all three plants also inhibited fungus growth by 2 to 28% (depending on fungus) but differently from the ethanol extracts. Growth inhibition from the water extracts was never as great as with the ethanol extracts (Figures 3B, 4B and 5B). This confirms the greater antifungal activity of the ethanol than water extracts.

Fungus growth inhibition was dose dependent for all the fungus/extract combinations, but the inhibitory effects of extracts varied for extracts from the different plants. In general, extracts from *E. hirta* gave greater inhibition than those from *S. cayennensis*, which were more active than the extracts from *O. barrelieri*.

An effect was observed on the morphology of *F. oxysporum* grown on PDA plates amended with WEs. Mycelium colour and texture were greatly modified on amended PDA plates, as shown in Figure 6. These changes in morphology were not apparent for the other two fungi growing on WEs-amended media.

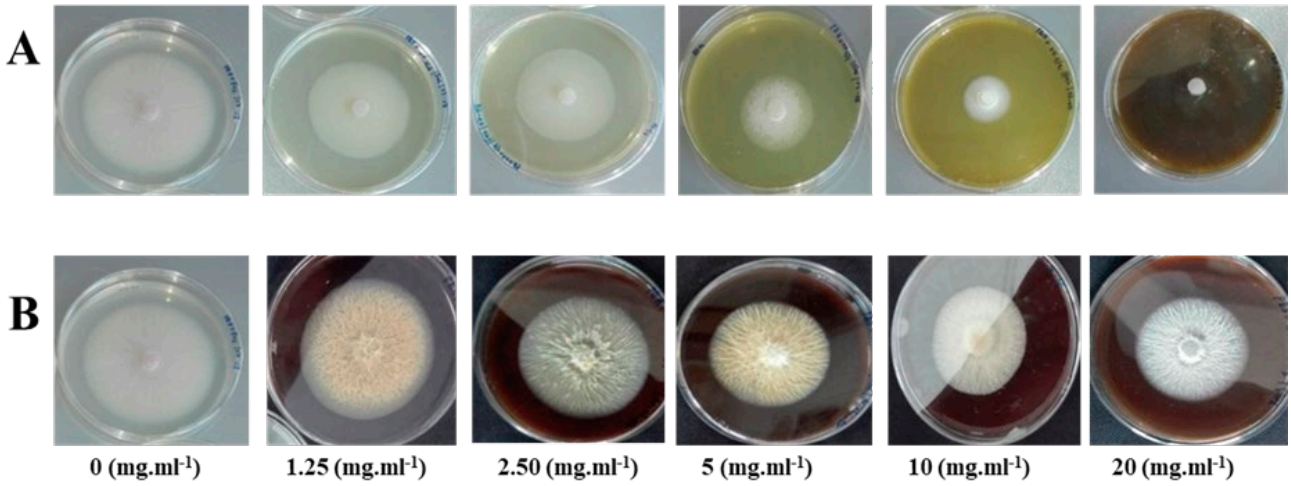

Figure 2. Inhibition of mycelium growth of *Fusarium oxysporum* f. sp. *vasinfectum* after 7 d on PDA supplemented with increasing concentrations of ethanol extract (A) and water extract (B) from *Euphorbia hirta*.

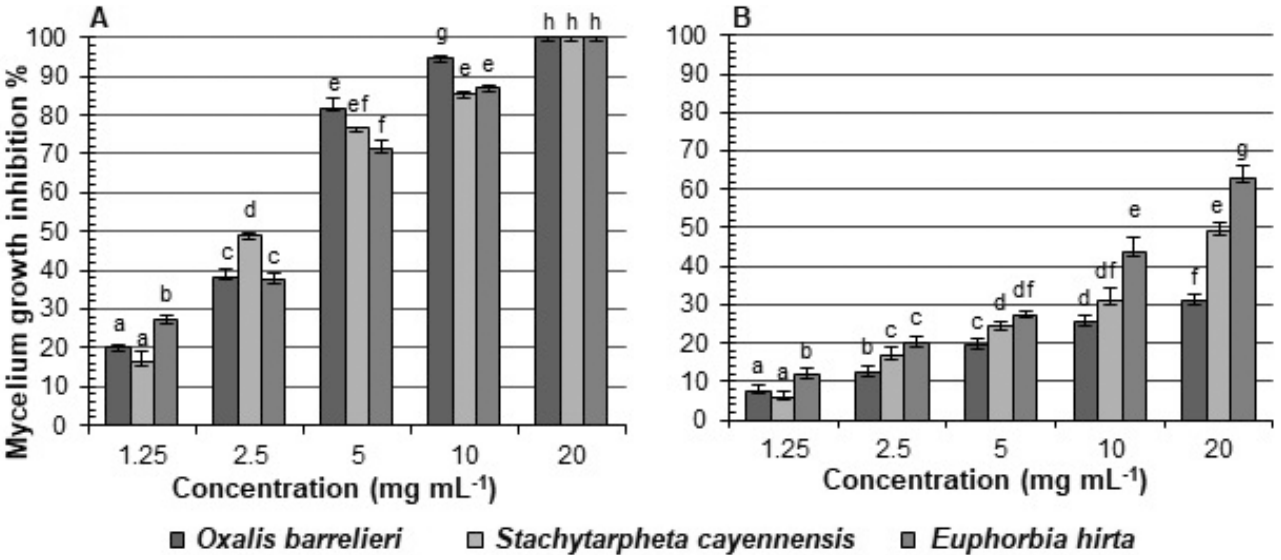

Figure 3. Mycelium growth inhibition (%) of *Fusarium oxysporum* f. sp. *vasinfectum* at 1.25, 2.50, 5, 10 or 20 mg mL^{-1} concentrations of the ethanol extracts (A) or water extracts (B) for extracts from three different plant species. Data are means ± standard deviation from three experiments, each with five replicates. At each extract concentration, the means accompanied by different letters are significantly different, according to ANOVA paired with Tukey's tests ($P \leq 0.05$).

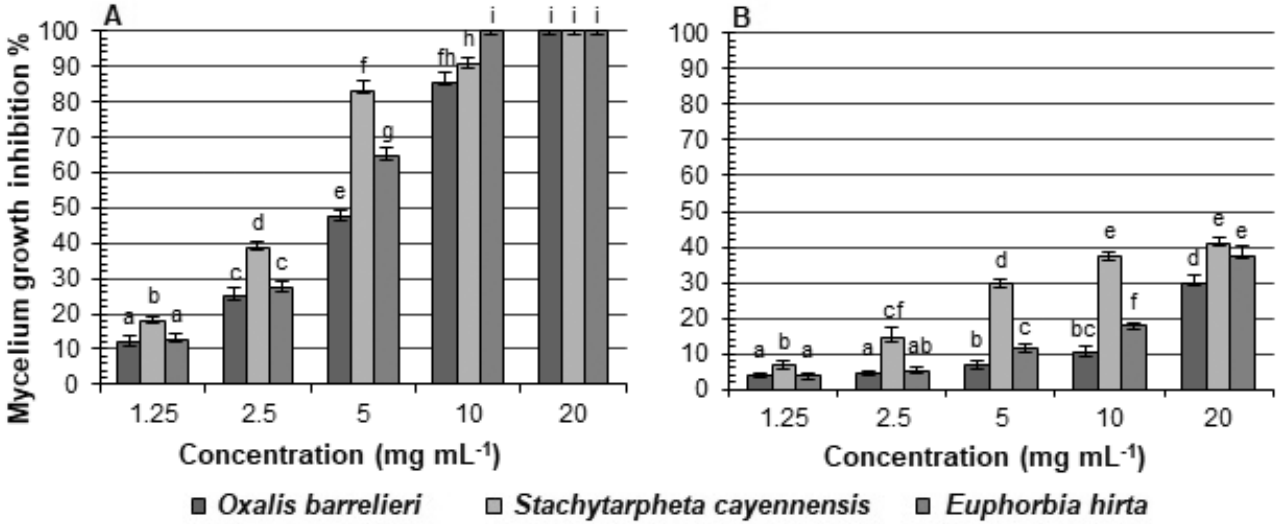

Figure 4. Mycelium growth inhibition of *Rhizoctonia solani* at 1.25, 2.50, 5, 10 and 20 mg mL^{-1} concentrations of the ethanol extracts (A) and water extracts (B) from three plant species. Data are means ± standard deviation from three experiments, each with five replicates. At each concentration, the means accompanied by different letters are significantly different, according to ANOVA paired with Tukey's tests ($P \leq 0.05$).

Effects of Euphorbia hirta *ethanol extract on tomato plant height*

The height of all tomato plants sprayed with EE*eu* (TE) was significantly increased after 7 d (Figure 7). Plant size increased during the following weeks and was greater ($P \leq 0.05$) in EE*eu*-treated plots, when compared to controls. Three weeks after spraying with EE*eu*, mean plant heights from the different extract treatments were: TE, 20.4 cm; TC, 14.5 cm; TE+R, 12.3 cm; and TR. 11.2 cm.

Disease reduction by Euphorbia hirta *ethanol extract*

Data of disease severity and reduction are summarized in Table 1. Tomato plants infected with *R.*

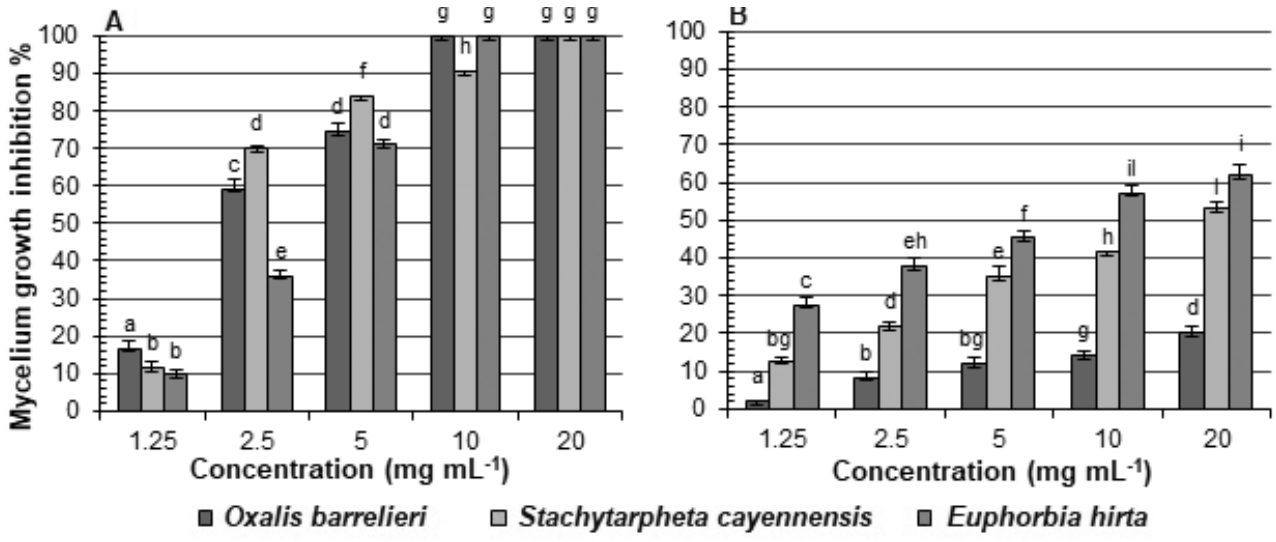

Figure 5. Mycelium growth inhibition of *Alternaria solani* at 1.25, 2.50, 5, 10 and 20 mg mL^{-1} concentrations of the ethanol extracts (A) and water extracts (B) from three plant species. Data are means ± standard deviation from three experiments, each with five replicates. At each concentration, the values accompanied by different letters are significantly different, according to ANOVA paired with Turkey's tests ($P \leq 0.05$).

solani (TR) showed greater disease severity, when compared to EE*eu*-treated plants inoculated with *R. solani* (TE+R). The final disease severity estimates gave 56.7 ± 5% from the TE+R treatment and 80 ± 2% from the TR treatment. This demonstrated that the *E. hirta* ethanol extract efficiently protected tomato plants from *R. solani* infections.

Phytochemical content and antioxidant capacity of the plant extracts

Means values of total phenolic, flavonoid, alkaloid, tannin, protein, and sugar contents and radical antioxidant activity of all the tested plant extracts are presented in Table 2. Except for the alkaloids, whose contents in plant extracts were very similar, the quantification of other chemical components was different for all tested plant extracts. EE*st* had the greatest amounts of phenolics (101.7 ± 11.8 mg gallic acid g^{-1}), flavonoids (33.5 ± 0.7 mg catechin g^{-1}), and tannins (7 ± 1.9 mg catechin g^{-1}). WE*ox* had the greatest amount of proteins (8.1 ± 0.8 mg BSA g^{-1}). The polysaccharide components were greater in water extracts compared to the ethanol extracts, except for EE*st* (179 ± 27.3 mg glucose g^{-1}) and WE*st* (94.8 ± 18.9 mg glucose g^{-1}).

Table 2 shows that WE*ox* and WE*st* were more effective in scavenging ABTS radical cation, with values, respectively, of 262.67 ± 41.48 and 250.33 ± 40.54 mg of L-ascorbic acid g^{-1}. Mean values for the other extracts were: for EE*st*, 168.33 ± 2.36; for EE*ox*, 157.22 ± 4.46; for WE*eu*, 112.53 ± 8.01, and for EE*eu*, 83 ± 26.40 mg of L-ascorbic acid g^{-1}.

The chemical nature of the extracts correlated with the extraction procedures. For instance, *O. barrelieri* was quite productive for total phenolics, when extracted with water (more than double the quantity of phenolics in WE than in EE), whereas *S. cayennensis* and *E. hirta* were much more productive when extracted with ethanol. *Euphorbia hirta* gave the maximum productivity using ethanol for all components, other than total polysaccharides. Total polysaccharides were more abundant in WEs of all three plants. This was expected, since polysaccharides are more soluble in water than in ethanol solutions (Guo *et al.*, 2017). Conversely, total alkaloids and total flavonoids were better extracted with ethanol than water from all three plants: in particular, *E. hirta* was very productive in total flavonoids when extracted with ethanol, compared to water extraction.

The cumulative percentage (74.7%) of the total variance explained by the first two principal components (PC1 and PC2) allowed designing the bidimensional plot represented in Figure 8. The distribution of extracts along PC1 and PC2 shows a clear distinction between ethanol and water extracts, respectively, positive and negative scores on PC1. In order to understand which variables accounted most for this distribution, they were added to the bidimensional plot. The water extracts had negative scores on PC 1 and were characterized by greater polysaccharide contents than the respective ethanol extracts, and the greatest

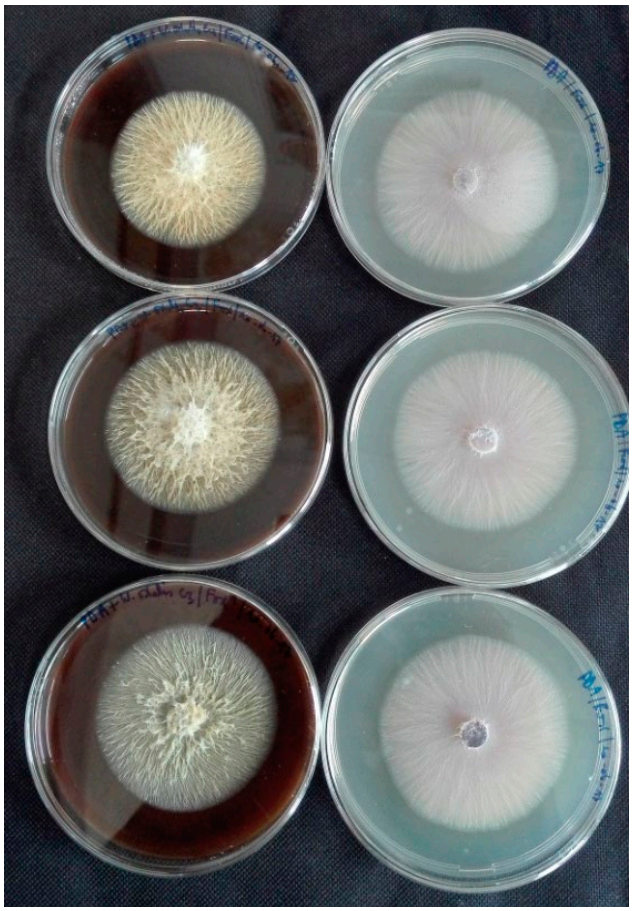

Figure 6. Morphological modification (colour, structure and texture) of *Fusarium oxysporum* f. sp. *vasinfectum* growing on modified PDA amended with water extract from *Oxalis barrelieri*. Left, PDA amended with 5 mg mL^{-1} of water extract; right, PDA without plant extract.

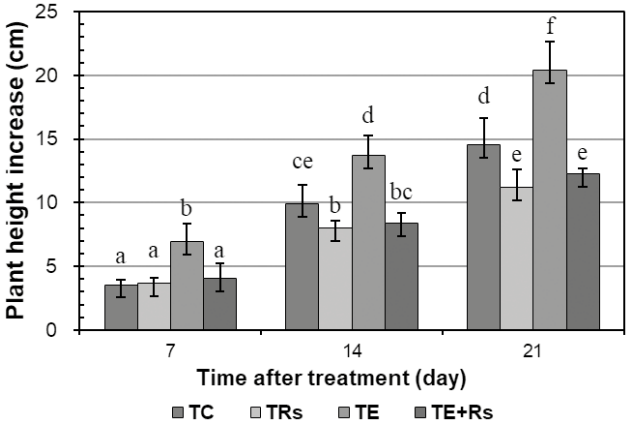

Figure 7. Height (cm) of tomato plants, with or without inoculation of *Rhizoctonia solani*, after treatment with *Euphorbia hirta* ethanol extract, for plants measured at 7, 14 or 21 days after treatment. TC: Negative control, sprayed with water; TRs: positive control, inoculated with *R. solani*; TE: Plants treated with *E. hirta* extract; TE+Rs: Plants treated with *E. hirta* extract and inoculated with *R. solani*. Values are means ± standard deviation, calculated for 30 plants per treatment. Values accompanied by different letters are significantly different, according to ANOVA paired with Tukey's tests ($P \leq 0.05$).

antioxidant activities (WE*ox* and WE*st*, with positive scores on PC 2). Otherwise, ethanol extracts, which were positively linked to PC 1, were characterized by the greatest phenolic, tannin and flavonoid contents (particularly EE*st* and EE*eu*), and were more effective for growth inhibition of the fungi than the respective water extracts, depicted by the positive scores and positive correlation on PC 1.

DISCUSSION

The potential of plant-derived molecules as effective compounds for management of plant pests and diseases raised considerable attention during the last 10-15 years (Reignault and Walters, 2007; Martinez, 2012). This has been particularly important in areas where chemical pesticides may have deleterious impacts on the sustainability of agricultural systems and on food safety (Tripathi and Dubey, 2004; Shuping and Eloff, 2017).

Table 1. Influence of *Euphorbia hirta* ethanol extract on fungal disease caused by *Rhizoctonia solani* on tomato plants.

Treatment	Disease index (%)			
	5 d	10 d	15 d	20 d
R. solani (T_{Rs})	16 ± 2.00^{b}	37.33 ± 3.06^{d}	60.67 ± 7.57^{e}	80 ± 2.00^{f}
Extract + *R. solani* (T_{E+Rs})	8 ± 2.00^{a}	24.67 ± 5.77^{c}	38.67 ± 6.11^{d}	56.67 ± 5.03^{e}
	Disease reduction (%)			
	50.53 ± 6.37	33.43 ± 17.74	35.47 ± 13.59	29.24 ± 4.52

Values are the means ± standard deviation, calculated for 30 plants per treatment.
Different letters indicate significant differences in disease indices, according to ANOVA paired with Tukey's tests ($P \leq 0.05$).

Table 2. Total phenolics, flavonoids, alkaloids, tannins, proteins, polysaccharides content, and antioxidant capacity of three tropical plant extracts from *Oxalis barrelieri*, *Stachytarpheta cayennensis* and *Euphorbia hirta.*

Components	*Oxalis barrelieri*		*Stachytarpheta cayennensis*		*Euphorbia hirta*	
	Ethanol extract	Water extract	Ethanol extract	Water extract	Ethanol extract	Water extract
Total phenolics (mg GAE g^{-1})	34.24 ± 2.65^{b}	70.19 ± 6.80^{d}	101.71 ± 11.84^{e}	49.99 ± 4.62^{c}	71.58 ± 2.34^{d}	24.99 ± 3.87^{a}
Total flavonoids (mg CE g^{-1})	7.52 ± 0.52^{b}	7.03 ± 0.35^{b}	33.54 ± 0.69^{e}	22.95 ± 1.6^{d}	15.16 ± 0.1^{c}	3.47 ± 0.68^{a}
Total tannins (mg CE g^{-1})	0.96 ± 0.44^{ab}	3.39 ± 0.04^{c}	6.96 ± 1.89^{d}	0.94 ± 0.29^{a}	5.00 ± 1.18^{d}	1.73 ± 0.38^{b}
Total alkaloids (mAbs g^{-1})	76.75 ± 21.75^{b}	39.55 ± 12.09^{a}	73.00 ± 12.73^{b}	70.10 ± 16.83^{b}	68.25 ± 11.67^{b}	41.70 ± 18.95^{ab}
Total proteins (mg BSA g^{-1})	4.14 ± 0.28^{b}	8.08 ± 0.82^{e}	5.28 ± 0.22^{c}	4.41 ± 0.27^{b}	6.32 ± 0.27^{d}	1.75 ± 0.11^{a}
Total polysaccharides (mg glucose g^{-1})	46.40 ± 19.24^{a}	83.12 ± 4.01^{b}	94.76 ± 18.88^{b}	179.27 ± 27.25^{c}	59.81 ± 5.07^{a}	84.65 ± 10.71^{b}
Antioxidant activity (mg L-ascorbic acid g^{-1})	157.22 ± 4.46^{c}	262.67 ± 41.48^{e}	168.33 ± 2.36^{d}	250.33 ± 40.54^{e}	112.53 ± 8.01^{b}	83.00 ± 26.4^{a}

Values means ± standard deviation of three replicates.
Uppercase letters in the same row indicate significant differences according to ANOVA paired with Tukey's tests ($P \leq 0.05$).

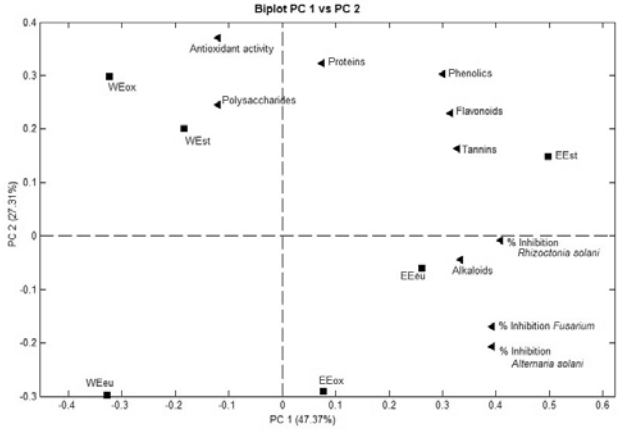

Figure 8. Main biplot (loadings and scores) obtained from principal component analysis for data from ethanol and water extract evaluations, along principal components 1 (PC1) and 2 (PC2). WE and EE indicate, respectively, values for water and ethanol extracts. ox: *Oxalis barrelieri* L., st: *Stachytarpheta cayennensis* L., and eu: *Euphorbia hirta* L. Symbol ▪ depicts the different extracts (scores), whereas ◂ represents the bioactivities and biochemical properties of the extracts (loadings).

In the present study, leaves from three pan-tropical plants, *O. barrelieri*, *S. cayennensis*, and *E. hirta*, were used as potential sources of bioactive molecules against three important phytopathogenic fungi, affecting tomato in Cameroon and worldwide. The three plant species were chosen as sources of possible bioactive/antimicrobial compounds, since they are very common as ruderal herbs in young fallows (Tchiengué, 2012). Additionally, preliminary ethnopharmacological studies showed that these species are relevant as medicinal plants (FAO, 1999).

Our results highlighted the potential fungicidal/fungistatic activity of these plant extracts *in vitro*. Antifungal effects were always more pronounced using hydro-ethanol extracts (EEs) than water extracts (WEs), and were concentration and extract-pathogen-interaction dependent. This confirms the results of Kotze and Eloff (2002), who reported that, in most cases, water extracts had a low antimicrobial efficacy. Therefore, for several plant species, the use of less polar solvents increases the extraction efficiency and concentration of antimicrobial molecules (Eloff, 1998), such as flavonoids and phenolics (Table 2). The complete inhibition of fungal growth was recorded using a concentration ranging from 10 to 20 mg mL^{-1} of EEs obtained from the three plants tested. The antifungal efficacy of such extracts increased linearly ($P \leq 0.05$) in relation to the concentration used, both for EEs and for WEs. This indicates the presence of antifungal molecules in both extracts. The preliminary phytochemical composition of WEs and EEs of the three plants species used in our experiments showed various concentrations of active biomolecules. The concentration of phenolics was greatest in EE*st* and EE*eu* and least in WE*eu*. Conversely, for *O. barrelieri*, more phenolics were found in WE*ox* than EE*ox*. This is possibly due to the diverse nature of phenolics produced by *O. barrelieri* compared, for instance, with *E. hirta* (Mekam *et al.*, 2019). Phenolics (or phenols) are a

large class of compounds classified as simple phenols or polyphenols. They are found in all plants and consist of simple phenols, benzoic and cinnamic acids, coumarins, tannins, lignins, lignans and flavonoids (Khoddami *et al*., 2013). Therefore, it is expected that different plants may produce phenolics in different quantities and of different chemical structures. Phenolics are frequently synthesized in plants in response (or as protection) to stress, such as pathogen infection, insect attack, UV radiation or wounding. Therefore, these compounds have important roles in plant defense (Mandal *et al*., 2010). In most plant species, the key step in phenolic syntheses is the conversion of phenylalanine. This reaction, catalyzed by the phenylalanine ammonia-lyase enzymes, leads to the production of various hydroxycinnamic acids, benzoic acids and derivatives of polyphenols, which are important antifungal biomolecules often found in plants (Raymond Chia and Dykes, 2010).

As highlighted in Table 2, phenolic contents of the different plant extracts were: EE*st* > EE*eu* > WE*ox* > WE*st* > EE*ox* > WE*eu*. Nevertheless, their antifungal activities did not follow this order, since the EEs gave greater antifungal activity than WEs, when tested at equivalent concentrations. EE*eu* gave the greatest antifungal activity, completely inhibiting mycelium growth of *F. oxysporum* f. sp. *vasinfectum* and *A. solani*, at a concentration of 10 mg mL^{-1}. At the same concentration, WE*eu* exhibited moderate antifungal activity, inhibiting mycelium growth of *F. oxysporum* f. sp. *vasinfectum* by 18% and *A. solani* by 57%. In general, hydro-ethanol treatments extracted more (and possibly more diverse) antifungal compounds than water, and this may be related to the less polar nature of ethanol as an extractant, when compared to water. Considering the minimum effective concentration of EEs that completely inhibited fungal growth, the measured EE activity was twice to three times greater than for WEs. These results agree with those of other reports that have showed the efficiency of some hydro-ethanolic plant extracts have the greatest and widest range of *in vitro* activities, resulting in complete inhibition of fungal growth. Galani *et al*. (2013) reported that the EEs of *Ageratum conyzoides* and *Callistemon citrinus* completely inhibited *Phythophthora infestans* at 5,000 ppm, and that of *Ocimum gratissimum* at 10,000 ppm. Dakole *et al.* (2016) reported that the EEs of *Ageratum conyzoides* and *Callistemon citrinus* were the most active for inhibiting radial growth of *Phytophthora infestans*. *Cymbopogon citratus* and *Ocimum gratissimum* were the most active against radial growth and conidia germination of *Fusarium oxysporum* f. sp. *lycopersici* at a concentration of 6,250 µg mL^{-1}. This variability in antifungal activity of extracts suggested that a correlation exist between the nature of plant extracts and their concentrations of active phytochemicals. The amounts, biochemical nature and activity of phenolics (including flavonoids and tannins) and alkaloids contained in the present study extracts was dependent on: i) their solubility in water or ethanol; ii) the absence of inhibitors; iii) their synergism with polysaccharides and proteins present in the extracts; iv) the differences in modes of action; and, v) the structure and biology of the phytopathogenic fungi (Lapornik *et al*., 2005). Therefore, the phenolics present in these plant extracts displayed important and direct inhibition of growth of three tomato pathogens and may be considered as prospective compounds for management of plant diseases. In particular, the extracts from *E. hirta* used in our experiments showed the greatest amounts of phenolic compounds, mainly gallotannins, and hydroxybenzoic and hydroxycinnamic acids (Mekam *et al*., 2019). These phenolics are known bioactive molecules with antifungal properties (Alves Breda *et al*., 2016).

Plant-derived alkaloids were extensively studied for their antifungal properties against human pathogenic fungi, such as *Candida albicans* (Mollataghi *et al*., 2012). Plant alkaloids are also reported to have activity against phytopathogenic fungi. For instance, allosecurinine from *Phyllanthus amarus* (*Euphorbiaceae*) was able to inhibit the growth of *Alternaria* spp. (including *A. solani*), *Fusarium* spp. and other important fungi affecting crop plants (Singh *et al*., 2008). Liu *et al*., (2009) found that sanguinaine, an isoquinoline alkaloid from *Macleaya cordata* (*Papaveraceae*) reduced the growth of *R. solani* at a concentration of 0.45 µg mL^{-1}. Nonetheless, the specific roles of alkaloids extracted from the tested plants remain uncertain, as compared to the possible inhibitory role of phenolics present in the same extracts. This role (if any) does not appear to be important since no significant difference in content was found among the three tested plants.

Regarding polysaccharides, one of the best known with confirmed antifungal effects is laminarin (or laminaran), a glucan with different degrees of molecular branching at β-1,3 and β-1,6. For instance, grey mold (*Botrytis cinerea*) and downy mildew (*Plasmopara viticola*) of grapevine were inhibited by spraying laminarin onto vine canopies (Copping, 2004). Laminarin-based products are commercially available as biopesticides (Environmental Protection Agency, 2010). Activity of laminarin in plant tissues is more related to induced resistance than from direct antifungal effects (Aziz *et al*., 2003). In the present study, polysaccharides were particularly abundant in the WE of *S. cayennensis*, but WE was not more active than EE from this plant. This

suggests that polysaccharides may not have inhibitory effects on fungal growth as great as other compounds in the tested extracts – especially the EEs.

The greater antioxidant capability of a plant extract may be correlated with phenolic composition and concentration. The greater the phenolic content the greater is the antioxidant activity. This is due to the phenolic hydroxyl groups, which stop radical chain reactions via radical scavenging (Shahidi and Chandrasekara, 2010).

Principal component analyses for exploration of relationships between plant extracts and biochemical properties assisted description of variance in the set of multivariate data we obtained (polyphenol, tannin, alkaloid contents and associated fungal growth inhibition). Sample splitting showed in the PC bi-plot clearly reflected the main differences due to extraction method, the plant origins, and the phenolic and alkaloid compositions, which influenced antifungal activity of the different extracts.

Under greenhouse condition, foliar application of EE*eu* on tomato before a challenge pathogen inoculation resulted in increased plant height and the reduction of disease severity caused by *R. solani*. Increased plant height was a beneficial effect of foliar application of EE*eu* during the *in planta* experiments. EE*eu* stimulated plant height more than water in treated tomato plants (Figure 7). This growth promoting activity of EE*eu* was possibly attributable to low molecular weight components, such as plant hormones (gibberellic acids), and to major components such as polysaccharides, amino acids and polyphenols that are involved in many aspects of plant physiology and development. These include seed germination, stem and leaf elongation, flower induction, and fruit and seed development (Kamiya and Garcia-Martinez, 1999). This result is similar to those of Nguefack *et al*. (2013), who reported that spraying rice plants with a 2% ethanol extract, followed by a 2% (w/v) aqueous extract of *Callistemon citrinus* or *Cymbopogum citratus*, increased seedling emergence, tillering, panicles/plant and the grain yields by 25–55%. Zakiah *et al*. (2017) reported that low concentrations of a crude extract of *Cassava asiatica* (25 mg L^{-1}) increased height and leaf area of soybean plants. However, in the present study the tomato-EE*eu*-*R. solani* interaction showed less plant height increase, when compared to the tomato-water interaction. Infection by a root/stem pathogen probably interfered with host metabolism and contributed to reduced plant development.

Management of tomato fungal diseases is primarily achieved through breeding resistant cultivars. Many recently developed cultivars are moderately or very resistant to Fusarium wilt, whereas no tomato variety is currently available that shows acceptable tolerance to damping-off/crown rot. Therefore, we chose *R. solani* as the challenging pathogen for the *in vivo* experiments. Disease reduction reached 29 ± 5% in the tomato-EE*eu*-*R. solani* interaction, in comparison to the tomato-water-*R. solani* interaction, at 20 d after inoculation (Table 1). This suggests that EE*eu* contained natural compounds that possibly act alone or in synergy, stimulating plant defense to provide disease control through induced systemic resistance in tomato against damping-off. This is particularly important since damping-off cannot be easily managed, especially in areas where appropriate crop rotations are not followed. Although extracts from tropical plants are reported to provide crop disease control through stimulation of plant defense systems (Baraka *et al*., 2011; Nashwa and Abo-Elyousr, 2012), *E. hirta* extracts have not been previously examined.

CONCLUSIONS

Natural flora is a source of several biologically active compounds, and some of these have been formulated as botanical agrochemicals and are currently used in the management of agricultural pests (Dubey *et al*., 2011). The present study established that water and hydroethanol extracts obtained from leaves of *O. barrelieri*, *S. cayennensis*, and *E. hirta* are sources of phytochemicals, and these molecules demonstrated inhibitory activity against phytopathogenic fungi. Application of these extracts is, therefore, a promising and environmentally friendly strategy for crop disease control that could contribute to minimizing the risks and hazards of posed by conventional fungicides.

Economic advantages from the use of plant extracts could be particularly relevant in African rural areas, where these pan-tropical plants are common and adapted. Biomolecules may be developed into commercial products by local companies, thus contributing to rural and agro-industrial development, together with increased sustainability for local cropping systems. Current research is devoted to identifying the most effective phenolics and other antifungal compounds (Mekam *et al*., 2019), and compounds showing effective plant growth promotion, to develop and implement innovative plant disease biocontrol strategies.

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Research Paper

Crosstalk between the cAMP-PKA pathway and the β-1,6-endoglucanase in *Verticillium dahliae*

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Summary. In plant pathogenic fungi, different signalling pathways operate to control responses to nutrient availability during plant infection. A candidate from the cAMP-PKA signalling pathway, the cAMP-dependent protein kinase A gene, *pka*C1, and the β-1,6-endoglucanase gene, *veg*B, involved in cell wall degradation, were studied in *V. dahliae*. Double mutants of the fungus were constructed, with insertional inactivation in the *pka*C1 and *veg*B genes. Different developmental traits and virulence towards eggplant were evaluated in single and double disruption mutants. In all media tested, double mutants showed better radial growth but less conidia and microsclerotia than the wild type. An interaction between *veg*B and *pka*C1 in controlling virulence on eggplants was recorded, as double mutants were slightly less virulent than the single mutant *veg*B$^-$, but more virulent than the single mutant *pka*C1$^-$. Concomitant or independent function of the two genes and the signaling pathways they operate in for the different growth parameters and virulence are discussed.

Keywords. Protein kinase A, cAMP-mediated PKA, cell wall degrading enzyme.

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INTRODUCTION

Fungi, and especially plant pathogens, can produce many plant cell wall degrading enzymes (CWDEs) for penetration and successful infection of their hosts (Kubicek *et al*., 2014). Involvement of these enzymes in penetration, plant defense induction, and symptom expression has been studied extensively (Cooper, 1987; Walton, 1994; Di Pietro *et al*., 2003). However, although found to contribute to pathogenesis, in most cases no specific roles have been directly attributed to specific genes coding for CWDEs, and since the activities of these enzymes from different fungi show preferences for different types of plant biomass and adaption to pathogen lifestyles, their roles in pathogenesis remains unclear (King *et al*., 2011; Couturier *et al*., 2012).

Probable explanations for this are: (a) that CWDEs are indirectly involved in pathogenesis, and (b) that due to the organization in large multigene families of CWDEs in fungal pathogens, and the functional specialization that each enzyme exhibits, the unequivocal identification of a particular gene involved in pathogenicity is prevented by the masking of its function by the corresponding function of other genes in the same family (Walton, 1994; Coutinho *et al*., 2003; Zhao *et al*., 2013; CAZy website (http://www.cazy.org/CAZY/)). There is sufficient evidence for masking from a number of studies with mutants of inactivated CWDE genes that always retained at least some residual enzyme activity. Examples are: the xylanase genes of *Cochliobolus carbonum* (Apel-Birkhold and Walton, 1996); the polygalacturonase genes of *C. carbonum* (Scott-Craig *et al*., 1990, 1998); the pectate lyase genes of *Nectria haematococca* (*Fusarium solani* f. sp. *pisi*), (Rogers *et al*., 2000); and the xylanase genes of the rice pathogen *Magnaporthe grisea* (Nguyen *et al*., 2011). In the case of *C. carbonum*, double or triple xylanase mutants were shown to retain full pathogenicity on maize (Apel-Birkhold and Walton, 1996). Similarly, in *V. dahliae*, disruption of a trypsin protease gene did not affect pathogenicity (Dobinson *et al*., 2004), while an insertional mutant with the single copy β-1,6-endoglucanase gene showed only a minor reduction in virulence (Eboigbe *et al*., 2014).

Beta-1,6-endoglucanase function has been little studied in plant pathogenic fungi. These enzymes are produced by several fungal species under conditions of carbon starvation, together with other hydrolytic enzymes to release carbon for survival by cell autolysis (Adams, 2004; Martin *et al*., 2007). In mycoparasitic fungi, the role of β-1,6-endoglucanases in degradation of chitin and cell wall β-glucan has been demonstrated using gene disruption studies (Amey *et al*., 2003). In mutualistic fungi (*Neotipodium* sp.) a β-1,6-endoglucanase protein was found to be secreted in the host plant apoplast, conferring a role in fungal nutrition. Based on the absence of β-1,6-glucosidic bonds (the main substrate of β-1,6-endoglucanases) in plants, Moy *et al*., (2002) hypothesized that β-1,6-endoglucanases may be involved in the degradation of cell walls of other fungi to prevent them from infecting host plants. However, in plant pathogenic fungi β-1,6-endoglucanases are considered to also break β-1,3-glucosidic bonds present in plant cell wall callose during parasitic attack (Martin *et al*., 2007). Furthermore, β-glucanases secreted by fungi or produced by hosts aid β-glucan degradation, releasing hydrolytic enzymes from fungal hyphae. In support of this hypothesis, growth of *Botrytis cinerea* on media containing β-glucanases (glucanex, β-1,3-glucanase) resulted in altered morphology of the mycelium and increased activity of hydrolytic enzymes, including peroxidases, laccases, and catalases (Gil-ad *et al*., 2001).

To avoid the problems caused by the masking effects of gene expression in multigene families, attention was focused on the transcriptional regulation of CWDE genes expression and the signals that control whole sets of pathogenicity genes (Roncero *et al*., 2008; Kubicek *et al*., 2014). Tonukari *et al*. (2000) earlier reported that one way to overcome the problem of functional redundancy was to abolish expression of an entire class of CWDE enzymes by knocking out components of signal transduction pathways or transcriptional activators. The sucrose non-fermenting gene *SNF1* that regulates catabolite repression in *C. carbonum* was found necessary for the expression of several CWDEs (Tonukari *et al*., 2000). Mutation of *SNF1* in this fungus led to varying levels of repression of CWDE genes, reduced growth on complex polymers such as xylan and pectin, and also reduced virulence on its maize host. Similarly, the disruption of *SNF1* in the vascular wilt fungus *F. oxysporum* resulted in strongly impaired pathogenicity that was attributed to the low induction of CWDE genes (Ospina-Giraldo *et al*., 2003). Thus, the *VdSNF1* gene was essential for the induction of CWDEs and virulence in *V. dahliae*, confirming the significant role of these genes in pathogenicity of the fungus (Tzima *et al*., 2011).

Although the implication of the *SNF1* pathway in regulation of CWDEs in plant pathogenic fungi is well documented, there is little information on whether CWDEs are also influenced by the cyclic AMP- Protein kinase A (cAMP-PKA) and/or mitogen-activated protein kinase (MAPK) signalling pathways. Both pathways are necessary for fungal pathogenesis and play important roles in the formation of appressoria (Tudzynski and Tudzynski, 2001; Lee *et al*., 2003). The implication of the MAP kinase pathway in modulation of CWDEs was clearly indicated by reduced production of polygalacturonases and pectate lyase enzymes in the *Fusarium oxysporum* f. sp. *lycopersici* MAP kinase disruption mutant *fmk*1 (di Pietro *et al*., 2003). In *Magnaporthe grisea,* activity of the cAMP-dependent protein kinase (PKA) increased during germination of conidia and appressorium formation on hydrophobic surfaces, whereas in mutants lacking the catalytic subunit of PKA (*cpk*a), appressorium formation was impaired (Kang *et al*., 1999). Similarly, disruption of the catalytic subunit protein kinase A gene *VdPKAC1* (hereafter, *pka*C1) of *V. dahliae* caused reduced virulence, although mutants caused typical disease symptoms (Tzima *et al*., 2010). Therefore, since *V. dahliae* does not possess specialized infection structures that in other fungi (including *M. grisea*) are essential for

host invasion (Madhani and Fink 1998), we have tested if cAMP-dependent genes including *pka*C1 influence host penetration by *V. dahliae*, in combination with the β-1,6-endoglucanase gene (*veg*B) that was recently shown to affect the virulence of the fungus (Eboigbe *et al*., 2014).

MATERIALS AND METHODS

Fungus isolates and culture conditions

A *V. dahliae* race 2 isolate from tomato, obtained in Greece, (isolate ID number 123wt-r2; hereafter referred to as wild type or wt), and the *veg*B$^-$ disruption mutant derived from the wild type (Eboigbe *et al*., 2014) were used in this study. For long term storage, the wild type and mutant strains were stored at -80°C, as conidial suspensions in 25% glycerol. Cultures were reactivated on freshly made potato dextrose agar (PDA).

Fungus growth experiments on different carbon sources were performed using a basal medium containing mineral salts and trace elements as in minimal medium (Puhalla and Mayfield, 1974). Carbon sources were added individually to the basal medium (at final concentrations of 2% w/v) and the amended media were adjusted to pH 6.5. Radial growth assays were performed on basal medium containing 2% agar, supplemented with 2% carboxycellulose, pectin, glucose and sucrose. Growth experiments were also performed on PDA (provided by two different suppliers; Difco and BP), that was prepared according to the manufacturers' instructions. Plates were each inoculated by placing 10 μL of conidial suspension (10^7 conidia mL^{-1}) of each strain at the centre of each inoculated plate. Fungal cultures were incubated at 23°C and the colony diameters were recorded at intervals of 2 to 3 d, while colony morphology was also observed and recorded.

For conidia production, 5 mm culture plugs from the edge of actively growing mycelia of each fungal strain were transferred to the centre of PDA plates. After 10 d of incubation, 3 mL of sterilized water was added to each plate to harvest conidia. Conidia were released by scraping off fungal cultures with a glass rod. The plates were then shaken gently and a small aliquot (10 μL) of the suspension from each plate was placed on a microscope slide haemocytometer to determine number of conidia.

Construction and verification of vegB/pkaC1 double disruptants

For the construction of *veg*B$^-$/*pka*C1$^-$ double disruptants, the catalytic subunit of the protein kinase A gene, *pka*C1, was disrupted in the *veg*B$^-$ mutant strain by *Agrobacterium tumefaciens* mediated transformation (ATMT), as described by Tzima *et al*. (2010). Mutant *veg*B$^-$, is a race 2 *V. dahliae* strain in which the β-1,6-endoglucanase gene has been disrupted by insertional inactivation, using hygromycin B as a selection marker gene (Eboigbe *et al*., 2014). For ATMT of mutant *veg*B$^-$, the *Agrobacterium* strain AGL1 was used, which carries the binary vector pGK-Gen, harbouring the geneticin resistance cassette. The binary vector pGPK-Gen was constructed in a previous study, and consists of a pGKO2 backbone (Khang *et al*., 2005), in which the *V. dahliae pka*C1 mutant allele was cloned. The *pka*C1$^-$ mutant allele was constructed from a 3 kb fragment including the *pka*C1 gene, in which the 0.73 kb *Bsr*GI/*Bbv*CI portion, located 274 nucleotides downstream from the start codon, was replaced with the 1,480 bp geneticin resistance gene cassette by blunt ending both vector and insert.

Double disruptants were constructed by ATMT of the *veg*B$^-$ strain as previously described (Mullins *et al*., 2001; Tzima *et al*., 2010). Colonies of double mutants appeared after 3-4 d incubation on PDA supplemented with 50 μg mL^{-1} geneticin, 50 μg mL^{-1} hygromycin B and 50 μM F2dU (5-Fluoro-2-deoxyuridine; Sigma), and were subjected to single conidium isolation. The binary vector pGPK-Gen harbours the HSV*tk* gene (Herpes Simplex Virus Thymidine Kinase). The nucleoside analog F2dU is converted by the HSV*tk* gene product to a toxic compound against *V. dahliae*, selecting against ectopic transformants, thus increasing transformation efficiency (Khang *et al*., 2005).

The confirmation of *pka*C1 disruption in the *veg*B$^-$ mutant, thus creating *pka*C1$^-$/*veg*B$^-$ double disruptants, was achieved by PCR using primers VdPK1400bp-up (5'-AGCCCAACAGCCCCATTACCC-3') and VdPK-1400bp-dn (5'-GCCCAGGCGCTTCGTCAGA-3').

Pathogenicity assays on eggplants

Virulence of the *V. dahliae* wt, *pka*C1- and *veg*B$^-$ single disruption mutants and double disruptants 123Δ*VP1*, 123Δ*VP2*, 123Δ*VP3*, 123Δ*VP4* was evaluated on eggplant ('Black Beauty'). Although the wt was a tomato strain, we carried out the pathogenicity assays on eggplant because this species is more susceptible than tomato to *V. dahliae* (e.g. more rapid disease development, and increased incidence and severity of disease), thus allowing precise evaluation of plant responses to the wt and mutant isolates.

For inocula preparation, fungal cultures grown for 5 to7 d in SSN medium (Sinha and Wood, 1968) were

passed through several layers of cheesecloth (to remove mycelia). The concentration of conidia in resulting suspensions was adjusted to approx. 1×10^7 mL^{-1}. Ten plants at the second true leaf stage were inoculated by drenching the roots with 10 mL conidialsuspensions of each isolate. Virulence assays were performed twice.

Disease severity at each observation was expressed by the percent of leaves that showed wilting symptoms and was recorded for 39 d post inoculation. Disease ratings were plotted over time to generate the disease progress curves, and the areas under the disease progress curve (AUDPC) were calculated using the trapezoidal integration method (Campbell and Madden, 1990). Disease severity was expressed as percentage of the maximum possible AUDPC for the whole period of the experiment, and is referred to as relative AUDPC (Korolev *et al*., 2001). Relative AUDPC values calculated for each treatment were subjected to ANOVA, and means were separated by Duncan's multiple range test.

RESULTS

*Construction of double mutants pka*C1$^-$/*veg*B$^-$

Gene *pka*C1 was previously cloned from a *V. dahliae* tomato race 1 strain and found to have a coding sequence of 1,787 bp (Tzima *et al*., 2010). Since the *veg*B$^-$ knockout mutant contained the hygromycin resistance gene, the binary vector pGPK was altered to carry the geneticin resistance cassette (pGPK-Gen), and was used for ATMT transformation of *veg*B$^-$ (Figure 1A; Tzima *et al*., 2010). Double resistant to hygromycin and geneticin transformants were isolated on appropriate selective media and were examined for stability of the genotypes for several generations. Single colony derived stable transformants were subsequently tested by PCR to examine the sizes of amplicons for the *pka*C1 gene. Representative putatively double-disruptants producing a larger amplicon (2,183 bp) in comparison with the wild

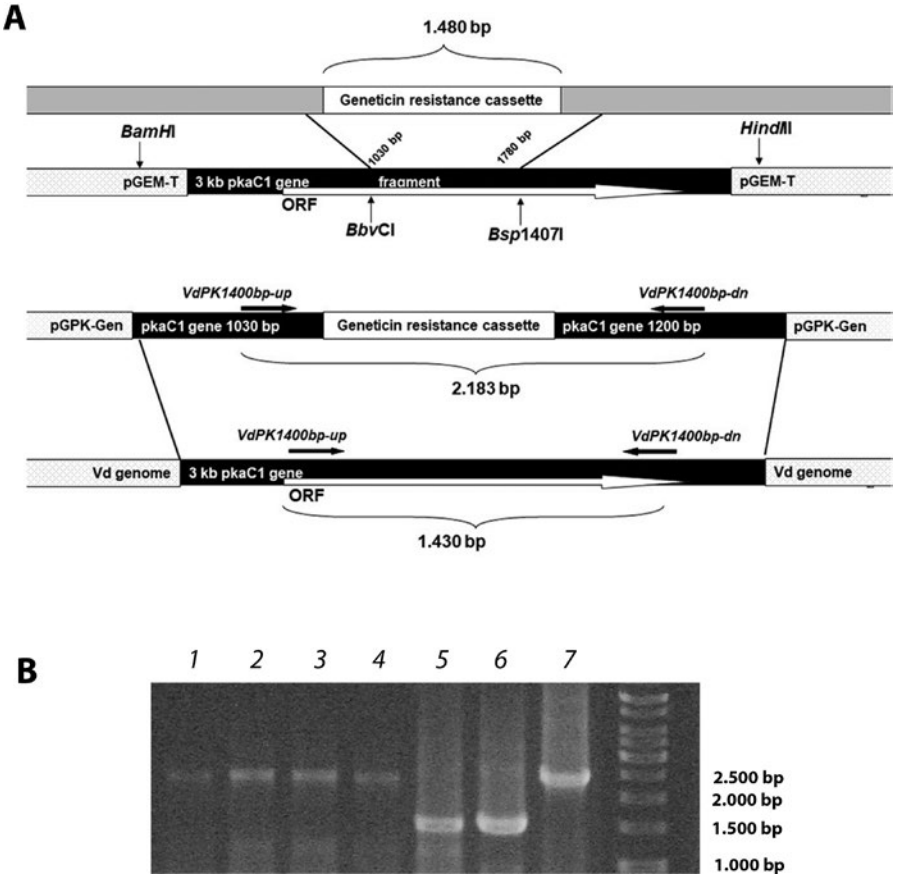

Figure 1. Generation of *Verticillium dahliae pka*C1/*veg*B double disruption mutants. A) Schematic presentation of *pka*C1 mutant allele construction and integration in the fungal genome by a double homologous recombination event. B) Disruption of *pka*C1 verified by amplification of the gene from four double disruptants 123Δ*VP1,* 123Δ*VP2,* 123Δ*VP3,* and 123Δ*VP4* (lanes 1-4; 2.183 bp), genomic DNA (lane 5) and plasmid DNA (lane 6) of clone pPKA3kb, with native fragment 1430 bp), and plasmid DNA of clone pGPK-Gen (lane 7).

type strain 1,430 bp amplicon are shown in Figure 1B. Transformants 123Δ*VP1*, 123Δ*VP2*, and 123Δ*VP4* were further examined for the possible genetic interaction between *veg*B with *pka*C1 in double knock-out *V. dahliae* mutants (*veg*B-/*pka*C1-), and for their pathogenicity in comparison with that of the single knock-out mutants and the wild type strain.

Production of conidia and utilization of carbon sources

The physiology and development of wild type *V. dahliae, veg*B$^-$ and double disruptants were examined by estimating conidium production, and their ability to degrade different carbon sources. Growth of mutant *pka*C1$^-$on glucose, sucrose and xylose was reduced compared to the wild type strain, while on pectin, carboxycellulose and Difco PDA this reduction was less apparent. Mutant *veg*B$^-$ and the double disruptants 123Δ*VP1*, 123Δ*VP2*, and 123Δ*VP4* showed slightly increased radial colony growth compared to the wild type strain and mutant *pka*C1$^-$, which was more pronounced on pectin, carboxycellulose and sucrose (Figure 2).

Colony morphology of all strains was similar in all carbon sources, except growth on Difco PDA. The *vegB*$^-$ mutant and the double disruptants formed abundant microsclerotia after 1 week of growth, while colonies of the wild type and *pka*C1$^-$ showed no microsclerotia at the same time and very few after 20 d. The use of PDA from another supplier (Scharlau) affected colony morphology of all strains with variable intensities of pigment formation. Mutant *veg*B$^-$ displayed increased and resuscitate growth compared to all other strains, while the double disruptants showed a fluffy growth phenotype with different patterns of microsclerotium production between the double disruptants. Colonies of 123Δ*VP1* were white, while 123Δ*VP2* produced microsclerotia at the colony centres and edges, and 123Δ*VP4* produced microsclerotia in internal rings within the colonies. This indicated the presence and absence of microsclerotium formation stimuli in the different PDA preparations.

In comparison with the wild type strain, production of conidia was reduced in all the other strains, at about 40-50%. The exception was *pka*C1,$^-$which showed an approx. 30% reduction.in production of conidia.

Pathogenicity assays on eggplant

Infection assays on eggplants were performed to determine the effects of single mutants *pka*C1$^-$, *veg*B$^-$, and double disruption mutants 123Δ*VP1*, 123Δ*VP2* and

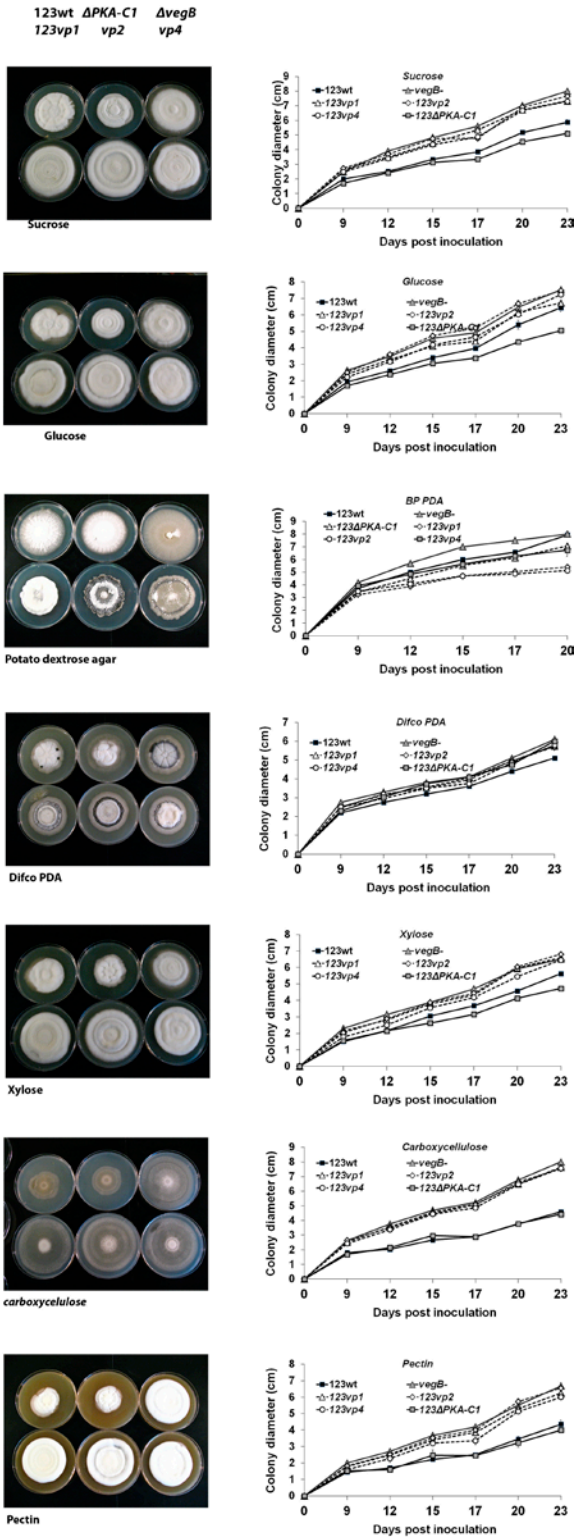

Figure 2. Radial growth of *Verticillium dahliae* wild type (123wt-r2), mutant *veg*B$^-$ and double disruptants 123Δ*VP1*, 123Δ*VP2* and 123Δ*VP4* on different carbon sources. Left: Images of fungal colonies on carbon sources after 23 d of growth. Right: Graphs presenting radial growth of the strains at specific time points.

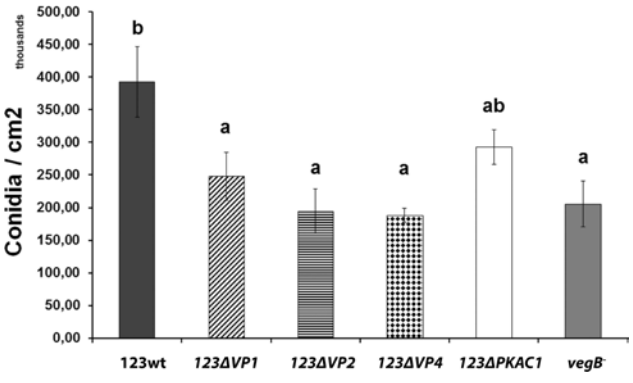

Figure 3. Production of conidia by *Verticillium dahliae* wild type (123wt-r2), mutant *veg*B- and double disruptants 123Δ*VP1*, 123Δ*VP2* and 123Δ*VP4*. Error bars indicate standard errors calculated for five replicates. Values calculated for each treatment were subjected to analysis of variance and means were separated by Fishers's least significant deifference (LSD) procedure. Columns with different letters are statistically different at $P \leq 0.05$.

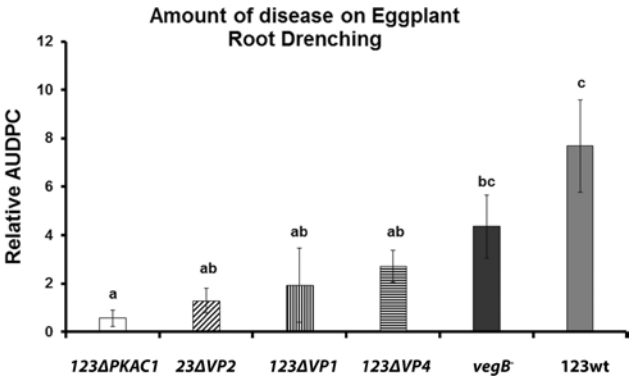

Figure 4. Disease severity caused by the *Verticillium dahliae* disruption mutants *veg*B-, *pka*C1-, double disruptants 123Δ*VP1*, 123Δ*VP2*, 123Δ*VP4* and the wild type 123wt-r2 on eggplants infected by root drenching. Disease severity was expressed as relative AUDPC. Bars indicate standard errors calculated for 20 replicates. Relative AUDPC values calculated for each treatment were subjected to analysis of variance and means were separated by Fishers's least significant deifference (LSD) procedure. Columns with different letters are statistically different at $P \leq 0.05$.

123Δ*VP4* on pathogenicity of *V. dahliae* in comparison with the wild type strain.

Mutant *pka*C1- caused significantly less disease (relative AUDPC of 0.55%) compared to the wild type strain (7.7% relative AUDPC). Disruption of *veg*B caused a smaller non-significant reduction in virulence of the *veg*B- mutant (4.4% relative AUDPC) compared to the wild type strain. Double disruptants 123Δ*VP1*, 123Δ*VP2* and 123Δ*VP4* caused intermediate amounts of disease compared to that caused by mutants *pka*C1- and *veg*B- (relative AUDPCs were 1.9% for 123Δ*VP1*, 1.3% for 123Δ*VP2* and 2.7% for 123Δ*VP4*; Figure 4).

DISCUSSION

The cAMP-PKA pathway has been extensively studied in yeast, but to a much lesser extent in plant pathogenic fungi. In plant pathogens, this signaling pathway has been implicated in morphological and physiological traits (conidiation, branching, growth), and in virulence (Yamauchi *et al*., 2004; Tzima *et al*., 2010; Kim *et al*., 2011). Disruption of the catalytic subunit protein kinase A gene, *pka*C1, in a *V. dahliae* tomato race 1 strain resulted in reduced conidiation and growth, increased branching and reduced invasive growth (Tzima *et al*., 2010).

Beta-1,6-endoglucanases, secreted by fungi, degrade mainly β-1,6-glucosidic bonds (present in fungus cell walls). However, there is accumulating evidence that these enzymes can also degrade β-1,3-glucosidic (laminarin and cell wall callose) and β-1,4-glucosidic bonds (chitin) in the environment, to achieve survival of the fungi under conditions of carbon starvation, by cell wall autolysis, as well as during plant- and myco- parasitism (Stahmann *et al*., 1992; Amey *et al*., 2003; Martin *et al*., 2007). In plant pathogenic fungi, β-1,6-endoglucanases probably aid in plasticity of hyphae during plant-microbe interactions, and release of hydrolytic enzymes entrapped in the cell walls of hyphae by the presence of β-glucan (Gil-ad *et al*., 2001). Disruption of the β-1,6-endoglucanase gene *veg*B in a *V. dahliae* race 2 isolate was reported by Eboigbe *et al*. (2014). Thus, to detect possible crosstalk between the β-1,6-endoglucanase gene and the PKA signalling pathway in *V. dahliae* race 2, double disruption mutants were created by a sequential approach.

In agreement with previous results (Tzima *et al*., 2010; Eboigbe *et al*., 2014), both single disruption mutants in *V. dahliae* race 2 that were examined here, *veg*B- and *pka*C1-, showed marked reduction in the production of conidia, which was more pronounced in *veg*B- than in *pka*C1-. However, no additional differences in the production of conidia were observed in the double mutants that were constructed. The *pka*C1- mutant had reduced growth on the different carbon sources tested, both in comparison with the wild type and also with the *vegB*- and the double disruption strains. V*eg*B- and the three double mutants showed similar growth on fermentable (sucrose, glucose) and alternative (xylose, pectin, carboxycellulose) carbon sources, which was increased in comparison with the wild type strain. This

indicates that the *veg*B$^-$ disruption phenotype was dominant over the *pka*C1$^-$ phenotype. A possible explanation for this reduced growth and conidiation phenotype of *pka*C1$^-$ is that the mutant fungal strain had impaired perception of appropriate signals from the environment, thus failing to initiate conidiation and growth processes. This has been demonstrated in several other similar cases (Kronstad *et al*., 1998; Casas-Flores *et al*., 2006; Doehlemann *et al*., 2006; Skamnioti and Gurr, 2007). In such mutants, the additional absence of β-1,6-endoglucanase may elevate the composition of fungal hyphae in its substrate, β-glucan (Martin *et al*., 2007). As endoglucanases have been shown to participate in hydrolysis and re-construction of the fungal cell walls during growth and morphogenesis (Adams *et al*., 2004), this may also further interfere with perception and response to environmental signals, thus explaining the dominant phenotype of *veg*B$^-$ over *pka*C1$^-$ disruption.

Glucose signalling in yeasts and filamentous fungi is controlled by different pathways including the cAMP-PKA pathway that regulates growth in response to glucose availability, whereas sucrose non-fermenting gene product (SNF1), induces transcriptional changes in the presence of low glucose concentrations or during growth on alternative carbon sources (Tonukari *et al*., 2000; Hong *et al*, 2003; Santangelo, 2006; Zaman *et al*., 2009). In yeasts, it has been shown that PKA activity controls the localization of the β subunit isoform (Sip1) of the Snf1 protein kinase and its complex with the Snf1 catalytic subunit in response to carbon source availability, indicating interference between the two pathways (Hedbacker *et al*., 2004). Furthermore, microarray analysis in yeast revealed that the PKA pathway centrally controls cell growth and transcriptional changes of the majority of genes involved in response to glucose availability. SNF1 modulates the expression of a small number of genes, a significant portion of which is independent of the PKA pathway, including the majority of glucose repressed genes (Zaman *et al*., 2009). The expression of *veg*B was reduced in *VdSNF1* mutants (Eboigbe *et al*., 2014), indicating that *veg*B is under catabolite repression controlled by SNF1 in *V. dahliae*. Therefore, similar to its role in yeast, the SNF1 ortholog gene may also operate in *V. dahliae* and *veg*B may belong to a group of genes that function independently from the PKA pathway.

Previous results for plant–*V. dahliae* interactions (Tzima *et al*., 2010; Eboigbe *et al*., 2014) have shown that *pka*C1 is implicated in invasive growth of *V. dahliae* race 1, whereas *veg*B has only a minor effect on virulence in *V. dahliae* race 2. It was therefore expected that the disruption of *pka*C1 in *V. dahliae* race 2 would cause reduction in virulence. Disruption of both genes in race 2 of *V. dahliae* resulted in double mutants causing less disease in the sensitive eggplant-*V. dahliae* pathosystem than the single mutant *veg*B$^-$, and more disease than the single mutant *pka*C1$^-$. These results, though not strongly supported statistically, indicate a possible interaction between *pka*C1 and *veg*B in controlling virulence. Pathogenicity is complex, which includes growth on alternative carbon sources (e.g. pectin induces the production of hydrolytic enzymes), ramification into plant tissues, and confrontation with host defense mechanisms (Agrios, 2005). It is therefore difficult to speculate in which of these pathogenicity factors the functions of *pka*C1 and *veg*B may overlap, to fully explain the phenotypes observed in the double disruptant mutants of *V. dahliae* race 2. However, as disruption of *veg*B is thought to affect the activity of other hydrolytic enzymes (Gil-ad, 2001), and disruption of *pka*C1 possibly causes deregulation of the expression profiles of genes involved in similar processes, the combined action of these genes may form the basis for an explanation of the phenotypes observed. In support of this hypothesis, as well as to add to the complexity of the mechanisms involved, the disruption of a Ga subunit gene in *Botrytis cinerea* caused reduced expression of endoxylanase and endoglucanase genes, and increased expression of a pectate lyase gene, all of which aided plant cell wall degradation (Gronover *et al*., 2001). Further investigation of the interaction of *pka*C1 and *veg*B in *V. dahliae* and the downstream targets affected is required to clarify pathogenicity mechanisms of this important plant pathogen.

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Research Paper

Development and validation of a severity scale for assessment of fig rust

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Summary. Standardized methods for quantifying rust severity (*Cerotelium fici*) on fig leaves (*Ficus carica* L.) are required, so this study aimed to develop and validate a diagrammatic scale to assessment the severity of this disease. Fig leaves that exhibited varying severities of rust symptoms were collected in the field. The actual severity, maximum and minimum limits, and intermediate levels of the scale were determined based on the frequency distribution of the severity values found in the field. In validation of the scale, eight evaluators estimated the severity in 50 leaves with different levels of symptoms with and without the use of the diagrammatic scale. Accuracy and precision of the data were evaluated, and linear regression was used to assess the repeatability and reproducibility of the estimates. The use of the diagrammatic scale provided adequate results for the parameters analysed when compared assessments made without use of the scale, confirming reliability of the estimates to evaluate rust severity on fig leaves.

Keywords. *Ficus carica*, *Cerotelium fici*, Lin's method.

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INTRODUCTION

Fig (*Ficus carica* L.) is among the most important cultivated world fruit species. The use of fig fruit as food, and of fig plants for ornamental purposes, have been recorded for thousands of years including in the Bible (Eisen, 1901). Turkey is the largest producer of figs in the world, producing 305,450 tonnes per year, followed by Egypt, Morocco, Algeria and Iran (FAO, 2018). European countries, including Portugal, Spain and Italy, are also major producers and exporters of figs (Khemira and Mars, 2017). In subtropical regions, fig crops are grown to produce ripe figs to supply fresh fruit markets, or unripe fruit for the production of sweets, compotes and crystallized figs (Dalastra *et al.*, 2009).

Although fig originated in temperate regions (Pio *et al.*, 2019), it can adapt to different climates and soil conditions, which has boosted expansion

of fig production to Brazilian tropical and subtropical regions (Chalfun *et al*., 2012). In the last 10 years, Brazil's cultivated fig area has remained steady at approx. 2,591 ha (IBGE. 2019). The primary fig-producing states in Brazil include Rio Grande do Sul (11,918 tonnes), São Paulo (10,903 tonnes) and Minas Gerais (1,698 tonnes). Recent decades have seen increased exploitation of fig crops in Brazil and Chile, the produce from which is destined for export to North African and European countries during the production off-season in those regions (Pio *et al*., 2017). However, when grown in subtropical regions, some diseases affect fig crops.

Fig rust (*Cerotelium fici* (Cast.) Arth.) is the principal disease that affects fig crops (Galleti and Rezende, 2005). Symptoms of the disease on adaxial surfaces of fig leaves appear as angular yellow-green spots that progress to brown. On abaxial leaf surfaces orange-red pustules develop that contain powdery masses of spores. In severe infections, the leaves fall, and growth and ripening of the figs are halted. With the premature fall of leaves, there is a reduction in the accumulation of carbohydrates, which compromises the next fruit production cycle (Galleti and Rezende, 2005; Solano-Báez *et al*., 2017).

Due to the losses associated with fig rust, appropriate disease management methods are required. These include development of resistant cultivars, fungicide applications, development of biological control agents, resistance inducers, appropriate pruning techniques or crop management. However, to measure the effectiveness of these techniques and to identify which can be integrated into crop management, it is necessary to quantify the disease (Gomes *et al*., 2004).

Quantifying disease enables control measures to be evaluated for whether they will be effective and therefore recommended for application in the field. For producers, the benefits of disease quantification include the assistance for efficient crop management decisions and prioritization of resources to enable low environmental impacts in sustainable disease management (Bergamin Filho and Amorim, 1996).

Among the methods for assessment of plant diseases, the most commonly implemented are those that are visual. These are simple because they do not require the use of sophisticated equipment, and they are accurate and precise (Campbell and Madden. 1990). Key tools for employing these techniques are diagrammatic disease severity scales. This method helps to define disease severity using photographs or diagrams of symptomatic plants or their organs. However, although this approach is simple, development must meet criteria to ensure the correct quantification of disease severity (Bergamin Filho and Amorim, 1996).

The primary aspects to be evaluated in the development of a diagrammatic disease severity scale are the minimum and maximum limits of the scale corresponding to the disease levels found in the field, and use of images that display a pattern compatible with the symptoms representing the levels of disease. A further important consideration is the limits of visual acuity of the human eye, according to Weber-Fechner's Law, assigning scores with respective severity intervals, as the human eye has difficulty seeing points or precise percentage values (Horsfall and Barratt, 1945; Nutter and Schultz. 1995).

The present study aimed to develop and validate a diagrammatic scale for accurate and precise assessment of fig rust, because no standardized methods were available for quantifying severity of the disease, which is the most important disease affecting fig orchards in subtropical conditions.

MATERIALS AND METHODS

Diagrammatic scale development

To develop the diagrammatic scale, 190 fig leaves from the field were randomly collected, that displayed different levels of disease severity. The leaves, naturally infected, were from several fig trees in an experimental orchard at the Federal University of Lavras, Brazil. The municipality is located at 21° 13' 40" south latitude and 44° 57' 42" west longitude, at an average altitude of 970 m above sea level. According to the Köppen climate classification, Lavras has a tropical climate of the Cwa type, characterized by dry winters and hot, humid summers (Alvares *et al*., 2014). To confirm the causal agent of the disease on the leaves, anatomical sections were prepared from a diseased leaf and analysed for the pathogen morphology.

All plant material was photographed on a white background, using a Nikon d3100 digital camera, in automatic mode, with 18–55 mm lens focal length. Subsequently, the diseased and total leaf area were determined for each leaf using the Assess® software (American Phytopathological Society). Pustules and the areas with necrotic and chlorotic tissue caused by the disease were considered as diseased areas.

According to the minimum and maximum levels found, a frequency plot was constructed, plotting the percentage of damaged leaf area (x-axis), in severity intervals of 5% (y-axis). These values were then fitted to a simple linear model and to non-linear exponential and logarithmic models (Campbell and Madden. 1990). The model that best fitted the frequency plot was chosen

as indicated from the largest R^2 and the significance of the parameters of the equations in the t-test. The disease severity scale was created according to the intervals with the greatest concentration of leaves having the same percentage of damaged area. The severity intervals for each score were established according to Weber-Fechner's visual acuity law (Horsfall and Barrat, 1945; Nutter and Schultz, 1995) and according to the shape and distribution of the lesions. Photographs of leaves with disease lesions were then used to develop the scale.

Diagrammatic scale validation

To validate the diagrammatic scale, 50 leaves of fig showing symptoms of rust were used, representing all variation levels of dis-ease severity. In three evaluations, 8 evaluators without experience in quantification of plant disease observed images of diseased leaves using Microsoft PowerPoint 2010. The first evaluation was performed without using the scale. After an interval of 7 days, a second evaluation was performed aided by the diagrammatic scale. To assess the repeatability of the observed values, a third evaluation was performed after 7 days, also using the proposed scale.

Based on the data obtained from each evaluator the accuracy and precision the developed scale were determined using Lin's method. Lin's concordance correlation coefficient (Pc) (Lin. 1989), to assess agreement between pairs of observations, was used to measure adjustment between the actual values and estimated disease severities. The method also includes other variables to aid in validation. The scale shift factor, where 1 = perfect agreement between x and y, measures the difference between actual and estimated values, and is calculated as the difference between the slope of the fitted regression lines and the concordant line. The location shift factor, where 0 = perfect agreement between x and y, estimates the change of the fitted regression line relative to the concordant line, by measuring the difference in height between the two lines. The BIAS correction factor, which measures how far the fitted line deviates from the concordant line, was calculated from the location shift factor and the scale shift factor, derived from the means and standard deviations of x and y. In addition to these factors, Pearson's correlation was used to evaluate the precision of the assessments. The confidence interval (CI) ($P < 0.05$) between the groups of evaluators, with and without the use of the scale, was calculated to determine if there were significant differences between the evaluations.

The repeatability of the estimates from each evaluator was determined by R^2 values of the linear regression between two assessments using the scale (Nutter *et al.*, 1993). The reproducibility of the estimates was evaluated by R^2 values obtained from linear regressions between the estimated severities of the same sample unit using different evaluators in pairs (Kranz. 1988; Campbell and Madden. 1990; Nutter and Schultz. 1995).

The data were tabulated and the statistical analyses performed using the RStudio software (R Core Team, 2018), and the epi.ccc function of the epiR package (Stevenson *et al.*, 2018) to determine the Lin's concordance correlation coefficient.

RESULTS

Scale development

The minimum and maximum severity of fig rust was 0% and the maximum severity was 89.3%. A high proportion (43%) of leaves were in the frequency intervals up to 5% severity (Table 1). Based on the disease severity found in natural infections, the scale had a maximum level of 89.3%, with clorotic and necrotic areas.

The best model adjusted for the frequency values in the severity intervals was logarithmic, in this case in according of Weber-Fechner's law, with the greatest R^2 (87%) and significance of the parameters of the equations in the t-test (Table 2).

The severity scale was developed using six scores or percentage intervals (Figure 1), three of which were distributed into intervals ranging up to 15.0% of diseased leaf area. The interval up to 1% included 11.6% of the total leaves, constituting the greatest frequency unit interval. The six percentage severity intervals of the scale were 0, 0.1–5.0%, 5.1–15.0%, 15.1–25%, 25.1–50.0% and >50%.

Scale validation

According to Lin's method, estimates of disease severity assessments improved with the use of the proposed scale (Table 3). According to the concordance coefficient and correlations between the actual and estimated values, greater estimation efficiency was obtained with use of the scale (a = 0.80) compared to evaluations without use of the scale (a = 0.71). The evaluators overestimated disease severity when not using the scale (c = 0.33), and underestimated severity when they used the scale (c = -0.28). The confidence interval between the two evaluations did not differ significantly, however, proving that there was no significant improvement in the variable under analysis. The Pearson's correlation

Table 1. Frequency distribution, in unit intervals, of disease severity values (%) of rust on fig leaves.

Interval (Severity %)	Frequency	Percentage (%)	Cumulative frequency	Cumulative Percentage (%)
0-1	22	11.6	22	11.6
1-2	2	1.1	24	12.6
2-3	7	3.7	31	16.3
3-4	5	2.6	36	19.0
4-5	7	3.7	43	22.7
5-6	2	1.1	45	23.7
6-7	4	2.1	49	25.8
7-8	4	2.1	53	27.9
8-9	4	2.1	57	30.0
9-10	3	1.6	60	31.6
10-11	1	0.5	61	32.1
11-12	1	0.5	62	32.6
12-13	2	1.1	64	33.7
13-14	3	1.56	67	35.3
14-15	4	2.1	71	37.4
15-16	6	3.2	77	40.5
16-17	2	1.1	79	41.6
17-18	5	2.6	84	44.2
18-19	3	1.6	87	45.8
19-20	1	0.5	88	46.3
20-21	4	2.1	92	48.4
21-22	5	2.6	97	51.0
22-23	3	1.6	100	52.6
23-24	1	0.5	101	53.3
24-25	5	2.6	106	55.8
25-26	2	1.1	108	56.8
26-27	0	0.0	108	56.8
27-28	3	1.6	111	58.4
28-29	2	1.1	113	59.5
29-30	1	0.5	114	60.0
30-31	1	0.5	115	60.5
31-32	4	2.1	119	62.6
32-33	1	0.5	120	63.1
33-34	1	0.5	121	63.7
34-35	4	2.1	125	65.8
35-36	1	0.5	126	66.3
36-37	2	1.1	128	67.4
37-38	1	0.5	129	67.9
38-39	1	0.5	130	68.4
39-40	0	0.0	130	68.4
40-41	3	1.6	133	70.0
41-42	2	1.1	135	71.0
42-43	3	1.6	138	72.6
43-44	3	1.6	141	74.2
44-45	0	0.0	141	74.2
45-46	0	0.0	141	74.2
46-47	2	1.1	143	75.3
47-48	1	0.5	144	75.8
48-49	0	0.0	144	75.8
49-50	1	0.5	145	76.3
>50	45	23.7	190	100.00

Table 2. Parameters of the linear and non-linear models for the frequency of severity of fig rust, in severity intervals.

Model	R^2 [a]	r [b]	y_0 [c]
Exponential	0.75	0.04***	39.42***
Logarithmic	0.87	-0.0004***	0.02***
Linear	0.57	-0.28***	22.95***

[a] Coefficient of determination (R^2).
[b] Progress rate (r).
[c] Initial inoculum (y_0).
*** Significant according to the t-tests ($P = 0.001$).

coefficient indicated increased precision of the evaluators when using the scale (e = 0.86), compared to the evaluations without the scale (e = 0.76). However, the value of the BIAS correction factor without the use of the scale (d = 0.94) was greater than that of the estimates obtained using the scale (d = 0.93). This indicated that there was no increase in the accuracy of the evaluators. Considering the confidence intervals, the assessments for fig rust with and without the use of the diagrammatic scale differed significantly at the 95% confidence interval, except for the location shift factor.

For reproducibility, without using the diagrammatic scale the value of the determination coefficient (R^2) ranged from 64 to 88%, with a mean of 81.1% (Table 4). With use of the scale, R^2 values ranged from 71 to 91% (mean = 80.7%) for the first evaluation, and from 61 to 81% (mean = 72.2%) in the second evaluation, with $R^2 \geq 70\%$ in approximately 82% of the combinations of evaluators.

There was good repeatability between the estimates of the same evaluator (Table 5). Between the two evaluations with the use of the scale, only one evaluator (A) exhibited a slope significantly different from 1, with good precision of the estimates of 87.5% of the evaluators. The evaluators all presented good repeatability in the estimates of leaf rust severity, as the mean variation between the first evaluation and the second evaluation was approx. 70%.

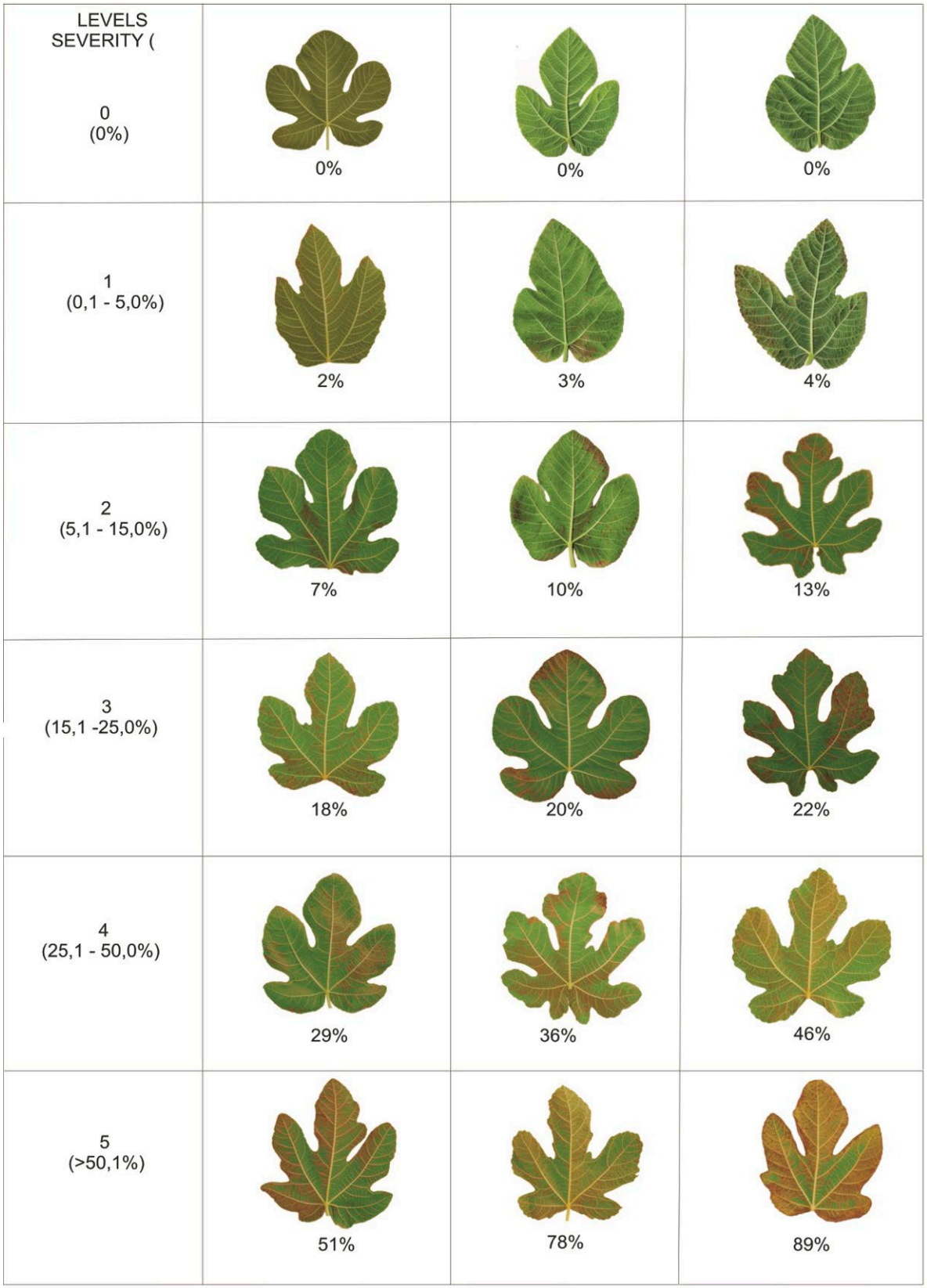

Figure 1. Diagrammatic scale for assessment of rust severity on fig leaves. The numbers represent percentages of leaf area diseased. leaf area.

Table 3. Lin's concordance correlation coefficients for eight evaluators without or with the diagrammatic disease severity scale, used to estimate rust severity on fig leaves.

Lin´s statistic	Without scale	With scale	95% CI[f]
Lin's concordance correlation coefficient [a]	0.71	0.80	**0.6872*; 0.8123***
Scale shift factor [b]	1.16	0.76	**0.8738*; 1.4020***
Location shift factor [c]	0.33	-0.28	-0.1705; 0.3112
Bias correction factor [d]	0.94	0.93	**0.7948*; 0.9328***
Pearson's correlation [e]	0.76	0.86	**0.8603*; 0.8837***

[a] Lin's concordance correlation coefficient.
[b] Scale shift factor relative to perfect agreement.
[c] Location shift factor relative to perfect agreement.
[d] Bias correction factor.
[e] Pearson's correlation.
[f] Upper and lower limits of the 95% confidence intervals.
Bold* represents a significant difference ($P \leq 0.05$) between the two evaluations, according to the t-tests.

Most evaluators presented good precision, regardless of whether the scale was used. Sixty-three percent of the participants presented R^2 values in the second evaluation that were greater or equal to those for the first evaluation, suggesting equal or greater precision with the second evaluation. Absolute errors were reduced when the scale was used, decreasing the range of values between the first and second evaluations (Figure 2). However, in the second evaluation, using the scale, the minimum and maximum values observed for the residuals of all the evaluators were, respectively, -49.67 and 70.90, increasing the range of the determined values.

DISCUSSION

The diagrammatic scale developed in here allowed the evaluators to obtain accurate and precise estimates of fig rust severity, according to the validation analyses.

Linear and non-linear models were fitted to the data to determine if the scale levels should increase logarithmically or linearly. The particulars of each pathosystem are considered for determination of scale intervals, and this fitting is required to assess the accuracy of the assessments. The model with the best fit was the logarithmic model, and with this the intermediate levels of the scale were determined based on the highest frequency intervals of disease levels on the leaves, combined with the logarithmic increase in severity, in accordance with Weber Fechner law (Campbell and Madden, 1990).

Each level of the scale was defined according to the frequency distribution of the number of leaves with a

Table 4. Coefficients of determination (R^2) of the linear regression equation between pairs of different evaluators, with or without the use of the disease severity assessment scale in two evaluations, estimating rust severity on fig leaves.

Evaluator	Without scale						
	B	C	D	E	F	G	H
A	0.73	0.76	0.75	0.68	0.64	0.77	0.73
B		0.83	0.78	0.88	0.75	0.85	0.71
C			0.82	0.80	0.81	0.86	0.77
D				0.80	0.74	0.81	0.74
E					0.82	0.83	0.66
F						0.87	0.84
G							0.84
	With scale – 1st assesment						
	B	C	D	E	F	G	H
A	0.74	0.79	0.71	0.86	0.87	0.78	0.81
B		0.78	0.79	0.82	0.79	0.75	0.76
C			0.91	0.78	0.85	0.80	0.82
D				0.79	0.79	0.77	0.78
E					0.83	0.81	0.85
F						0.86	0.89
G							0.83
	With scale – 2nd assesment						
	B	C	D	E	F	G	H
A	0.70	0.72	0.65	0.79	0.74	0.65	0.78
B		0.61	0.63	0.80	0.81	0.77	0.72
C			0.69	0.64	0.74	0.65	0.72
D				0.78	0.73	0.75	0.69
E					0.75	0.79	0.68
F						0.78	0.77
G							0.69

Table 5. Intercept (β_0), slope (β_1) and coefficient of determination (R^2) of the linear regression equations relating the first to second estimates of rust severity on fig leaves, for estimates performed by eight evaluators using the disease severity scale.

Evaluator	Coefficients		
	β_0	β_1	R^2
A	10.55*	0.80 [ns]	0.62
B	8.67 [ns]	0.69 [ns]	0.64
C	2.63 [ns]	0.71 [ns]	0.51
D	0.56 [ns]	1.04 [ns]	0.73
E	2.82 [ns]	0.90 [ns]	0.80
F	1.47 [ns]	0.85 [ns]	0.79
G	2.61 [ns]	0.80 [ns]	0.74
H	6.22 [ns]	0.83 [ns]	0.75

* ns represent situations where the null hypothesis ($\beta_0 = 0$ or $\beta_1 = 1$) was, respectively, rejected and not rejected according to t-tests ($P = 0.05$).

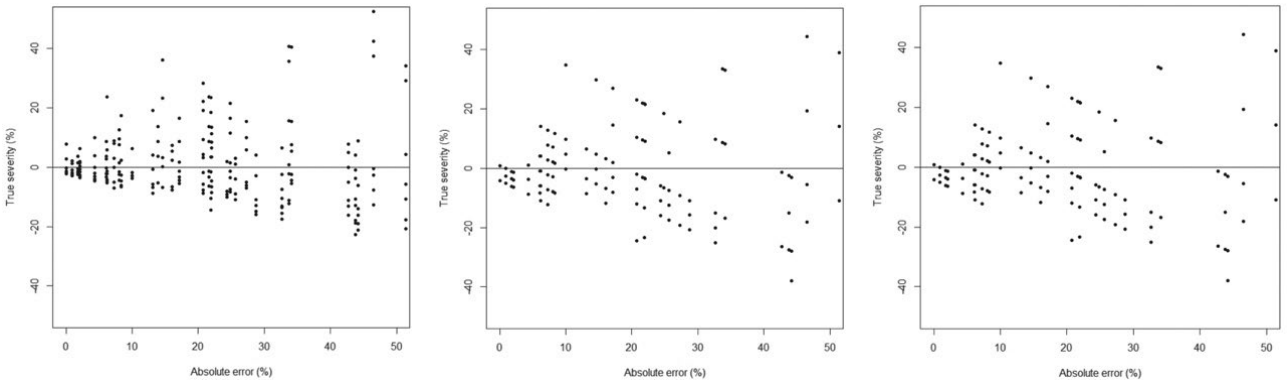

Figure 2. Distributions of residuals (estimated severity – actual severity) of estimates of rust severity on fig leaves, with or without the use of the disease severity scale, in two assessments by eight evaluators.

specific disease leaf area found in the field. Although the greatest unit interval of diseased leaves was between 0 and 1%, this interval was not used in the scale due to the difficulty in visually locating a lesion of that size on a fig leaf. Lorenzetti (2008) proposed a diagrammatic scale to quantify the severity of the same rust disease. However, their scale was expressed in percentages, with no values less than 4% of disease severity, and with large intervals between the percentages, factors that increase the subjectivity of the estimates of actual disease severity.

Based on the severity of fig rust found in natural infections, the scale had a maximum level of 89.3%, with chlorotic and necrotic areas. The absence of leaves with severities greater than 89% in the current study could be characteristic of the pathogen–host interaction of this disease. At greater disease intensities, leaf tissue necrosis was present that led to early leaf abscission.

Severity values greater than 50% were combined in the proposed scale because the human eye has difficulty distinguishing disease severity greater than this percentage (Campbell and Madden. 1990), and few leaves have been found in the unit intervals above this value due to defoliation caused by the disease (Pastore *et al.* 2016). The scale developed by Angeloti *et al.* (2011) for assessment of grapevine rust found a maximum level of severity in leaves of 75% for a similar reason. Dolinski *et al.* (2017), when developing a scale to quantify severity of peach rust severity, defined a maximum level of 30%. Although these authors found leaves with greater severity levels, as the variation in the disease severity can be due to cultivar susceptibility differences, cultivation practices and climate variations.

For the construction of the fig rust scale, photographs were used instead of graphical representations, which is a common practice. Belan *et al.* (2014) noted that this method increases the precision and accuracy of disease assessments. Using the real images or photographs, rather than black and white or colour diagrams, draws evaluators to the reality, facilitating the disease assessments.

In most studies involving validation of diagrammatic scales to determine disease severity in plant leaves, evaluators have exhibited tendency to overestimate the severity of particular diseases (Capucho *et al.*, 2011; Belan *et al.*, 2014; Freitas *et al.*, 2015). In some cases, such as early blight in potato, leaf disease severity was underestimated (Michereff *et al.*, 2000; Gomes *et al.*, 2004). In the present study, it is not possible to make such an inference because there was no significant difference between the evaluations.

In the validation of other rust severity evaluation scales, increased accuracy and precision by evaluators has been observed with their use. Capucho *et al.* (2011), using a diagrammatic scale for coffee leaf rust, validated the results using Lin's method, and the mean Pearson's correlation coefficient increased from 0.77 to 0.87 when using the proposed scale. In a study of diagrammatic scale validation for sugarcane orange rust, Klosowski *et al.* (2013) determined the indices by simple linear regression and obtained satisfactory results, with 100% of the evaluators obtaining intercepts statistically equivalent to zero and slope values equal to 1, indicating the absence of systematic deviations.

Although it is the most commonly used method for validating scales, linear regression does not detect the values of intercept 0 ($\beta 0$) and slope ($\beta 1$), when the data are scattered (Bock *et al.*, 2010), and this may lead to erroneous conclusions. Lin's method provides a single index ("Lin's concordance correlation coefficient"), and the accuracy and precision of severity estimates. This method has been used to analyse how disease severity data behave, and how they relate to actual estimates and

with evaluations of the repeatability of estimates (Nita *et al.*, 2003; Bock *et al.*, 2008).

The reproducibility of the disease severity estimates among the evaluators was analysed using paired linear regression (Nutter and Schultz, 1995), and greater standardization was observed in the estimations with the use of the described here. However, in some pairs the coefficient of determination reached values between 61 and 69% in the second assessment using the scale. It is possible that these results were due to inexperience among some evaluators with disease quantification.

More than 75% of the evaluator pairs presented R^2 values greater than 70% using the diagrammatic scale, similar to that found in the validation of scales for other pathosystems, such as rust (Capucho *et al.*, 2011) and bacterial blight (Belan *et al.*, 2014) of coffee leaves, with mean values, respectively, of 87% and 99%.

The range of the residuals in the assessments using the scale described here were -57.50 to 72.03 for the first evaluation, and -49.67 to 70.90 for the second. The high values are explained by the difficulty in evaluating the disease. Due to the characteristics of the lesions, which are individual and small, and scattered on fig leaf surfaces, this causes evaluators to underestimate or overestimate the diseased leaf areas. This can influence the quality of disease estimation through psychological stimuli and responses, including the complexity of the sample units, size and shape of the lesions, colour and number of lesions, evaluator fatigue or difficulty to concentrate on the task (Sherwood *et al.*, 1983; Kranz, 1988).

Disease severity evaluation results are considered satisfactory when the means of the absolute errors are between 10 and 15%. This was described by De Paula *et al.* (2016), proposing and validating diagrammatic scales to assess brown eye spot in red and yellow coffee cherries, and also by Godoy *et al.* (2006) validating a scale for quantification of soybean rust. Belan *et al.* (2014) reported mean absolute errors between -20.95 and 20.01 in two evaluations, with a scale for assessment of bacterial blight in coffee leaves, especially at high severity levels, which is contrary to the observations in the present study.

In conclusion, we have developed and validated a diagrammatic scale for assessment of rust severity on fig leaves. The disease severity scale outlined here provides good accuracy, precision, repeatability and reproducibility, for evaluation of this disease.

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Research Paper

Diversity of genes for resistance to stripe rust in wheat elite lines, commercial varieties and landraces from Lebanon and Syria

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Summary. Stripe (yellow) rust, caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*), is a major threat to wheat production in Central and West Asia and North Africa (CWANA). Effective fungicides are available, but host resistance remains the most economical, effective and ecologically sustainable method for stripe rust control. Understanding the genetic diversity of resistance to *Pst* is a key element in breeding for durable rust resistance. Multipathotype tests were performed on 87 elite lines of bread wheat from the spring wheat breeding programme at the International Center for Agricultural Research in Dry Areas (ICARDA), 23 Lebanese bread and durum wheat varieties, and 28 Lebanese landraces, with 11 *Pst* pathotypes. Low and high infection types were identified for the resistance genes *Yr1*, *Yr3*, *Yr4*, *Yr6*, *Yr7*, *Yr9*, *Yr17*, *Yr25*, *Yr27*, and *Yr32*. All but one of these genes (*Yr32* being the exception) were postulated. ICARDA elite lines displayed greater diversity for *Yr* genes than the Lebanese varieties and landraces. *Yr27* was the most frequent *Yr* gene postulated singly in the Lebanese varieties. *Yr7*, together with other unidentified *Yr* genes, was the most frequent gene in the ICARDA elite lines. Combinations of two *Yr* genes were common in ICARDA elite lines. These results confirm that the landraces consist of several genotypes. Seventy-five percent of landraces were susceptible to all pathotypes, but they displayed resistance diversity, with different proportions of resistant seedlings. In two landraces, some plants were resistant to the Warrior pathotype, which has recently spread in CWANA regions, and to other pathotypes. This indicates the presence of new resistance genes in these landraces. Some landraces, elite ICARDA lines and Lebanese varieties were completely resistant to all pathotypes, and are therefore potential sources of new resistances.

Keywords. Stripe (yellow) rust, wheat, gene postulation, seedling resistance.

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INTRODUCTION

In the context of global warming and the food insecurity, it is likely to cause, feeding the expanding world population through sustainable agricultural practices is a major challenge. With predicted world population of nine

billion by 2050, the demand for wheat is expected to increase by 60%. Annual increases in wheat yields will need to increase from the current level of 1% to at least 1.6% to meet this demand (Lucas, 2012).

Stripe rust, caused by the biotrophic fungus *Puccinia striiformis* f. sp. *tritici* (*Pst),* is a common wheat disease of economic importance in all world wheat production regions (de Vallavieille-Pope *et al*., 2012). In most of these areas stripe rust causes yield losses of 10-70% (Chen, 2005), depending on the time at which initial infections occur, host population density, susceptibility and nutritional status, disease development, and the duration of the epidemics. The last 40 years have seen five major stripe rust epidemics in the Central and West Asia and North Africa region (CWANA), in 1973, 1978, 1995, 2005 and 2010 (Solh *et al*., 2012). The two most recent epidemics were due to the successive emergence of *Pst* pathotypes with new virulence factors overcoming the widely used *Yr9* and *Yr27* resistance genes (Yahyaoui *et al*., 2002; Hodson and Nazari, 2010; Sharma-Poudyal *et al*., 2013). According to Hovmøller *et al*. (2011), PstS2, an aggressive strain with virulence against *Yr* genes *2*, *6*, *7, 8*, *9*, *25*, and *27,* was present at high frequency in the Red Sea area, East Africa and in Western and Central Asia between 2003 and 2008. The PstS2 strain was first detected in North America in 2000 (Milus *et al*., 2009). It was present in 50% of the virulence profiles of rust isolates surveyed in Syria in 2011 (El Amil *et al*., in press). The recent spread of Warrior pathotypes to this region (Mert *et al*., 2016) has added an additional dimension to the widespread stripe rust epidemics in wheat-growing areas in CWANA.

This damaging fungus causes losses of wheat grain yield and quality, by reducing tillering and causing grain to shrivel (Roelfs *et al.,* 1992), unless it is controlled by the use of resistant cultivars or timely fungicide applications (Hau and de Vallavieille-Pope, 2006). The deployment and use of resistant cultivars is the most economical and environmentally-friendly measure for controlling this disease (Pathan and Park, 2007). Effective deployment of resistance genes for the management of stripe rust in wheat requires knowledge of the resistance status and diversity of the resistance genes in available cultivars (Nazari *et al*., 2008). The durability of resistance may be threatened by the frequent emergence of new pathotypes. An understanding of the *Pst* population is therefore crucial in gene deployment strategies (McDonald and Linde, 2002). These strategies involve: i) the deployment of new resistance genes in a controlled manner and over a restricted geographic scale; ii) the combination of several resistance genes within a single cultivar to slow the emergence of new virulent pathotypes; and iii) the combination of race-specific resistance with non race-specific or partial resistance within a single cultivar. These approaches require a knowledge of the resistance genes present in the breeding germplasm and commercial cultivars. It is, therefore, important to identify the resistance genes present in different cultivars, because cultivars may have resistance genes in common, even if they originated from genetically different sources. Knowledge of the resistance genes present makes it possible to prevent the release of mega-cultivars containing the same resistance genes or profiles (Statler, 1984).

Gene postulation is based on the theory of a gene-for-gene relationship (Flor, 1956), according to which it is possible to postulate the existence of race-specific genes for resistance in a cultivar provided with an array of pathotypes bearing diverse combinations of avirulence and virulence genes. This approach can be used for the rapid identification of probable race-specific rust resistance genes (*Yr*) in a large group of wheat lines. This method has traditionally been used for the three rust diseases (Perwaiz and Johnson, 1986; Nazari *et al*., 2008). Stripe rust resistance genes have been postulated in wild emmer wheat derivatives and advanced wheat lines from Nepal (Sharma *et al*., 1995), French wheat lines (de Vallavieille-Pope *et al*., 1990; Robert *et al*., 2000), Danish wheat cultivars (Hovmøller, 2007), Chinese wheat cultivars and advanced lines (Xia *et al*., 2007), and Ethiopian bread wheat cultivars (Dawit *et al*., 2012).

Landraces of cultivated plants were the principal focus of agricultural production until the end of the nineteenth century and the advent of formal plant breeding (Harlan, 1975). According to Camacho Villa *et al*. (2005), "a landrace is a dynamic population of a cultivated plant that has a historical origin, distinct identity and lacks formal crop improvement, as well as often being genetically diverse, locally adapted and associated with traditional farming systems". Modern cereal cultivars are derived from narrow germplasm pools and are mostly adapted to high-input agriculture. A distinction is made between landraces and the modern, so-called "elite" lines generated by formal crop breeding programmes (Newton *et al*., 2010). Landraces may be good reservoirs of non race-specific or partial resistances capable of conferring durability when combined with major resistance genes commonly exploited in modern cultivars. Given that landraces are adapted to local edaphic and climatic conditions and display tolerance or resistance to many pests and diseases, their use could expand the narrow genetic basis of elite lines (Beharav *et al*., 1997). Zhang (1995) showed that nine Chinese wheat landraces expressed slow rusting or quantitative

resistance to stripe rust. Lebanon is located in the Fertile Crescent, the area in which wheat and its wild relatives are most diverse (Harlan and Zohary, 1966). Lebanese landraces are, therefore, also likely to be promising sources of novel resistance genes with major and partial effects. The identification of seedling rust resistance genes in Lebanese landraces is, therefore, an important first step towards further wheat improvement in the CWANA region.

The genes conferring resistance to wheat stripe rust in ICARDA elite breeding lines, Lebanese cultivars and Lebanese landraces, remain largely unknown. The present study aimed to provide detailed information about specific resistance to wheat stripe rust, detectable at the seedling stage, in 87 elite lines of bread wheat from the spring wheat breeding programme at ICARDA, 23 Lebanese bread and durum wheat varieties and 28 Lebanese landraces. Gene postulation was performed with an array of 11 *Pst* pathotypes that distinguished between low and high infection types for the resistance genes *Yr1*, *Yr3*, *Yr4*, *Yr6*, *Yr7*, *Yr8*, *Yr9*, *Yr17*, *Yr25*, *Yr27*, *Yr32*, *YrSD*, *YrSu* and *YrSP*. Adult plant resistance was also evaluated in some ICARDA lines.

MATERIALS AND METHODS

Pathogen material

The virulence combinations and pathotype codes of the *Pst* isolates used for resistance gene postulation were determined with the European and World sets of 15 differential varieties (Johnson *et al*., 1972), 13 Avocet lines near-isogenic for *Yr1*, *Yr5*, *Yr6*, *Yr7*, *Yr8*, *Yr9*, *Yr10*, *Yr15*, *Yr24*, *Yr26*, *Yr27*, *Yr32* and *YrSP* (Wellings *et al*., 2009), and the lines Kalyansona (*Yr2*), Federation 4*/ Kavkaz (*Yr9*), Clement (*Yr9+*), VPM1 (*Yr17+*), TP981 (*Yr25*) and Opata (*Yr27*). Each differential line carries at least one race-specific resistance gene (*Yr*) expressed at the seedling growth stage.

Resistance genes were postulated at the seedling stage at the INRA BIOGER rust facility, on the basis of infection types (IT), with a set of 11 French pathotypes displaying complementary virulences, including the recently propagated Warrior race (Table 1) (de Vallavieille-Pope *et al*., 2012). As most of these pathotypes present more than one avirulence factor, it was not always possible to infer precise resistance gene combinations.

All isolates from the INRA-Grignon collection were purified from single spores, and were stored in liquid nitrogen. Spore multiplication was performed in a controlled climate chamber. Reference isolate spores were used to inoculate 7-d-old seedlings of the susceptible cultivar 'Victo', which was then incubated in a dew chamber at 8°C for 16 h in the dark to ensure successful infection, before transfer to a controlled climate chamber (day: 16 h, 300 μmol m^{-2} s^{-1}, 17°C; night: 8 h, 14°C). Seedlings were exposed to high-intensity light treatment at 200 μE m^{-2} s^{-1} for at least 8 h before inoculation, to maximize infection efficiency (de Vallavieille-Pope *et al*., 2002). One week after inoculation, each pot was sealed in a cellophane bag to prevent cross-contamination. Uredospores were collected 18 d post-inoculation, were

Table 1. Pathotype code, name and avirulence/virulence formula of 11 French *Puccinia striiformis* f. sp. *tritici* pathotypes used for the postulation of stripe rust resistance genes in Syrian and Lebanese wheat genotypes.

Pathotype code	Pathotype nomenclature[a]	Avirulence formula[b]	Virulence formula
A	6E16	*1, 3, 4, 5, 9, 10, 15, 17, 24, 25, 26, 27, 32, SD, Su, ND, SP*	*2, 6, 7, 8*
B	6E16*v9v27*	*1, 3, 4, 5, 10, 15, 17, 24, 26, 32, SD, Su, ND, SP*	*2, 6, 7, 8, 9, 25, 27*
C	43E138	*4, 5, 6, 8, 9, 10, 15, 17, 24, 26, 27, 32, Su, SP*	*1, 2, 3, 7, 25, SD, ND*
D	45E140	*4, 5, 7, 8, 9, 10, 15, 17, 24, 26, 27, 32, Su, SP*	*1, 2, 3, 6, 25, SD, ND*
E	106E139	*1, 5, 6, 8, 9, 10, 15, 17, 24, 26, 27, 32, SP*	*2, 3, 4, 7, 25, SD, Su, ND*
F	169E136*v17*	*4, 5, 6, 7, 8, 10, 15, 24, 26, 27, 32, Su, SP*	*1, 2, 3, 9, 17, 25, SD, ND*
G	232E139	*1, 5, 6, 7, 8, 10, 15, 17, 24, 26, 27, 32, SP*	*2, 3, 4, 9, 25, SD, Su, ND*
H	237E140	*5, 7, 8, 10, 15, 17, 24, 26, 27, 32, SP*	*1, 2, 3, 4, 6, 9, 25, SD, Su, ND*
I	237E141*v17*	*5, 7, 8, 10, 15, 24, 26, 27, 32, SP*	*1, 2, 3, 4, 6, 9, 17, 25, SD, Su, ND*
J	237E173*v17 (Oakley/Solstice)*	*5, 7, 8, 10, 15, 24, 26, 27, SP*	*1, 2, 3, 4, 6, 9, 17, 25, 32, SD, Su, ND*
K	239E175*v17 (Warrior)*	*5, 8, 10, 15, 24, 26, 27*	*1, 2, 3, 4, 6, 7, 9, 17, 25, 32, SD, Su, ND, (SP)*[c]

[a] Pathotype nomenclature is based on Johnson *et al*. (1972).
[b] *YrSD*, *YrSu*, *YrND* and *YrSP*, correspond to, respectively, Strubes Dickkopf, Suwon 92 × Omar, Nord Desprez and Spaldings Prolific.
[c] *SP* infection types 5 to 6 were considered to be intermediate reactions, and are shown in parentheses.

dried in a desiccator containing silica gel at 4°C for 3 d, and were then stored in liquid nitrogen (LN). Any cold dormancy of samples stored in LN was broken by subjecting the uredospores to a heat shock (40°C for 10 min) before inoculation in *Yr*-gene postulation tests.

Host material

We tested 138 genotypes at the seedling stage. These included: 87 advanced lines of bread wheat from the spring wheat breeding programme at ICARDA (Table S1), 23 cultivars commonly grown in Lebanon (13 of bread wheat and ten of durum wheat) (Table S2), and 28 Lebanese landraces (21 accessions of bread wheat and seven of durum wheat; Table S2). Seed stocks for elite lines and landraces were obtained, respectively, from ICARDA and the Lebanese Agriculture Research Institute (LARI). Landraces are known to be highly diverse and heterogeneous. Landrace seeds were therefore collected from a number of different sites in Lebanon, to obtain a broad genetic pool. The seeds were then purified at LARI for morphological traits corresponding to the criteria for use in agriculture, before the resistance gene tests (Table 2).

Inoculation and scoring

All seeds were planted in square pots (7 × 7 × 8 cm) filled with standard peat soil. Five seeds of each elite line and variety and 15 seeds of each landrace were planted in two replicated pots. The pots were placed in air-filtered cabinets in a glasshouse, at temperatures between 15 and 25°C, with a 16-h photoperiod extended with sodium vapour lamps, for 10 d. The seedlings were inoculated with spores when they were 2 weeks old and with second leaves fully expanded. The inoculated spores had been stored at –80°C, then taken out of the freezer and immediately heat shocked at 40°C for 10 min before use. Spores (3 mg) were suspended in 600 µL of engineered fluid (NovecTM 7100) for inoculation onto seedlings. Inoculated plants were incubated for 24 h in the dark in a dew chamber at 8°C and 100% relative humidity, after which they were placed in cabinets with the conditions described above. The experiment was performed twice.

Seedling infection types were recorded 15–17 d after inoculation, using a 0–9 scale based on the presence of necrosis, chlorosis, size of sporulation areas and sporulation intensity (McNeal *et al*., 1971). Infection types (IT) 0 to 4 were considered to indicate various levels of incompatibility (host resistance and pathogen avirulence) between host and pathogen, whereas infection types 7 to 9 were considered to correspond to compatible (host susceptibility and pathogen virulence) interactions. Infection types 5 to 6 were considered to be intermediate reactions (Roelfs *et al.,* 1992). Resistance genes were postulated by comparing the low and high IT patterns obtained with the pathotype array on the tested entities with those of differential lines with known resistance genes. If a wheat variety had a low/high IT pattern similar to that of a differential line with a known resistance profile, the tested genotype was postulated to possess the same resistance genes as the differential. This method was applied successively to all *Yr* genes detectable with the array of 11 pathotypes used for the study.

The diversity of seedling resistance within the Lebanese wheat landrace collections was investigated by assessing the frequency of resistant plants in the overall susceptible landrace population and the frequency of susceptible plants in the overall resistant landrace population, for the 11 *Pst* pathotypes (Table 6).

Assessment of adult plant resistance

The ICARDA elite lines tested at the seedling stage against pathotype 6E16*v9v27* (the most prevalent *Pst* pathotype in Syria and Lebanon during the 2010–2011 cropping season) included 44 lines containing postulated resistance gene and/or gene combinations that were assessed for adult-plant resistance. The experiments on adult plants were performed with this pathotype at the ICARDA research stations in Tal Hadya, Syria in 2009-2011, and Terbol, Lebanon in 2009–2013 (Table 5).

At each site, 30 seeds of each genotype were planted in two 0.5 m rows in a field nursery in November in each year. The highly susceptible 'Morocco' and two cultivars known to carry *Yr9* (Seri-82) and *Yr27* (Cham-8) were planted as spreader rows bordering the trial areas, in all pathways, and at ten-row intervals within the trial. The inoculum used for this study comprised the dominant pathotypes collected separately in Syria or Lebanon from natural infections in the same fields during the previous year. The inoculated pathotypes carried virulence against the genes *Yr2*, *Yr6*, *Yr7*, *Yr8*, *Yr9*, *Yr25*, *Yr27* and *YrSD*. The trail fields were dusted in the evenings with a spore-talc mixtures (1 to 50), at the seedling, tillering, and flag leaf stages. Disease infection types were recorded as described by Roelfs *et al*. (1992), and the modified Cobb scale was used to assess disease severity (Peterson *et al*., 1948) at the host booting and flag leaf stages. Flag leaf scoring was used to assess the final disease responses of the tested genotypes at the adult plant growth stage.

Table 2. Resistance group, seedling infection types and postulated stripe rust resistance genes in 87 wheat elite lines from ICARDA, tested against 11 *Puccinia striiformis* f. sp. *tritici* pathotypes.

Group	Entry No.	Genotype	Pathotype code [a]											Postulated *Yr* genes
			A	B	C	D	E	F	G	H	I	J	K	
1	**3**	Tabeldi-1	1	1	3	6	1	5	1	3	2	2	5	Resistant[b]
	11	Babaga-3	1	2	3	6	1	5	1	3	2	2	4	Resistant
	20	Sale-6	1	1	2	2	1	2	1	2	2	2	3	Resistant
	32	Hashab-2	1	1	2	2	1	4	1	2	2	2	4	Resistant
	40	Usher-18	1	5	4	3	3	3	2	2	2	3	4	Resistant
	45	Saba/Flag-1	2	6	2	2	2	2	2	2	2	2	4	Resistant
	72	Naji-3	1	2	3	4	1	6	1	2	2	2	5	Resistant
	78	Shuha-8/Ducula	1	1	2	2	1	3	1	2	2	2	4	Resistant
2	**12**	Cham-6	8	7	8	8	9	8	8	8	8	8	8	Susceptible[c]
	75	Nesma*2/14-2//2*Safi-3	8	7	8	8	9	8	8	8	8	8	8	Susceptible
	79	Shuha-8/Ducula	8	6	8	8	8	8	8	8	8	6	8	Susceptible
3	6	Utique 96/Flag-1	4	7	3	8	9	5	9	8	8	8	8	Ni[d]
	7	Hamam-4	1	7	1	1	1	5	7	3	8	8	4	Ni
	16	Durra-8	2	8	3	6	9	5	8	3	8	8	2	Ni
	22	Fow-2/SD8036//SafiI-3/3/NS732/HER//Kauz'S'	3	8	5	8	8	8	8	3	8	5	2	Ni
	30	Sandall-5	2	3	2	2	2	9	2	2	8	8	8	Ni
	38	Temerind-8	1	8	1	8	-[e]	2	3	2	2	2	4	Ni
	39	Gonglase-4	2	2	8	1	2	9	2	8	8	8	8	Ni
	48	Bushraa-3	5	3	3	2	4	8	2	4	3	2	8	Ni
	54	Jasmin-5	2	2	3	7	8	8	8	8	4	3	3	Ni
	57	Qadanfer-5	7	8	4	3	8	5	8	6	2	3	8	Ni
	62	Tevee'S'/3/T.aestivum/SPRW'S'//CA8055/4/Pastor-2/5/Sunbri	1	1	8	8	1	8	1	8	4	8	8	Ni
	69	Manhal-4	2	2	8	8	1	1	6	6	2	8	8	Ni
	21	HD2206/Hork'S'/3/2*NS732/HER//Kauz'S'	4	6	5	8	7	9	8	7	7	8	8	Ni
	85	Girwill-13/2*Pastor-2	1	8	3	2	2	4	8	8	8	8	8	Ni
	14	Jawahir-14	6	1	8	8	1	9	1	8	8	8	8	Ni
		Chinese 166[f]	1	1	9	9	1	8	1	9	9	9	9	*Yr1*
4		**Avocet *Yr1*/6*Avocet S**	2	1	8	8	2	8	1	8	8	8	9	*Yr1*
	60	Crow'S'/Bow'S' -3-1994/95//Tevee'S'/Tadinia	1	1	8	8	1	9	1	8	7	8	7	*Yr1*
	61	Tevee'S'/3/T.aestivum/SPRW'S'//CA8055/4/Pastor-2/5/Sunbri	1	1	8	8	1	9	1	8	8	8	8	*Yr1*
	65	Qafzah-2/Ferroug-2	1	2	8	8	1	9	2	8	8	8	8	*Yr1*
	70	Usher-16	1	2	8	8	1	8	1	8	8	8	8	*Yr1*
	74	Settat-45	2	2	8	8	1	7	1	8	8	8	8	*Yr1*
		Vilmorin 23	2	3	8	8	9	8	8	9	9	9	9	*Yr3*
5	**46**	Bow #1/Fengkang15/3/HYS//DRC*2/7C	5	3	8	8	8	9	7	8	8	8	8	*Yr3*
6		**Hybrid 46**	2	1	1	2	9	2	8	9	9	9	8	*Yr4*
	33	Sanobar-1	2	3	2	2	8	2	8	8	8	8	8	*Yr4*
	18	Sanobar-6	2	2	2	2	8	2	8	6-7	8	8	8	*Yr4*
	82	ESWYT99#18/Arrihane	2	5	4	2	8	3	7	8	8	8	8	*Yr4*
7		**Avocet *Yr6*/6*Avocet S**	8	8	3	8	4	4	4	8	8	8	8	*Yr6*
		Heines Kolben	9	9	2	9	2	2	1	9	9	9	9	*Yr6, Yr2*
	67	Hamam-4/Angi-2	8	8	3	8	3	3	2	8	8	8	8	*Yr6*
	87	Hubara-16/2*Somama-3	7	8	2	8	2	2	3	8	6	8	8	*Yr6*
		Heines Peko	2	4	2	9	2	2	2	9	9	9	8	*Yr6, Yr+*
	2	Zafir-3	2	2	2	9	2	2	2	2	2	7	8	*Yr6, Yr+*

(Continued)

Table 2. (Continued).

Group	Entry No.	Genotype	Pathotype code [a]											Postulated *Yr* genes
			A	B	C	D	E	F	G	H	I	J	K	
	5	Aguilal/Flag-3	2	3	2	8	2	2	2	8	5	8	8	*Yr6, Yr+*
	47	Faisal-1	1	5	4	8	1	3	4	7	8	8	8	*Yr6, Yr+*
	63	Weebill-1/2*Qafzah-21	4	6	3	7	7	2	5	8	8	8	8	*Yr6, Yr+*
	64	Rebwah-12/Zemamra-8	2	1	2	2	1	3	1	8	8	8	8	*Yr6, Yr+*
8		**Avocet *Yr7*/6*Avocet S**	8	8	8	3	8	4	4	2	3	3	8	*Yr7*
		Lee	9	9	9	3	9	3	2	3	3	3	9	*Yr7, Yr+*
		Reichersberg 42	2	4	9	2	9	2	2	2	2	2	8	*Yr7, Yr+*
	43	Soonot-11	1	2	1	1	1	1	2	2	2	2	8	*Yr7, Yr+*
	23	Neem-2	1	2	1	1	1	1	2	2	2	2	8	*Yr7, Yr+*
	34	Cham-10	1	1	1	1	1	1	2	1	2	2	8	*Yr7, Yr+*
	19	Reyna-12	1	1	1	1	1	1	2	1	2	2	8	*Yr7, Yr+*
	36	Reyna-25	1	1	1	1	-	1	2	1	2	2	8	*Yr7, Yr+*
	41	Florkwa-2/Asfoor-5	1	2	1	1	1	1	2	2	2	2	8	*Yr7, Yr+*
	42	Settat-13	1	2	1	1	1	1	3	2	2	2	8	*Yr7, Yr+*
	44	Hubara-15/Zemamra-8	1	1	1	1	1	3	1	2	3	5	8	*Yr7, Yr+*
	37	Reyna-29	1	2	1	1	1	1	2	2	2	2	8	*Yr7, Yr+*
	68	Sisaban-3	1	1	1	1	1	1	2	1	2	2	8	*Yr7, Yr+*
	77	Achta*3//Kanz/KS85-8-4/3/Lakta-8/4/Zemamra-1	2	2	2	1	2	1	2	2	2	2	8	*Yr7, Yr+*
	52	Reyna-24	1	1	1	1	1	1	1	1	1	1	8	*Yr7, Yr+*
	17	Laloub-2	1	1	1	1	1	1	2	1	2	2	8	*Yr7, Yr+*
	81	Achtar/INRA 1764	2	8	2	5	2	3	2	2	4	6	8	*Yr7, Yr+*
	80	Achtar/INRA 1764	2	8	3	3	2	4	3	4	3	6	8	*Yr7, Yr+*
9		**Avocet *Yr9*/6*Avocet S**	2	8	2	2	2	8	8	8	8	8	8	*Yr9*
		Clement	1	5-6	2	1	2	8	8	8	8	8	9	*Yr9*
		Federation 4*/ Kavkaz	1	9	2	1	1	8	8	9	9	9	9	*Yr9*
	66	Haala-50	3	8	5	1	1	8	8	7	8	8	8	*Yr9*
	28	Battell-3	2	8	2	2	2	8	9	-	8	8	8	*Yr9*
	29	Sandall-3	1	8	2	2	2	8	9	-	8	8	8	*Yr9*
	35	ICARDA-SRRL-5	2	8	2	2	2	8	8	-	8	8	8	*Yr9*
10		**VPM1**	2	3	2	2	2	8	2	2	9	9	9	*Yr17*
	13	Ruth-1	1	1	1	2	1	7	1	2	8	8	8	*Yr17*
	49	Nouha-3	1	4	2	2	1	8	2	2	8	8	8	*Yr17*
	31	Samira-2	1	2	5	2	1	5	2	3	8	8	8	*Yr17+*
11		**TP981**	2	8	9	9	9	8	8	9	9	9	9	*Yr25*
	15	Nayzak-3	1	8	8	9	9	9	8	8	8	8	8	*Yr25*
12		**Avocet *Yr27*/6*Avocet S**	5	8	3	2	3	3	2	3	2	2	3	*Yr27*
		Opata	5	8	3	2	3	3	2	3	2	2	3	*Yr27*
	1	Cham-8	1	8	1	1	1	2	2	2	2	2	5	*Yr27*
	8	Inqualab 91/Flag-2	4	8	2	3	2	2	2	2	3	3	4	*Yr27*
	10	Bow#1/Fengkang15/3/HYS//DRC*2/7C	2	8	2	3	2	2	2	2	2	2	2	*Yr27*
	53	NS732/HER//SD8036/3/Saada	2	7	2	2	2	2	5	2	2	2	2	*Yr27*
	56	Loulou-16	1	8	1	1	1	2	2	2	2	2	3	*Yr27*
	73	HIJLEEJ-1	2	8	4	2	5	3	2	2	2	2	3	*Yr27*
	76	NS732/HER*2//Saada	2	8	2	2	3	2	2	2	2	2	2	*Yr27*
	58	Taleh-1	1	8	1	1	1	2	4	2	2	3	4	*Yr27*

(Continued)

Table 2. (Continued).

Group	Entry No.	Genotype	Pathotype code [a]										Postulated *Yr* genes
			A	B	C	D	E	F	G	H	I	J	K	
13	**51**	Sidraa-1	4	6-7	3	4	5	2	5	8	8	8	8	*Yr6 + Yr9*
	71	Latifa-2	1	8	1	1	1	5	4	8	8	8	8	*Yr6 + Yr9*
	27	Koukab-2	1	3	2	1	1	4	4	2	8	7	8	*Yr6 + Yr17*
	86	Hubara-3/Angi-2//Somama-3	1	1	2	1	1	2	1	4	7	8	8	*Yr6 + Yr17*
	24	Firdous-29	2	1	7	3	2	2	1	2	3	2	8	*Yr7 + Yr1*
	26	Saamid-2	2	2	2	1	7	1	2	2	2	2	8	*Yr7 + Yr4*
	4	Soonot-10	2	2	1	1	9	1	2	2	2	2	8	*Yr7 + Yr4*
	59	Nadia-13	2	2	2	1	8	2	2	2	2	2	8	*Yr7 + Yr4*
	84	ACSAD 529/Karawan'S'//Somama-3	1	1	3	2	1	9	1	8	8	8	8	*Yr9 + Yr1*
	83	Hubara-5/3/SHA3/Seri//SHA4/Lira	1	2	2	3	2	8	8	9	8	8	8	*Yr9 + Yr3*
	9	Qafzah-33/Florkwa-2	1	4	2	2	1	3	8	-	8	8	8	*Yr9 + Yr4*
	25	Jelmoud-1	1	1	1	1	2	1	8	-	8	8	8	*Yr9 + Yr4*
	50	Milan/SHA7//Potam*3KS811261-5	1	4	1	1	2	3	8	-	7	8	8	*Yr9 + Yr4*
	55	Fanoos-14	1	2	1	1	1	3	8	-	8	8	8	*Yr9 + Yr4*

[a]A = 6E16, B = 6E16*v9v27*, C = 43E138, D = 45E140, E = 106E139, F = 169E136*v17*, G = 232E137, H = 237E141, I = 237E141*v17*, J = 237E173*v17 (Oakley/Solstice)*, K = 239E175*v17 (Warrior)*. Pathotypes are coded according to Johnson *et al.* (1972).
The virulences and avirulences tested were 1, 2, 3, 4, 6, 7, 8, 9, 17, 25, 27, 32, SD, SP, Su. Scoring was performed as described by McNeal *et al.* (1971); Infection types IT0 = No visible uredia, IT1 = Necrotic flecks, IT2 = Necrotic areas without sporulation, IT3-4 = Necrotic and chlorotic areas with restricted sporulation, IT5-6 = Moderate sporulation with necrosis and chlorosis, IT7-8 = Sporulation with chlorosis, IT9 = Abundant sporulation without chlorosis.
[b] Resistant to all *Puccinia striiformis* f. sp. *tritici* pathotypes used.
[c] Susceptible to all *Puccinia striiformis* f. sp. *tritici* pathotypes used.
[d] Ni indicates non-identified resistance genes.
[e] Indicates missing data.
[f] The entries in bold font correspond to the infection type profiles of the tester lines confronted with the array of 11 *Puccinia striiformis* f. sp. *tritici* pathotypes.

Statistical analyses

Principal component analysis (PCA) was performed to illustrate the heterogeneity of the distributions of resistant and susceptible plants for each pathotype tested on landraces. The heterogeneity of resistance in landraces was assessed by determining the percentage of resistant plants in landraces mostly susceptible to one pathotype and the percentage of susceptible plants in landraces mostly resistant to one pathotype. Data were plotted for 11 variables, corresponding to the 11 pathotypes tested. Another PCA was performed on the percentage of plants in each landrace resistant to each of the 11 pathotypes. PCAs were performed with R software (http://www.R-project.org, 2008).

RESULTS

Postulation of seedling resistance genes

The seedling tests conducted on the 138 elite lines, varieties and landraces with 11 French *Pst* pathotypes made it possible to postulate genes for seedling resistance to stripe rust, either singly or in combinations (Tables 2, 3, and 4; Figure 1). The pathotypes used made postulation possible for *Yr1*, *Yr3*, *Yr4*, *Yr6*, *Yr7*, *Yr9, Yr17, Yr25*, *Yr27* and *Yr32*. Based on these postulations, the lines were attributed to 13 stripe rust resistance groups.

Tested lines resistant to all 11 pathotypes were classified into group 1, those susceptible to all 11 pathotypes were classified into group 2, and tested lines that could not be characterized with the set of 11 pathotypes were classified into group 3.

Resistance group 1 corresponded to genotypes resistant to all *Pst* pathotypes. This group included eight ICARDA elite lines of bread wheat (Table 2) and two Lebanese durum wheat varieties (Table 3). These genotypes had low to intermediate ITs for all pathotypes tested. They therefore possessed a *Yr* gene or combination of *Yr* genes without corresponding virulence in the 11 *Pst* pathotypes.

Resistance group 2 corresponded to the genotypes susceptible to all *Pst* pathotypes, which therefore harboured no *Yr* genes, although they may have harboured

Table 3. Resistance group, infection type and postulated seedling-stage stripe rust resistance genes against 11 *Puccinia striiformis* f. sp. *tritici* pathotypes, for 23 Lebanese wheat varieties.

Resistance group	Wheat line	Pathotype code[a]											Postulated *Yr* genes
		A	B	C	D	E	F	G	H	I	J	K	
1	Stork	1	3	2	2	3	4	4	2	2	2	1	Resistant[b]
	Azeghar	3	3	2	2	2	1	2	2	2	2	2	Resistant
2	Super X	8	8	8	8	-[c]	9	-	9	8	8	9	Susceptible[d]
3	Senatore Cappelli	4	4	2	3	8	3	7	3	2	2	1	Ni[e]
	Miki	5	5	4	8	8	5	8	5	3	3	8	Ni
	Tal Amara 2	3	2	3	8	8	5	8	8	8	8	8	Ni
	Icarasha	4	4	2	8	2	2	2	2	4	2	8	Ni
	Tal Amara 1	4	3	1	8	2	2	1	4	2	2	2-4	Ni
	Tal Amara 3	6	5	2	6	2	2	2	4	4	4	8	Ni
	Nab El Jamal	1	8	2	3	2	8	9	9	8	8	8	Ni
5	**Vilmorin 23** [f]	2	3	8	8	9	8	8	9	9	9	9	
	Genessi	3	3	7	8	6-7	9	8	8	8	8	8	*Yr3*
8	**Avocet *Yr*7/6*Avocet S**	8	8	8	3	8	4	4	2	3	3	8	*Yr7*
	Lee	9	9	9	3	9	3	2	3	3	3	9	*Yr7, Yr+*
	Reichersberg 42	2	4	9	2	9	2	2	2	2	2	8	*Yr7, Yr+*
	Haramoun	8	8	8	3	9	3	3	2	2	3	9	*Yr7*
	Tannour	1	8	2	1	2	2	2	2	2	2	8	*Yr7, Yr+*
	885	2	8	2	2	1	2	3	2	2	2	8	*Yr7, Yr+*
11	**TP981**	2	8	9	9	9	8	8	9	9	9	9	
	Florence Aurore	1	8	8	8	9	9	9	9	8	8	8	*Yr25*
12	**Avocet *Yr*27/6*Avocet S**	5	8	3	2	3	3	2	3	2	2	3	*Yr27*
	Opata	5	8	3	2	3	3	2	3	2	2	3	*Yr27*
	MR 1009	3	8	2-3	2	2	3	2	2	2	2	5	*Yr27*
	Katilla	1	8	1	1	1	2	2	2	2	2	2	*Yr27*
	Bouhouth 6	3	8	3	2	3	2	2	2	2	2	4	*Yr27*
	Aammoun	1	8	1	1	1	2	3	2	2	2	5	*Yr27*
	Sham 8	2	8	2	1	2	2	2	3	2	2	5	*Yr27*
13	Lahn	8	7	1	5	2	1	2	6	3	4	8	*Yr6 + Yr7*
	A1103	8	8	3	2	2	3	2	3	2	2	7	*Yr6 + Yr7*
	Naama	1	4	2	2	2	2	2	5	8	8	8	*Yr6 + Yr17*

[a]A = 6E16, B = 6E16*v9v27*, C = 43E138, D = 45E140, E = 106E139, F = 169E136*v17*, G = 232E137, H = 237E141, I = 237E141*v17*, J = 237E173*v17 (Oakley/Solstice)*, K = 239E175*v17 (Warrior)*. Pathotypes are coded according to Johnson *et al.* (1972).
The virulences and avirulences tested were 1, 2, 3, 4, 6, 7, 8, 9, 17, 25, 27, 32, SD, SP, Su. Scoring was performed as described by McNeal *et al.* (1971); Infection types IT0 = No visible uredia, IT1 = Necrotic flecks, IT2 = Necrotic areas without sporulation, IT3-4 = Necrotic and chlorotic areas with restricted sporulation, IT5-6 = Moderate sporulation with necrosis and chlorosis, IT7-8 = Sporulation with chlorosis, IT9 = Abundant sporulation without chlorosis.
[b] Resistant to all *Puccinia striiformis* f. sp. *tritici* pathotypes used.
[c] - indicates missing data.
[d] Susceptible to all *Puccinia striiformis* f. sp. *tritici* pathotypes used.
[e] Ni indicates non-identified resistance genes.
[f] The entries in bold correspond to the infection type profiles of the tester lines confronted with the array of 11 *Puccinia striiformis* f. sp. *tritici* pathotypes.

resistance genes corresponding to the virulence profiles of the 11 tester pathotypes resulting in compatible reactions. This group included three elite lines (Table 2), one Lebanese variety (Table 3) and 21 Lebanese landraces (Table 4; 14 populations of Salamouni wheat, three populations of Abou Shwereb wheat, two populations of Ukranian wheat, and one population each of Bekaii and Haurani wheat).

Table 4. Resistance group, infection type and postulated seedling-stage stripe rust resistance genes against 11 *Puccinia striiformis* f. sp. *tritici* pathotypes, for 28 Lebanese wheat landraces.

Resistance group	Wheat landrace	Pathotype code[a]											Postulated *Yr* genes
		A	B	C	D	E	F	G	H	I	J	K	
1	Waha	1	2	2	2	2	1	1	2	1	2	1	Resistant[b]
2	Abou Shwereb	8	8	8	7	9	9	9	9	8	8	8	Susceptible[c]
	Abou Shwereb	8	8	8	8	9	9	9	9	8	8	9	Susceptible
	Salamouni	8	8	8	8	9	9	9	9	8	8	9	Susceptible
	Salamouni	8	8	8	9	9	8	9	8	8	8	9	Susceptible
	Salamouni	8	8	8	8	9	8	9	8	8	8	8	Susceptible
	Salamouni	8	8	8	8	9	8	9	9	8	8	8	Susceptible
	Salamouni	9	8	8	8	9	8	9	9	8	8	8	Susceptible
	Ukranian	8	8	8	8	9	8	9	9	8	8	9	Susceptible
	Ukranian	8	8	8	8	9	8	9	7	8	8	8	Susceptible
	Salamouni	9	8	8	9	9	9	9	9	7	8	9	Susceptible
	Salamouni	8	8	8	9	9	8	9	9	8	8	8	Susceptible
	Abou Shwereb	9	8	8	9	9	9	9	9	8	8	9	Susceptible
	Salamouni	9	8	8	9	9	9	9	9	8	8	9	Susceptible
	Haurani	8	6-7	8	9	9	9	9	9	8	8	9	Susceptible
	Bekaii	7	8	6-7	7	9	8	9	8	8	8	9	Susceptible
	Salamouni	9	8	8	9	9	8	9	9	8	8	8	Susceptible
	Salamouni	8	8	8	9	9	8	9	9	8	8	8	Susceptible
	Salamouni	8	8	8	8	9	9	9	9	8	8	9	Susceptible
	Salamouni	8	8	8	8	9	9	9	9	8	8	9	Susceptible
	Salamouni	9	8	8	8	9	9	9	9	8	8	9	Susceptible
	Salamouni	8	8	8	8	9	9	9	9	8	8	9	Susceptible
3	Bekaii	8	8	4	8	9	9	9	8	4	3	6	Ni[e]
	Abou Shwereb	3	4	8	8	9	9	9	9	8	8	8	Ni
5	**Vilmorin 23[d]**	2	3	8	8	9	8	8	9	9	9	9	
	Awnless variety	1	3	8	8	9	9	8	9	7	7	8	*Yr3*
6	**Hybrid 46**	2	1	1	2	9	2	8	9	9	9	8	
	Nessr	2	4	2	5	9	5	9	9	8	8	8	*Yr4*
11	**TP981**	2	8	9	9	9	8	8	9	9	9	9	
	Salamouni	1	8	8	8	9	9	9	9	8	8	9	*Yr25*
	Salamouni	1	8	8	8	9	9	9	9	8	8	9	*Yr25*

[a]A = 6E16, B = 6E16*v9v27*, C = 43E138, D = 45E140, E = 106E139, F = 169E136*v17*, G = 232E137, H = 237E141, I = 237E141*v17*, J = 237E173*v17 (Oakley/Solstice)*, K =239E175*v17 (Warrior)*. Pathotypes are coded according to Johnson *et al*. (1972).
The virulences and avirulences tested were 1, 2, 3, 4, 6, 7, 8, 9, 17, 25, 27, 32, SD, SP, Su. Scoring was performed as described by McNeal *et al.*(1971); Infection types IT0 = No visible uredia, IT1 = Necrotic flecks, IT2 = Necrotic areas without sporulation, IT3-4 = Necrotic and chlorotic areas with restricted sporulation, IT5-6 = Moderate sporulation with necrosis and chlorosis, IT7-8 = Sporulation with chlorosis, IT9 = Abundant sporulation without chlorosis.
[b] Resistant to all *Puccinia striiformis* f. sp. *tritici* pathotypes used.
[c] Susceptible to all *Puccinia striiformis* f. sp. *tritici* pathotypes used.
[d] The entries in bold correspond to the infection type profiles of the tester lines inoculated with the array of 11 *Puccinia striiformis* f. sp. *tritici* pathotypes.
[e] Ni indicates non-identified resistance genes.

Resistance group 3 included 15 ICARDA elite lines (Table 2), one bread and six durum wheat varieties from Lebanon (Table 3), and two Lebanese durum wheat landraces that did not display clear differential responses to the 11 pathotypes used in this study. The genotypes in this group, therefore, could not be used for gene postulation (Table 4).

The tested genotypes in resistance group 4 had high ITs (7 to 9) for the seven *Pst* pathotypes virulent against

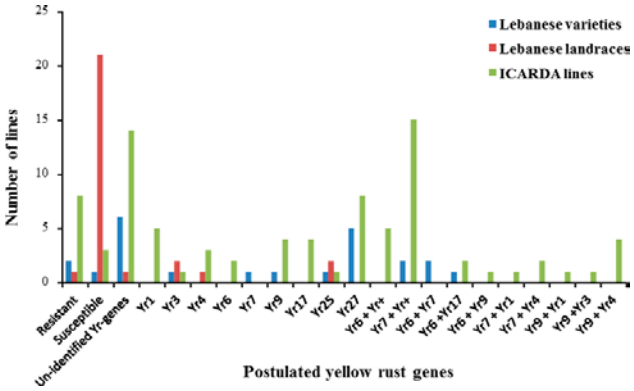

Figure 1. Postulated *Yr* seedling-stage stripe rust resistance in Lebanese wheat varieties, Lebanese wheat landraces and ICARDA wheat lines.

Yr1, and low ITs (1 to 3) for the four pathotypes avirulent against *Yr1*. This led us to postulate the presence of *Yr1* in the five ICARDA elite lines, with resistance profiles similar to those of the tester genotypes Chinese 166 and Avocet *Yr1* (Table 2). This is the first postulation of *Yr1* in Lebanese varieties and landraces.

Genotypes in resistance group 5 had high ITs (7 to 9) for the nine pathotypes virulent against *Yr3*, and low ITs (1 to 4) for pathotypes avirulent against *Yr3*. One ICARDA elite line, one Lebanese variety (Genessi), and one Lebanese bread wheat landrace (awnless variety) were postulated to have *Yr3*. These postulated lines had resistance profiles similar to that of the tester genotype Vilmorin 23 (Tables 2, 3 and 4).

Three elite lines and the bread wheat landrace Nessr were postulated to carry the *Yr4* gene (resistance group 6). The postulated lines had resistance profiles similar to that of the tester genotype Hybrid 46 (Table 1). The low to intermediate ITs (2 to 4, except for one line, ESWYT99#18/Arrihane, which had an IT of 5) for five pathotypes avirulent against *Yr4* and high ITs (7-9) for six pathotypes virulent against *Yr4* led to a postulation of *Yr4* in the tested genotypes in this resistance group (Tables 2 and 4). The IT profile of Hybrid 46 matched that of the tested lines except for the line with an intermediate IT of 5.

Two elite lines had resistance profiles similar to that of the Avocet *Yr6* tester line in tests against the 11 pathotypes (resistance group 7). Based on the high and low infection type patterns of the tested genotypes with the tester line Avocet *Yr6*, the tested genotypes were postulated to carry *Yr6* (Table 2). Five of the ICARDA elite bread wheat lines had high (7–8) and low (1–3) infection type patterns, similar to those of the tester variety Heines Peko when tested against the 11 pathotypes. Heines Peko is known to carry *Yr6* plus additional uncharacterized *Yr*-gene/s (Calonnec *et al*., 1997). These five elite lines (lines 2, 5, 47, 63, and 64), designated *Yr6+* in Table 2, were, therefore, also postulated to carry *Yr6* plus unknown additional gene/s.

Resistance group 8 included one Lebanese bread wheat variety with high ITs (8 to 9) for the pathotypes virulent against *Yr7* and low ITs (2 to 3) for the pathotypes avirulent against *Yr7*. The same infection type pattern was observed when the tester line Avocet *Yr7* was tested against the 11 *Pst* pathotypes (Table 3). Riechersberg 42 is known to carry *Yr7* and additional uncharacterized resistance genes (McIntosh *et al*., 1995). Based on the similarity of the infections patterns of Riechersberg 42, we postulated that *Yr7* and additional uncharacterized genes were present in the 15 elite lines and three Lebanese bread wheat varieties (Table 2).

The genotypes in resistance group 9 were postulated to carry *Yr9*. This group included four bread wheat elite lines. The postulation of *Yr9* in this group was based on similar infection type patterns for the tested lines Avocet *Y9* and Clement, in tests against the seven *Pst* pathotypes virulent against *Yr9*, and the four pathotypes avirulent against this gene (Tables 2 and 3).

Resistance group 10 included three elite lines postulated to carry *Yr17*. These lines had high ITs (IT = 8) against four pathotypes virulent against *Yr17*, and low ITs (2 to 3) for seven pathotypes avirulent against *Yr17*. These lines had resistance profiles similar to that of the tester line VPM1 (Table 2).

Resistance group 11 included one elite line, one Lebanese bread wheat variety (Florence Aurore) and two Lebanese bread wheat landraces (two accessions of Salamouni) which were postulated to carry *Yr25*, with low ITs (IT = 1) for the *Pst* pathotype avirulent against *Yr25*, and high ITs (7 to 9) against the other ten pathotypes virulent against *Yr25*. The resistance profiles of the genotypes postulated to carry *Yr25* were similar to that of the tester line TP981 used as source of *Yr25* in this study (Tables 2, 3 and 4).

Resistance group 12 included eight elite lines (Table 2) and five Lebanese bread wheat varieties (Table 3) postulated to carry *Yr27*. The tested genotypes had high ITs (7 to 8) for the only pathotype virulent against *Yr27*, and low ITs (1 to 5) for the remaining ten pathotypes avirulent against *Yr27*. These postulated lines had resistance profiles similar to that of the tester genotypes Avocet *Yr27* and Opata.

Resistance group 13 included the genotypes for which two *Yr* genes were postulated. Similarities in the infection type patterns of two tester genotypes tested against the 11 pathotypes (Table 1) and those of tested

elite lines, Lebanese varieties and landraces, were used to postulate gene combinations in the tested genotypes in this resistance group. In this group, combinations of *Yr6* and *Yr9* were postulated in only two ICARDA elite lines, *Yr6* and *Yr17* were postulated in three of these ICARDA lines, *Yr7* and *Yr1* were postulated in one ICARDA line, *Yr7* and *Yr4* were postulated in three ICARDA lines, *Yr9* and *Yr1*, were postulated in one ICARDA line, *Yr9* and *Yr3* were postulated one ICARDA line, and *Yr9* and *Yr4* were postulated in only four ICARDA elite lines (Table 2). Similarly, the combination of *Yr6* and *Yr7* was postulated in two Lebanese varieties, and the combination of *Yr6* and *Yr17* was postulated in one Lebanese variety (Table 3). With the 11 pathotypes used here, we were able to postulate all the *Yr* genes considered, except for *Yr32,* in the genotypes tested.

Evaluation of adult plant resistance

We evaluated the adult-plant responses of the tested genotypes by performing a field test with 44 ICARDA elite lines at two sites, one in Syria (Tal Hadya) and the other in Lebanon (Terbol). Table 5 shows the seedling and adult plant responses of the tested lines at the two sites, together with the postulated *Yr* genes. Genotypes were considered to carry only adult-plant resistance when the same genotype was susceptible (high ITs of 7 to 9) to pathotype 6E16*v9v27* at the seeding stage, but resistant at the adult-plant stage. Twenty nine out of 44 elites lines that were resistant at the seedling stage, showed low to moderate resistance at adult-plant stage. The elite lines 7, 12, 22, 29, 35, 51, 66, 67, 75, 85 and 87 (Table 5; Figure 2), which were susceptible at the seedling stage, were resistant at adult-plant stage. Elite line 70, which was resistant to the 11 pathotypes used in the gene postulation study, including pathotype 6E16*v9v27,* was also resistant in field tests.

Elite lines postulated to carry *Yr1* (lines 60, 61, and 65), *Yr3* (line 46), and *Yr4* (line 18) were resistant at both the seedling and adult-plant stages. The elite lines postulated to carry *Yr6* (lines 47, 63) and *Yr6*+ (64) were resistant in the field. The pathotypes used for inoculation in the field carried virulence against both *Yr6* and *Yr6*+. The field resistance responses of these lines can therefore be considered to indicate the presence of adult-plant resistance in these lines.

Eight lines carrying *Yr7* and additional uncharacterized seedling resistance genes (lines 25, 34, 36, 37, 41, 42, 44, and 68, Table 5) were resistant at both the seedling and adult-plant stages. The source of *Yr7* was susceptible at both the seedling and adult-plant stages and the *Yr7*+ source displayed intermediate field responses at the two sites. We therefore considered the strong field responses of the lines postulated to carry *Yr7*+ to be due to the combination of an uncharacterized seedling resistance gene and adult-plant resistance genes effective against the field pathotype at both sites.

The line postulated to carry *Yr17* (line 31) displayed an intermediate resistance response in field conditions. The seedling and field responses of the source of *Yr17* showed *Yr17* to be effective against the pathotype used for seedling and adult-plant assessment. Line 39 therefore probably carries *Yr17.* Three lines were postulated to carry *Yr27* (lines 1, 8 and 46), one of which, line 46, displayed moderate resistance at the adult-plant stage suggestive of the presence of adult-plant resistance genes in this line.

Line 51 displayed an adult-plant response of 5R in field conditions, but seedlings of this line had a high infection type with the pathotypes used. As the pathotypes tested were virulent against combinations of the *Yr6* and *Yr9* genes, the adult-plant resistance response of this line was considered to indicate the presence of adult-plant resistance in this line.

The *Yr6*+*Yr17* combination conferred resistance at both the seedling and adult-plant stages in the Lebanese field for lines 27 and 86, demonstrating the efficacy of *Yr17,* as virulence against *Yr6* was common in both the seedling and adult-plant tests. The line postulated to carry both *Yr7* and *Yr4* (line 4) was resistant at both the seedling and adult-plant stages, demonstrating the efficacy of *Yr4* in field tests. As *Yr1, Yr3*, and *Yr4* were effective against the pathotypes used in both seedling and adult-plant tests, the resistance response of combinations of *Yr9* with *Yr1* (line 84), *Yr3* (line 83), and *Yr4* (line 9) was due to the efficacy of *Yr1*, *Yr3*, and *Yr4* against the pathotypes used in the seedling and adult-plant tests.

Three of the tested lines for which the seedling resistance could not be postulated by multipathotype testing (lines 7, 22, and 85) were postulated to carry adult-plant resistance, given the high level of infection observed at the seedling stage.

Resistance diversity in Lebanese landraces

The landraces generally displayed considerable diversity in their response to the 11 pathotypes (Table 6; Figure S1). Many landraces displayed variation in their resistance responses to each pathotype, with 0 to 50% susceptible plants in resistant landraces and 0 to 50% resistant plants in susceptible landraces, for any given pathotype. The number of resistant plants was highest with the 6E16, 43E138 and 237E173*v17 (Oakley/ Solstice)* pathotypes, and lowest with 239E175*v17 (War-*

Table 5. Pedigree, postulated seedling-stage stripe rust resistance genes and field responses to stripe rust of 44 advanced bread wheat lines from ICARDA at the Tel Hadya (Syria) and Terbol (Lebanon) research stations.

Entry	Pedigree	Postulated *Yr* genes	Seedling infection type[a]	Adult-plant resistance[b]
11	Tracha'S'//CMH76-252/PVN'S'	Resistant[c]	2	20-30MR
20	Achtar*3//Kanz/KS85-8-4/3/Zemamra-5	Resistant	2	10MR
32	Blass-1/4/CHAT'S'//KVZ/CGN/3/BAU'S'	Resistant	1	50RMR
70	Crow'S'/Bow'S' -1994/95//Asfoor-5	Resistant	5	10R
12	W3918A/JUP	Susceptible[d]	7	10R
75	Nesma*2/14-2//2*Safi-3	Susceptible	8	5R
60	Crow'S'/Bow'S' -3-1994/95//Tevee'S'/Tadinia	*Yr1*	1	10MR
61	Tevee'S'/3/T.aestivum/SPRW'S'//CA8055/4/Pastor-2/5/Sunbri	*Yr1*	1	10MR
65	Qafzah-2/Ferroug-2	*Yr1*	2	10MR
46	Bow #1/Fengkang15/3/HYS//DRC*2/7C	*Yr3*	3	10R
18	SHA3/Seri//Yang87-142/3/2*Towpe	*Yr4*	2	20MR
67	Hamam-4/Angi-2	*Yr6*	8	10R
87	Hubara-16/2*Somama-3	*Yr6*	8	30R
47	MON'S'/ALD'S'//Towpe'S'	*Yr6*	5	10R
63	Weebill-1/2*Qafzah-21	*Yr6*	6	10RMR
64	Rebwah-12/Zemamra-8	*Yr6* +[f]	1	1R
34	Kauz//Kauz/Star	*Yr7* +	1	10MR
36	Cham-4/Shuha'S'/6/2*Saker/5/RBS/Anza/3/KVZ/HYS//YMH/TOB/4/Bow'S'	*Yr7*+	1	5R
37	Cham-4/Shuha'S'/6/2*Saker/5/RBS/Anza/3/KVZ/HYS//YMH/TOB/4/Bow'S'	*Yr7*+	1	5R
41	Florkwa-2/Asfoor-5	*Yr7*+	2	30MR
42	Ferroug-2/Potam*2KS811261-8//Zemamra-8	*Yr7*+	2	10R
44	Hubara-15/Zemamra-8	*Yr7*+	1	10R
19	Cham-4/Shuha'S'/6/2*Saker/5/RBS/Anza/3/KVZ/HYS//YMH/TOB/4/Bow'S'	*Yr7*+	1	5R
68	Shuha-5/Asfoor-1	*Yr7*+	1	10R
66	MON'S'/ALD'S'//Aldan'S'/IAS58/3/Safi-1/4/Zemamra-1	*Yr9*	8	10MR
29	Clement/ALD'S'//Zarzour/5/AU//KAL/BB/3/BON/4/KVZ//CNO/PJ62 (Sandall 3)	*Yr9*	8	5R
35	ICARDA-SRRL-5	*Yr9*	8	20R
31	Shuha-8//Vee'S'/Saker'S'	*Yr17*	2	40MRMS
1	Kauz = JUP/BJY//URES	*Yr27*	8	100S
8	Inqualab 91/Flag-2	*Yr27*	8	10S
10	Bow#1/Fengkang15/3/HYS//DRC*2/7C	*Yr27*	8	30MRMS
51	GV/ALD'S'/5/ALD'S'/4/BB/G11//CNO67/7C/3/KVZ/TI/6/2*Towpe	*Yr6* + *Yr9*	7	5R
27	DVERD-2/*Aegilops squarrosa* (214)//2*ESDA/3/NS732/HER	*Yr6* + *Yr17*	3	5R
86	Hubara-3/Angi-2//Somama-3	*Yr6* + *Yr17*	1	20R
4	Samar-8/Kauz'S'//Cham-4/Shuha'S'	*Yr7* + *Yr4*	2	5R
84	ACSAD 529/Karawan'S'//Somama-3	*Yr9* + *Yr1*	1	5R
83	Hubara-5/3/SHA3/Seri//SHA4/Lira	*Yr9* + *Yr3*	2	5R
9	Qafzah-33/Florkwa-2	*Yr9* + *Yr4*	4	5R
7	*T. aestivum*/SPRW'S'//CA8055/3/Bacanora86	Ni[e]	7	1R
22	Fow-2/SD8036//SafiI-3/3/NS732/HER//Kauz'S'	Ni	8	20MR
38	NS732/HER//Arrihane/3/PGO/Seri//BAU	Ni	8	40S
39	IZAZ-2//Tevee'S'/Shuha'S'	Ni	2	20R
54	Sakha73/5/IAS 58/4/KAL/BB//CJ'S'/3/ALD'S'/6/Goumria-12	Ni	2	20R
85	Girwill-13/2*Pastor-2	Ni	8	10R

[a] Infection type with 6E16*v*9v27, the predominant pathotype in the CWANA region for the 2010-2011 season, carrying the *v*2, 6, 7, 8, 9, 25, 27, and SD, based on pathotype surveys conducted by ICARDA for the same year.

[b] R = resistant, MR = moderately resistant, MS = moderately susceptible, and S = susceptible, according to the modified Cobb scale of Peterson *et al*. (1948). The numbers 5-100 are the percentages of the leaf area covered by stripe rust. The score reported is the mean for two seasons in Syria, and three seasons in Lebanon.

[c] Resistant to all *Puccinia striiformis* f. sp. *tritici* pathotypes used.

[d] Susceptible to all *Puccinia striiformis* f. sp. *tritici* pathotypes used.

[e] Ni indicates non-identified resistance genes.

[f] Additional and uncharacterized *Yr* genes.

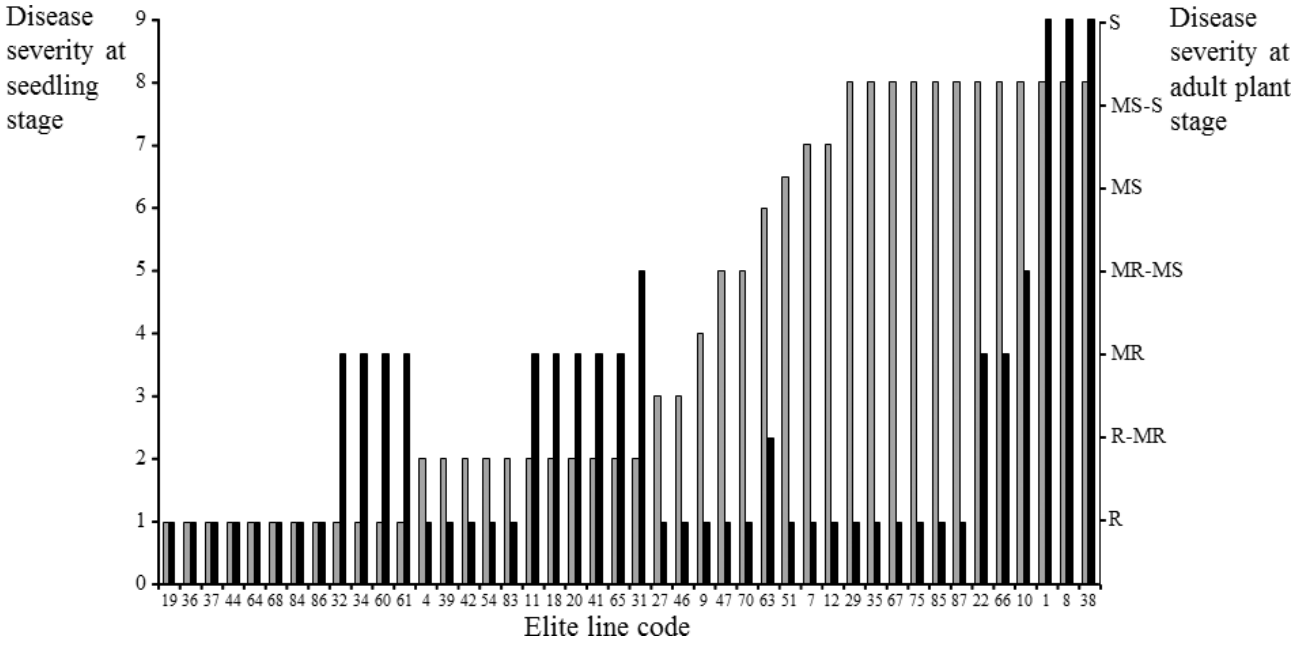

Figure 2. Disease severity at the seedling (gray columns) and adult-plant (black columns) growth stages, for 44 ICARDA bread wheat elite lines inoculated with the predominant pathotype for the 2010-2011 season in Lebanon and Syria. This pathotype carries *v2*, *6*, *7*, *8*, *9*, *25*, *27*, and *SD*, according to the pathotype surveys conducted by ICARDA for the same year. Disease severity at the seedling stage is scored from 0 to 9, where 0 was considered fully resistant and 9 fully susceptible (McNeal *et al.* 1971). At the adult-plant stage, disease severity was recorded as described by Roelfs *et al.* (1992); R = resistant, MR = moderately resistant, MS = moderately susceptible, and S = susceptible. The codes of the elite lines are given in Table 5.

rior), which has the largest number of virulence factors. Interestingly, two durum wheat Bekaii landraces (landraces 24 and 27) from two different sites had 100% and 11% resistant plants, respectively, when tested with the pathotype 239E175*v17 (Warrior)*. These landraces thus carry new resistance genes that could not be detected with the pathotype arrays used here, but the uncharacterized genes in the resistant plants contributed to the resistance of the landraces, particularly in tests against the most virulent race, 239E175*v17 (Warrior)*.

PCA with 11 variables, including the percentage of plants differing from the most frequent reaction with each of the 11 pathotypes, separated the landraces in terms of their heterogeneity of reaction to all 11 pathotypes (Figure 3, Table 7). The two first axes accounted for 54.3% of the variance, with the most heterogeneous landraces to the right of axis 1 (landraces 27, 21, 24, 5, 2, 4, 23) and the most homogeneous ones to the left (landraces 10, 17, 18, 11, 15). Four of the seven most heterogeneous landraces were durum wheat landraces. Axis 2 separated landraces in terms of their heterogeneity of reaction to four pathotypes (three carrying *v7*: 6E16, 43E138, and 106E139). PCA with 11 variables, including the percentage of plants resistant to each of the 11 pathotypes, separated the landraces in terms of their level of resistance for their reaction to all 11 pathotypes (Figure 4; Table 8). The two first axes accounted for 70.1% of total variance, with the most resistant landraces to the right of axis 1 of the PCA (landraces 28, 24, 19, 27) and the most susceptible landraces to the left (landraces 10, 15, 17, 25). Five of the nine most resistant landraces were durum wheat landraces. Axis 2 separated landraces in terms of their resistance to four pathotypes (6E16, 6E16*v9v27*, 45E140 and 169E136*v17*).

DISCUSSION

Host resistance-based approaches remain the most economical and environmentally friendly method of controlling wheat rust diseases. Most of the characterized resistance genes are race-specific and conform to the well-described gene-for-gene model (Flor, 1956). A knowledge of the genetic structure of breeding lines and genetic resources is crucial for the breeding of more durable resistant genotypes and the efficient use of genetic resources. We postulated the *Yr* genes in 138 wheat genotypes from ICARDA, Lebanese varieties and landraces, using an array of 11 *Pst* pathotypes at the seedling stage. Using this set of pathotypes with

Table 6. Assessment of resistance heterogeneity in the wheat landraces, expressed as the percentage of resistant plants (R) among the susceptible landraces, and the percentage of susceptible plants (S) among the resistant landraces, for each of the 11 *Puccinia striiformis* f. sp. *tritici* pathotypes.

Landrace name	Location	Landrace Code	Species[b]	Pathotype[a]																					
				A		B		C		D		E		F		G		H		I		J		K	
				R[c]	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
Salamouni[d]	Qamouaa, Akkar	1	TA	33[e]	-[f]	0	-	25	-	14	-	8	-	0	-	8	-	4	-	4	-	13	-	0	-
Salamouni	Fneidik, Akkar	2	TA	-	48	17	-	26	-	8	-	17	-	13	-	26	-	0	-	29	-	30	-	0	-
Salamouni	Qamouaa, Akkar	3	TA	0	-	28	-	19	-	-	46	0	-	0	-	0	-	0	-	19	-	26	-	0	-
Salamouni	Laklouk, Jbeil	4	TA	8	-	9	-	19	-	-	41	0	-	29	-	0	-	17	-	-	42	-	36	0	-
Salamouni	Laklouk, Jbeil	5	TA	50	50	32	-	13	-	7	-	9	-	36	-	0	-	0	-	32	-	50	50	0	-
Ukranian Variety	Tel Akhdar, Bekaa	6	TA	-	46	0	-	5	-	3	-	0	-	9	-	0	-	50	-	0	-	0	-	0	-
Ukranian Variety	Tel Akhdar, Bekaa	7	TA	41	-	13	-	16	-	0	-	12	-	18	-	0	-	38	-	4	-	0	-	0	-
Salamouni	Jeb Janine, Bekaa	8	TA	13	-	0	-	-	28	0	-	0	-	0	-	0	-	0	-	32	-	35	-	0	-
Salamouni	Jeb Janine, Bekaa	9	TA	0	-	11	-	22	-	0	-	4	-	16	-	0	-	11	-	48	-	20	-	0	-
Salamouni	Aarida, Akkar	10	TA	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Salamouni	Northern Bekaa	11	TA	0	-	0	-	-	0	0	-	4	-	5	-	0	-	0	-	0	-	0	-	0	-
Salamouni	Nabha, Bekaa	12	TA	50	50	0	-	40	-	8	-	4	-	10	-	0	-	0	-	0	-	0	-	0	-
Salamouni	Nabha, Bekaa	13	TA	-	0	0	-	4	-	4	-	4	-	0	-	0	-	4	-	4	-	4	-	0	-
Salamouni	Ham, Bekaa	14	TA	7	-	0	-	7	-	0	-	0	-	7	-	4	-	0	-	4	-	4	-	0	-
Salamouni	Ham, Bekaa	15	TA	7	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Salamouni	Aarsal, Bekaa	16	TA	0	-	0	-	4	-	0	-	23	-	0	-	4	-	0	-	0	-	0	-	0	-
Salamouni	Northern Bekaa	17	TA	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Salamouni	Northern Bekaa	18	TA	-	0	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Nessr	Tal Amara, Bekaa	19	TA	-	0	-	0	-	25	-	0	8	-	-	0	0	-	0	-	0	-	0	-	0	-
Awnless Variety	Qamouaa, Akkar	20	TA	-	0	-	0	19	-	10	-	0	-	0	-	9	-	4	-	5	-	4	-	0	-
Abou Shwereb	Qamouaa, Akkar	21	TD	26	-	-	26	34	-	19	-	16	-	13	-	23	-	9	-	30	-	50	50	0	-
Abou Shwereb	Qamouaa, Akkar	22	TD	0	-	0	-	15	-	0	-	8	-	10	-	8	-	4	-	9	-	8	-	0	-
Abou Shwereb	Fneidik, Akkar	23	TD	-	0	-	42	0	-	0	-	0	-	47	-	0	-	0	-	43	-	36	-	0	-
Bekaii	Jeb Janine, Bekaa	24	TD	19	-	38	-	-	31	35	-	4	-	37	-	12	-	50	-	-	0	-	20	-	0
Abou Shwereb	Aarida, Akkar	25	TD	5	-	8	-	0	-	0	-	4	-	0	-	0	-	0	-	0	-	0	-	0	-
Haurani	Tal Amara, Bekaa	26	TD	0	-	-	4	17	-	0	-	0	-	0	-	0	-	0	-	0	-	22	-	0	-
Bekaii	Tal Amara, Bekaa	27	TD	-	33	-	13	-	26	-	43	4	-	36	-	0	-	12	-	-	39	39	-	11	-
Waha	Jeb Janine, Bekaa	28	TD	-	13	-	8	-	0	-	0	-	0	-	8	-	0	-	0	-	0	-	0	-	0

[a]A = 6E16, B = 6E16*v9v27*, C = 43E138, D = 45E140, E = 106E139, F = 169E136*v17*, G = 232E137, H = 237E141, I = 237E141*v17*, J = 237E173*v17 (Oakley/Solstice)*, K = 239E175*v17 (Warrior)*. Pathotypes are coded according to Johnson *et al.* (1972).
The virulences and avirulences tested were 1, 2, 3, 4, 6, 7, 8, 9, 17, 25, 27, 32, SD, SP, Su.
[b] TA = *Triticum aestivum* (L), TD = *T. durum*.
[c] R = seedling resistant (low infection type of 0-6), S = seedling susceptible (high infection type of 7-9).
[d] 30 seedlings per landrace and per pathotype were tested.
[e] Percentage of resistant (R) plants in a mainly susceptible landrace and percentage of susceptible (S) plants in a mainly resistant landrace.
[f] indicates missing data.

complementary virulence spectra, we were able to infer the resistance profiles of most of the lines tested. The 11 pathotypes used here discriminated between the *Yr1*, *Yr3*, *Yr4*, *Yr6*, *Yr7*, *Yr8*, *Yr9*, *Yr17*, *Yr25*, *Yr27*, *Yr32*, *YrSD*, *YrSu* and *YrSP* genes, and we were able to postulate *Yr1*, *Yr3*, *Yr4*, *Yr6*, *Yr7*, *Yr9*, *Yr17*, *Yr25* and *Yr27* singly or in combination in the tested genotypes. These results highlight the utility of this pathotype array for the detection of *Yr* genes. However, a group of genotypes (Group 3) remained for which seedling resistance could not be explained with the pathotypes, and for which the resistance genes present remained unknown. Based on the resistance responses of these genotypes to the wide array of virulence factors of the 11 pathotypes, we postulated that these genotypes might be sources of new stripe rust resistance genes.

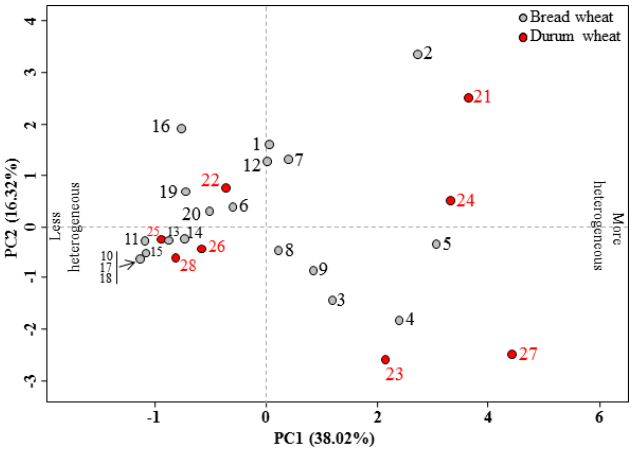

Figure 3. Plot of the first (PC1) and second (PC2) principal component means from an analysis of the 11 variables (percentage of resistant plants in susceptible landraces and percentage of susceptible plants in resistant landraces, for each of the 11 *Puccinia striiformis* f. sp. *tritici* pathotypes, for 28 Lebanese landraces (1-28)), as described in Table 7. Gray symbols = bread wheat, red symbols = durum wheat.

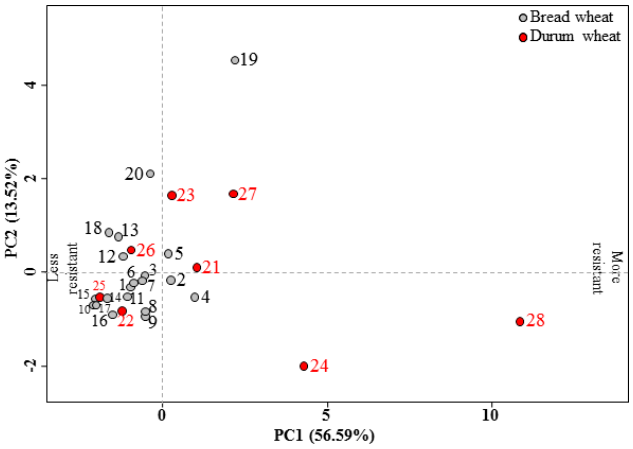

Figure 4. Plot of the first (PC1) and second (PC2) principal component means from an analysis of the 11 variables (percentage of plants resistant to each of the 11 *Puccinia striiformis* f. sp. *tritici* pathotypes, for 28 Lebanese landraces (1-28)), as described in Table 7. Gray symbols = bread wheat, red symbols = durum wheat.

In resistance group 1, 9% of ICARDA elite lines and 9% of Lebanese varieties displayed complete resistance to all pathotypes. Among the resistant Lebanese varieties, only two durum wheat genotypes displayed full resistance. None of the Lebanese bread wheat varieties were completely resistant to all pathotypes. Only one durum wheat landrace was completely resistant to all pathotypes.

With the exception of a few uncharacterized elite lines and landraces, the ICARDA elite lines and Lebanese varieties generally carried at least one effective *Yr* gene providing resistance to at least one of the 11 *Pst* pathotypes, whereas 75% of landraces were susceptible to all

Table 7. Eigen vectors of the two principal components axes (PC1 and 2) for the 11 variables assessed (resistance heterogeneity in the landraces expressed as the percentage of resistant plants in susceptible landraces and the percentage of susceptible plants in resistant landraces, for each of the 11 *Puccinia striiformis* f. sp. *tritici* pathotypes), and their contributions to the variance.

Variables[a]	PC1	PC2
A	0.53	0.42
B	0.76	-0.15
C	0.63	0.37
D	0.67	-0.23
E	0.28	0.75
F	0.80	-0.30
G	0.43	0.71
H	0.33	0.10
I	0.74	-0.31
J	0.86	-0.15
K	0.42	-0.36
Eigen value	4.18	1.80
Percentage of variance (%)	38.02	16.32

[a]A = 6E16, B = 6E16*v9v27*, C = 43E138, D = 45E140, E = 106E139, F = 169E136*v17*, G = 232E137, H = 237E141, I = 237E141*v17*, J = 237E173*v17 (Oakley/Solstice)*, K =239E175*v17 (Warrior)*.

Table 8. Eigen vectors of the two principal components axes (PC1 and 2) for the 11 variables assessed (percentage of plants resistant to each of the 11 *Puccinia striiformis* f. sp. *tritici* pathotypes for Lebanese landraces), and their contributions to the variance.

Variables[a]	PC1	PC2
A	0.53	0.42
B	0.76	-0.15
C	0.63	0.37
D	0.67	-0.23
E	0.28	0.75
F	0.80	-0.30
G	0.43	0.71
H	0.33	0.10
I	0.74	-0.31
J	0.86	-0.15
K	0.42	-0.36
Eigen value	4.18	1.80
Percentage of variance (%)	38.02	16.32

[a]A = 6E16, B = 6E16*v9v27*, C = 43E138, D = 45E140, E = 106E139, F = 169E136*v17*, G = 232E137, H = 237E141, I = 237E141*v17*, J = 237E173*v17 (Oakley/Solstice)*, K = 239E175*v17 (Warrior)*.

pathotypes. Nine of the resistance genes tested (*Yr1*, *Yr3*, *Yr4*, *Yr6*, *Yr7*, *Yr9*, *Yr17*, *Yr25* and *Yr27*) were postulated singly in ICARDA elite lines; *Yr7*, *Yr9*, *Yr25* and *Yr27* were detected in Lebanese varieties and only *Yr3*, *Yr4* and *Yr25* were detected in some landraces. *Yr27* was the most frequent *Yr* gene postulated singly in the Lebanese varieties. *Yr7* in combination with other unidentified *Yr* genes was postulated with the highest frequency in ICARDA elite lines. Combinations of two *Yr* genes were found in 16% of ICARDA elite lines: *Yr6+Yr9*, *Yr6+Yr17*, *Yr7+Yr1*, *Yr7+Yr4*, *Yr9+Yr1*, *Yr9+Yr3*, and *Yr9+Yr4*. The *Yr6+Yr7* combination was found in two Lebanese varieties and *Yr6+Yr17* was found in one landrace only. Only one awnless landrace (BW) contained *Yr3*. Only one Lebanese bread wheat landrace (Nessr) had *Yr4* and two Salamouni bread wheat landraces had *Yr25*. The postulated *Yr* genes, either singly or in combination, were more frequent in Lebanese varieties than in landraces. ICARDA elite lines displayed greater diversity for the postulated *Yr* genes than the Lebanese varieties and landraces (Figure 1).

In general, our study revealed a narrow genetic basis of resistance in the genotypes tested for seedling resistance genes in the absence of effective adult-plant resistance genes, but we did not investigate or characterize such adult-plant resistance genes in this study. With the exception of *Yr3* and *Yr4,* and, to some extent, *Yr1,* which are effective in most of the wheat growing areas in CWANA, the rest of postulated genes were not effective against current pathotypes. The *Yr1*, *Yr3*, and *Yr4* genes will also cease to be effective if the North Western European pathotypes spread to the CWANA region. With the recent incursion of the Warrior pathotype into CWANA, the efficacy of these three *Yr* genes is dwindling, and their use in breeding for rust resistance cannot, therefore, be recommended unless they are used in combination with effective seedling resistance genes and/or adult-plant resistance genes.

Yr1 was postulated in only 6% of ICARDA elite lines. Despite the efficacy of *Yr1* in most of the wheat-growing areas of CWANA, virulence against *Yr1* has been reported in East Asia (Stubbs, 1985), Central Asia and the Caucasus region (Yahyaoui *et al.,* 2002) and Syria (K. Nazari unpublished data), highlighting the race specificity of this gene. Considering the specificity of *Yr1* and the presence of pathotypes virulent against this gene in the *Yr27*-virulent group (Mogens Hovmoller, personal communication), together with the recent spread of the Warrior pathotype to North Africa, Turkey, and Azerbaijan from Europe, the use of elite lines and commercial cultivars bearing only *Yr1* should be restricted in CWANA.

Yr3 and *Yr4* were infrequent in the lines tested. These two resistance genes are very common in winter wheat cultivars and breeding lines in North Western Europe (de Vallavieille-Pope *et al*., 1990; 2012). However, despite the low frequency of virulence against these two genes in most of the wheat-growing areas of CWANA, sources of *Yr3* and *Yr4* have not been widely used in breeding for stripe rust resistance in spring wheat genotypes. Virulence against these two genes is very common within the *Pst* population in Europe and Australia (de Vallavieille-Pope *et al*., 2012; Wellings, 2011). *Yr3* and *Yr4* can no longer be recommended as sources of resistance in CWANA, due to the recent spread of the Warrior race to some of the wheat-growing areas of North Africa and West Asia.

Yr6 was postulated singly or in combination with *Yr9* or *Yr17* in 13% of ICARDA elite lines and in combination with *Yr7* in 9% of Lebanese varieties. Varieties carrying *Yr6* were introduced into the CIMMYT wheat breeding program and, hence, into ICARDA germplasm, as sources of leaf rust resistance, including *Lr13* and *Lr34* (Wellings, 1986). However, *Yr6* was not frequent in the ICARDA lines tested and virulence against *Yr6* has been reported to be fixed in all isolates from Asia, Africa and South America tested (GRRC, 2017).

Yr7 was postulated singly in only one Lebanese bread wheat variety (Haramoun) and in combination with additional genes in other two Lebanese bread wheat varieties, Tannour and 885, and 15 elite lines. *Yr7* originated from the durum wheat cv. Iumillo. The gene was transferred to Thatcher wheat, from which the differential variety Lee was derived (McIntosh *et al*., 2012). *Yr7* is present in a range of winter and spring wheat cultivars (McIntosh *et al*., 2012). This gene has been defeated in the CWANA region and is no longer effective against the prevalent pathotypes in this region.

Yr9 was postulated singly in four ICARDA elite lines (5%) and in combination with *Yr1*, *Yr3* and *Yr4* in six elite lines (7%). This gene originated from *Secale cereale* and is linked to *Lr26* and *Sr31* in the 1BL.1RS translocation (McIntosh *et al*., 2012). During the 1990s, most of the adapted wheat germplasm generated and distributed by CIMMYT in spring wheat production areas at low latitudes carried the 1BL.1RS translocation (Bimb and Johnson, 1997). This translocation was also identified in European wheat germplasm by Mettin *et al*. (1973) and Zeller (1973). Virulence against *Yr9* has been common in wheat-growing areas in CWANA and sub-Saharan countries since the 1980s, particularly in countries in which 1B.1R-containing genotypes were distributed, including Ethiopia (Badebo and Bayu, 1992), Syria (Mamluk and El-Naimi, 1992), Turkey (Dusunceli *et al*., 1996), Iran (Torabi *et al*., 1995), Pakistan (Bahri *et al.,* 2011), and in Central Asia and Caucasian countries (Yahyaoui, 2005).

The use of this gene in breeding materials should therefore be restricted.

Yr17 was postulated singly in three ICARDA elite lines (3%) and in combination with *Yr6* in one Lebanese bread wheat variety (Naama). The *Yr17*, *Lr37* and *Sr38* gene cluster was transferred to wheat in a translocation from *Aegilops ventricosa* (Dousssinault *et al*., 1998). It was originally transferred to the VPM1 line (a cross of *Ae. ventricosa*, *Triticum persicum* and *cv.* Marne Desprez) (Bariana and McIntosh, 1993). Virulence against *Yr17* has been detected in the USA (Line *et al*., 1992), and in North Western Europe (Bayles *et al*., 2000; Hovmøller *et al*., 2002), where it remains frequent (de Vallavieille-Pope *et al*., 2012). The emergence of virulence against *Yr17* with the incursion of the Warrior race into North Africa (Hovmoller *et al*., 2016) and Turkey (Mert *et al*., 2016) should restrict the use of *Yr17* sources in isolation. Most of the French isolates studied here carry virulence against *Yr9* and *Yr17,* and it is therefore difficult to postulate these two genes when they are present singly. The use of diagnostic molecular markers for these genes can be very useful.

Yr25, which is common and ineffective in North Western European wheat varieties, was postulated in ICARDA elite line Nayzak-3 (line 15), one Lebanese variety (Florence Aurore), and two accessions of the Salamouni landrace. Virulence against *Yr25* is frequent in CWANA (Yahyaoui *et al*., 2002, Nazari, unpublished data).

Yr27 was postulated in five Lebanese varieties (22%) and eight ICARDA elite lines (9%). This gene originated from the wheat cultivar Selkirk and derivatives of the cultivar 'McMurachy' (Wellings, 1992), a parent of 'Selkirk'. This gene is also present in many CIMMYT genotypes (Wellings, 2011). Virulence against *Yr27* has been detected in New Zealand (Wellings and Burdon, 1992), Pakistan (Bahri *et al*., 2011; Ali *et al*., 2014), India (Prashar *et al*., 2007), Tajikistan, Kyrgyzstan (Singh *et al*., 2004), Iran (Nazari and Torabi, 2000) and Syria (Nazari *et al*., 2011). In the last few years, major wheat stripe rust epidemics have occurred in CWANA, sub-Saharan Africa, the Caucasus region and the Indian subcontinents, mostly due to the widespread cultivation of *Yr27* genotypes (Atilla and Kauz derivatives). Interestingly, that the Warrior pathotype does not carry virulence against *Yr27* and *Yr27* wheat cultivars may therefore once again come to predominate in the region.

In Lebanon and Syria, in 2010/2011, the *Yr1*, *Yr3* and *Yr4* genes remained effective, but virulence against *Yr2*, *Yr6*, *Yr7*, *Yr9*, *Yr25*, and *Yr27* predominated, with virulence against *Yr8* and *Yr17* occurring at only low to moderate frequencies (El Amil *et al*., in press).

Adult-plant resistance is often race non-specific and more durable than race-specific seedling resistance. Adult-plant resistance is generally controlled by temperature-sensitive, minor or additive genes. The presence of adult-plant resistance genes has been reported in various winter and spring wheats (Johnson, 1980; Singh and Rajaram, 1994; Chen *et al*., 2014).

We therefore tested adult-plant resistance in Lebanese and Syrian fields, for a subset of elite lines. The three lines with *Yr1* were moderately resistant in the field and virulence against this gene (*v*1) was not detected in the survey conducted in 2010-2011; *v*1 may therefore have been present, at low frequency, at Terbol (LB). Lines 63 and 64, which carry *Yr6,* were resistant and moderately resistant, respectively, in the field, despite the presence of *v6* in Syria and Lebanon, indicating the presence of additional adult-plant resistance in these lines. Line 34, which carries only *Yr7,* was less resistant in the field than line 4, which carries the *Yr7*+*Yr4* combination. Given that the *Yr4* elite line 18 tested was moderately resistant when tested at Terbol (LB), the *Yr7*+*Yr4* combination seems to be responsible for conferring full resistance. The combination of two seedling resistance genes, *Yr7*+*Yr4,* was effective at both sites. Line 1, in which *Yr27* was postulated singly, was susceptible in Syria. Elite line 8 was susceptible in Lebanon and line 10 was moderately resistant to moderately susceptible, suggesting that line 10 displayed adult-plant resistance. The adult-plant resistance test confirmed the presence of *v27* in Syria and Lebanon in 2010 and 2012.

Seedling resistance is of short duration and is rapidly overcome by the pathogen population in the absence of adult-plant resistance. Combinations of seedling resistances prolong the efficacy of the genes, but are rarely durable. Quantitative trait loci (QTLs) for adult-plant resistance provide partial resistance, but seldom protect the plant early in its life. A combination of both types of resistance is, therefore, crucial for the protection of the plant throughout the entire growing season. Durable stripe rust resistance has been observed in four French cultivars and one English cultivar combining both seedling resistance genes and QTLs active at the adult plant stage: *cv.* Renan (Dedryver *et al*., 2009), *cv.* Camp Rémy (Mallard *et al*., 2005), *cv.* Apache (Paillard *et al*., 2012), *cv.* Soissons (de Vallavieille-Pope *et al*., 2012) and *cv.* Claire (Powell *et al*., 2013).

Landraces are considered to be potential sources of disease resistance and agronomic traits. Considerable heterogeneity has already been reported, for plant height and days to heading, in Israeli bread and durum wheat landrace populations (Beharav *et al*., 1997), and has been advocated as a potential source of stripe and

leaf rust resistance in nine Chinese landraces (Zhang, 1995). Our study confirms that landraces are composed of several genotypes, and it will be of particular interest to investigate resistance genes in the genotypes resistant to 239E175*v17 (Warrior)*, the most frequent pathotype in North Western Europe. Further studies may determine whether the resistance gene found in the landraces differs from the genes already identified, and whether resistant landraces could be exploited for rust resistance and other agronomic traits.

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Research Paper

Chlorine and mefenoxam sensitivity of *Phytophthora nicotianae* and *Phytopthora citrophthora* from South African citrus nurseries

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Summary. *Phytophthora nicotianae* and *P. citrophthora* isolates were subjected to mefenoxam and chlorine sensitivity evaluations at different concentrations, and for chlorine, different exposure times. Based on mefenoxam sensitivity, the isolates of the two species were divided in six sensitivity groups with EC_{50} values ranging from sensitive (0.04 ppm mefenoxam) to highly insensitive (greater than 123.69 ppm mefenoxam), with 86% of isolates being sensitive to mefenoxam. Chlorine sensitivity testing indicated strong interactions between chlorine concentration and exposure time for both species. Increased mortality was observed with increased concentration and exposure time to chlorine. For some isolates, close to 100% mortality was only reached at 6 ppm active chlorine and at an exposure time of 60 min. Because highly mefenoxam-insensitive isolates were detected from South African citrus nurseries, this fungicide should be used with care as a curative method for management of diseases caused by *Phytophthora* spp. It is recommended that chlorination of irrigation water, at 6 ppm active chlorine and exposure of more than 60 min, is used to eliminate *P. nicotianae* and *P. citrophthora* propagules from irrigation water as a preventative measure for these diseases.

Keywords. Irrigation water, soilborne pathogens.

INTRODUCTION

The South African citrus industry annually produces approx. 1.3 million tons of citrus fruit, of which about 1.1 million tons are exported, and these fruits are produced on 77,708 ha (Edmonds, 2018). New plantings and replacement of old orchards are important to maintain this level of production. This places heavy demand on continuous production of good quality, disease- and pest-free nursery produced young trees.

Soilborne pathogens, especially *Phytophthora nicotianae* (Breda de Haan) and *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian, have been shown to cause economic losses in citrus plantings in many countries (Meitz-Hopkins *et al.,* 2013). In established orchards, *P. nicotianae* causes collar and root rots of trees as well as infections of low hanging fruit, resulting in brown rot. This fruit rot can potentially spoil entire cartons of fruit during export if it spreads among the fruit in each carton (Graham and Feichtenberger, 2015). *Phytophthora citrophthora* can also attack aerial parts of citrus trees, such as trunks and limbs, ultimately causing tree death (Graham and Feichtenberger, 2015).

Phytophthora nicotianae has been reported to occur sporadically in citrus nurseries in many countries, including South Africa (Wehner *et al.,* 1986; Ahmed *et al.,* 2012). Diagnostic results from the Citrus Research International Diagnostic Centre (Nelspruit, South Africa) also showed that *P. citrophthora* occurs sporadically in South African citrus nurseries. These findings are important, because infected nursery trees are sources of infection of new citrus orchards (Ippolito *et al.,* 2004).

Water is a potential source of infections caused by *P. nicotianae* and *P. citrophthora* in nursery environments (Grech and Rijkenberg, 1992; Ippolito *et al.,* 2004). As a result, preventative control measures are employed to ensure that irrigation water is free from these pathogens. One of these measures is chlorination of irrigation water. This is routinely used in citrus nurseries in South Africa, and other citrus producing countries, to eradicate pathogen propagules that might be present. This practice is also employed by nurseries in other industries (Hong *et al.,* 2003; Ghimire *et al.,* 2011). In cases where nurseries are infested with one of these *Phytophthora* spp., curative fungicide treatments are applied. One such fungicide is mefenoxam, a systemic compound which has been widely studied for control of *Phytophthora* spp. in citrus, and in other tree and ornamental crops (Farih *et al.,* 1981; Davis 1982; Matheron and Matejka, 1988; Matheron *et al.,* 1997; Morales-Rodríguez *et al.,* 2014; Aiello *et al*., 2018).

Despite the application of preventative and curative measures by South African citrus nurseries, *P. nicotianae* and *P. citrophthora* continue to occur sporadically, which indicates ineffective control measures. Hong *et al.* (2003) reported that although zoospores of *P. nicotianae* in water did not survive exposure to 2 mg kg^{-1} active chlorine, mycelium fragments could, in some cases, survive exposure of up to 8 mg kg^{-1}. This is significant, as zoospores and mycelium fragments have been isolated from irrigation water (Hwang and Benson, 2005), with current treatment of citrus nursery water in South Africa carried out with active chlorine concentrations between 3 and 6 ppm. Mefenoxam resistance, has been reported for a number of *Phytophthora* spp., including *P. nicotianae* and *P. citrophthora*, occurring on a wide variety of crops (Hwang and Benson, 2005; Hu *et al.,* 2008, 2010).

The aims of the present study were to obtain *P. nicotianae* and *P. citrophthora* isolates from South African citrus nurseries, and characterize them with regards to sensitivity to chlorine and mefenoxam. The results obtained in this study could be used to develop effective preventative and curative control measures for these pathogens in citrus nurseries.

MATERIALS AND METHODS

Collection and purification of isolates

Isolates of *P. nicotianae* and *P. citrophthora* (Table 1) were collected from South African citrus nurseries, in different provinces of South Africa, by sampling plant propagation substrate from pots of young citrus trees. These samples were placed into different compartments

Table 1. Numbers of *Phytophthora citrophthora* and *Phytophthora nicotianae* isolates from different South African provinces used for mefenoxam and chlorine sensitivity testing.

Species	Province	No. of isolates per species	No. of isolates used for mefenoxam sensitivity testing	No. of isolates used for chlorine sensitivity testing
Phytophthora citrophthora	Eastern Cape	52	48	26
	Western Cape	8	7	4
Phytophthora citrophthora	Eastern Cape	27	27	11
	Limpopo	10	10	7
	Mpumalanga	9	8	5
	North West	4	4	3
	Western Cape	11	11	6

of ice trays, with one ice tray allocated per sample. The substrate in each compartment was covered with distilled water before placing two citrus leaf discs (each 5 mm diam.) in each compartment (Grimm and Alexander, 1973). Before cutting the leaf discs from the citrus leaves, they were washed thoroughly with distilled water. The leaves had been collected from trees not subjected to any fungicide treatments. The ice trays were covered to prevent light infiltration, and were incubated at ambient room temperature on a laboratory bench for 48 h. Following incubation, the leaf discs were removed, blotted dry on absorbent paper toweling, and plated onto 90 mm Petri dishes containing PARPH medium (Jeffers and Martin, 1986). Inoculated plates were then incubated in the dark at 29°C for 48 h before being inspected for *Phytophthora* spp. colonies. Isolates were selected from the inoculated plates and transferred to water agar (WA, Biological agar, Biolab), followed by additional incubation at 29°C for 48 h. Colonies were purified from WA by hyphal tipping onto 90 mm Petri dishes containing V8 agar (Galindo and Gallegly, 1960). Purified isolates were stored in molecular grade water in 2 mL capacity micro centrifuge tubes at 25°C.

Molecular identification of isolates

Selected isolates were grown on V8 agar at 29°C for 7 d before mycelia were harvested for genomic DNA extraction, using a modified CTAB-based extraction protocol (Allen *et al.,* 2006).

PCR-RFLP analyses. The ITS region of the isolates was amplified using the primers ITS 6 (Cooke and Duncan, 1997) and ITS 4 (White *et al.,* 1990). The PCR reaction consisted of 20.0 µL of GoTaq® G2 Hot Start Green Master Mix (Promega Corporation), 1.0 µL of each primer (concentration of 10 µM), 16 µL PCR grade water and 2 µL of genomic DNA, for a total volume of 40 µL. Amplifications were conducted in a 2720 thermal cycler (Applied Biosystems). Initial denaturation was at 94°C for 5 min, followed by 32 cycles of 94°C for 30 s, annealing for 30 s at 55°C, extension at 72°C for 30 s, with a final extension step at 72°C for 5 min. PCR products were resolved in a 1% agarose gel, and DNA fragments were visualized by staining with an ethidium bromide solution. The resulting PCR products were restriction digested with enzymes *Hin*fI and *Hha*I in a single reaction, according to manufacturer's instructions (Fermentas Inc.). The PCR-RFLP products were run on a 3% agarose gel, and isolates with the same RFLP banding patterns were assigned to each RFLP group.

ITS sequencing. The ITS regions of at least two isolates of each PCR-RFLP group were sequenced, and double stranded consensus sequences were obtained. The consensus sequences were subjected to BLAST analyses in Genbank (https://blast.ncbi.nlm.nih.goc/Blast.cgi), and were identified to species level based on similarity of at least 99% to existing *P. nicotianae* or *P. citrophthora* ITS sequences on Genbank (https://www.ncbi.nlm.nih.gov/genbank/).

Mefenoxam sensitivity testing

In vitro sensitivity testing

Sensitivity testing was conducted according to a slightly amended protocol described in Timmer *et al.* (1998). A total of 60 *P. citrophthora* and 61 *P. nicotianae* isolates, from different nurseries in different citrus production areas in South Africa (Table 1), were selected and were on 90 mm Petri dishes containing corn meal agar (CMA; Sigma-Aldrich). Plates were incubated at 29°C for 5 d. After incubation, 5 mm plugs were cut from the edges of the actively growing cultures and plated onto 90 mm Petri dishes containing CMA amended with mefenoxam (Ridomil Gold® 450 EC; Syngenta) at 0, 1.0, 2.0, 5.0, 10.0, 25.0, 50.0 or 100.0 ppm. These plates were then incubated at 29°C for 2 d.

Each isolate and concentration combination was repeated using two plates, while the whole trial was repeated twice at the same time. Colony diameters of growing isolates were each measured in two directions and the average colony diameter was calculated for each isolate at each concentration. The percentage inhibition for each plate at each concentration for all the isolates was calculated, and data were subjected to statistical analyses to group isolates, and determine EC_{50}, EC_{80} and EC_{90} values for each isolate. The percentage inhibition was calculated using the following equation:
Percentage inhibition (%) = mean colony diameter of control (0 ppm) – mean diameter ÷ mean diameter of control (0 ppm) × 100.

Data analyses

The Mitscherlich function [$y = a(1\text{-}e^{-bx})$] or % Inhibition = Maximum Inhibition [$1\text{-}e^{-(Rate)(Concentration)}$] fitted the data well, and was used throughout the study. Hereafter, percentage (%) inhibition will be referred to as %Inhb, and maximum inhibition, as MaxInhb. The function was fitted for the two Petri dishes representing each isolate mefenoxam concentration combination within each of the two trials.

EC_{50}, EC_{80}, and EC_{90} values were calculated from the estimated regression parameters (MaxInhb and Rate of

inhibition) for each isolate. Wherever MaxInhb < EC, these respective values could not be calculated according to the appropriate equations (EC_{50} = (-log (1 - (50 ÷ a))) ÷ b; EC_{80} = (-log (1 - (80 ÷ a))) ÷ b; EC_{90} = (-log (1 - (90 ÷ a))) ÷ b). MaxInhb did not always give realistic values, especially where the Rate of inhibition was very slow, because MaxInhb is a value at a theoretical concentration. An additional value, PInhbConc100 = $a[1\text{-}e^{-b(100)}]$, was therefore calculated. This represents the %Inhb at a fungicide concentration of 100 ppm, which gave a more realistic interpretation within the boundaries of the data than just MaxInhb. Regression parameters and EC_{50}, EC_{80} and EC_{90} values were subjected to analysis of variance (ANOVA), and cluster analysis using Ward's clustering method to cluster isolates. Principle component analysis (PCA) was also carried out for the 115 isolates and numbers of isolates in clusters as labels, to see if the grouping or clustering obtained from the cluster analyses made sense.

Chlorine sensitivity testing

Mycelium suspension preparation

Hong *et al.* (2003) found that *Phytophthora* spp. mycelium fragments were more insensitive to chlorine than zoospores. Mycelium fragment suspensions were therefore used in chlorine sensitivity tests. Ten percent V8 broth was prepared by adding 0.5 g $CaCO_3$ (Calcium carbonate; Merck) and 50 mL V8 juice (V8 Original Vegetable Juice, Campbell Soup Company) to each Schott bottle containing 450 mL filtered water, and bottles were then autoclaved at 121°C for 15 min. *Phytophthora* isolates of the two species (32 *P. nicotianae* and 30 *P. citrophthora*; Table 1), randomly selected from the populations used for mefenoxam sensitivity testing, were plated onto 90 mm Petri dishes containing CMA and incubated for 7–10 d at 29°C. After sufficient growth, the agar from each Petri dish was divided into smaller pieces using a scalpel, and placed into the prepared 10% V8 broth. The inoculated V8 broth was then placed on an orbital shaker (SHKO 20; FHM Electronics) running at 100 rpm and 29°C, for 21 d in the dark.

To prepare the mycelium broth, autoclaved filtered water was adjusted to pH 6.5, Adjustment of pH was achieved using sodium hydroxide (NaOH 40 g mol^{-1}, Merck; 2 g in 250 mL autoclaved filtered water) and hydrochloric acid (HCl 32%, Merck; 5 mL in 250 mL autoclaved filtered water). The mycelium masses harvested from the 10% V8 broth were drained using a 180-µm sieve before being each washed twice with 100 mL autoclaved filtered water. Excess water was then pressed out of the remaining fungal mycelium mass using two sterile stainless steel teaspoons. For trial purposes, a measured *Phytophthora* suspension was prepared by blending 1 g (wet mass) of mycelium in 100 mL filtered water (pH 6.5) for 30 s, followed by filtration (1,000 µm sieve) into a Schott bottle, which was then filled to 500 mL using deionized water (pH 6.5).

Chlorine sensitivity testing

Trial variables included chlorine concentration (0, 1.5, 3 or 6 ppm) and a range of chlorine exposure times (0, 5, 10, 30 or 60 min). A chlorine stock solution (SS) was prepared by adding 0.15 g chlorine granules (HTH®, South Africa) to 100 mL filtered, autoclaved water (pH 6.5). In order to achieve 0, 1.5, 3 and 6 ppm concentrations of active chlorine, 0, 0.75, 1.5 or 3.0 mL chlorine SS was added to different 500 mL Schott bottles. As a positive control, a 1.5 and 6 ppm active chlorine solution was tested using a chlorine photometer (Total Chlorine Ultra High Range Portable Photometer, HI 96771; Hanna Instruments Inc.) before the commencement of each trial set. These chlorine control solutions were also de-activated with sodium thiosulfate stock solution ($Na_2S_2O_3$*$5H_2O$; 248.21 g mol^{-1}; Merck) containing 1.47 g $Na_2S_2O_3$*$5H_2O$ in 1,000 mL of filtered, autoclaved water, and tested with Insta-Test® low range 90–10 ppm) free chlorine test strips (LaMotte) to determine whether the stock solution was still functional.

Additionally, the prepared *Phytophthora* mycelium suspension of each isolate was mixed on a magnetic stirrer plate for 10 min before being used to inoculate two PARPH plates as positive controls. Each mycelium suspension (two separate suspensions per *Phytophthora* isolate) was added (treated) to 0, 1.5, 3 or 6 ppm active chlorine and mixed for a further 30 s. Following each exposure time (0, 5, 10, 30 or 60 min), 40 mL of solution was dispensed into two containers and de-activated using the sodium thiosulfate SS. For deactivation, 0, 0.3, 0.6 or 1.2 mL of sodium thiosulfate SS was required to de-activate, respectively, 0, 1.5, 3 and 6 ppm active chlorine. De-activated solution (1 mL) from each container was used to inoculate two PARPH plates and was subsequently spread using a hockey stick and incubated for 2 d at 29°C. Free chlorine test strips were used to confirm de-activation.

Following incubation, *Phytophthora* spp. colonies were counted and percentage mortality determined using the following formula:
[(Cn-Tn)/Cn)]*100, where Cn is the number of colonies on control plates and Tn the number of colonies on treated plates.

The percentage mortality data were subjected to statistical analyses using SAS (SAS Institute Inc.). Fisher's LSD was calculated at the 5% level to compare means.

RESULTS

Molecular identification of isolates

The 121 isolates were divided into two distinct groups based on the ITS-RFLP analysis. ITS sequence analyses of representative isolates from each group identified 61 of the isolates obtained from citrus nurseries as *P. nicotianae*. The remaining 60 isolates were identified as *P. citrophthora*. The species identity was based on a 100% nucleotide homogeneity with *P. nicotianae* and *P. citrophthora* isolates lodged from previous studies on Genbank (https://www.ncbi.nlm.nih.gov/genbank/).

Mefenoxam sensitivity testing

Plotting of the mean EC_{50}, EC_{80} and EC_{90} values of all the isolates indicated that for a group of five isolates no EC_{50}, EC_{80} or EC_{90} values could be calculated. These isolates were grouped together in sensitivity group 1 (Table 2). A further two isolates had EC_{50} values that were greater than 100 ppm. These two isolates were placed in sensitivity group 2 (Table 2). The remaining isolates were subjected to Ward's cluster analysis and PCA to group them into sensitivity groups. Both the Ward's cluster analysis and PCA showed that the remaining *P. citrophthora* and *P. nicotianae* isolates could be aligned into four distinct sensitivity groups (Figure 1). This brought to six the total number of sensitivity groups (Table 2). The analysis of variance (ANOVA) comparing the groups using the regression parameters and EC_{50}, EC_{80} and EC_{90} data showed highly significant ($P < 0.0001$) sensitivity group effects for these three parameters. The ANOVA of the PInhbCon100 and Rate of inhibition data showed a significant sensitivity group × species interaction ($P < 0.0001$ for PInhbCon100 and $P = 0.0021$ for Rate of inhibition).

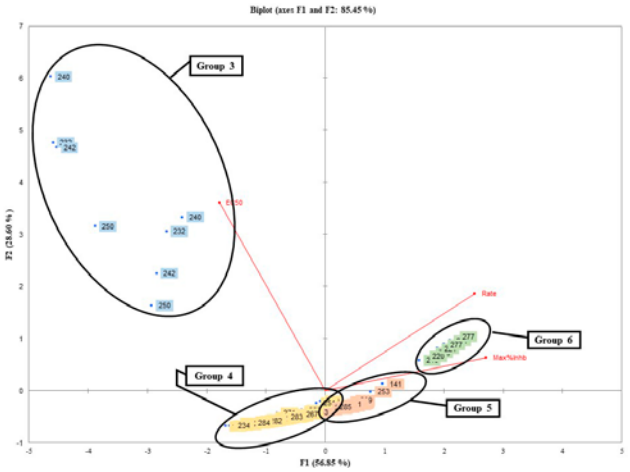

Figure 1. Principal component analysis (PCA) indicating separation of *Phytophthora nicotianae* and *Phytopthora citrophthora* isolates into four distinct mefenoxam sensitivity groups based on the regression parameters EC_{50}, rate of inhibition and Max%Inhb.

Table 2. Mean PInhbConc100 and rate of inhibition values for *Phytophthora citrophthora* and *Phytophthora nicotianae* isolates, grouped into mefenoxam sensitivity groups 1–6, following *in vitro* exposure to different mefenoxam concentrations.

Sensitivity group	Species	PInhbConc100 (%)	Rate of inhibition
1	*P. citrophthora*	39.36 h[1]	0.068 e
	P. nicotianae	30.74 i	0.036 e
2	*P. citrophthora*	44.30 g	0.002 e
	P. nicotianae	---	---
3	*P. citrophthora*	54.88 f	0.027 e
	P. nicotianae	---	---
4	*P. citrophthora*	70.29 e	1.811 d
	P. nicotianae	80.94 d	1.307 d
5	*P. citrophthora*	92.35 c	2.616 c
	P. nicotianae	96.02 b	1.858 c
6	*P. citrophthora*	100.00 a	17.752 a
	P. nicotianae	100.00 a	15.820 a
LSD		3.430	0.7200
P		< 0.0001	0.0021

[1] Means followed by the same letter are not statistically different at a 95% confidence level.

Within group 6, the isolates of *P. citrophthora* and *P. nicotianae* were 100% inhibited by a concentration of 100 ppm mefenoxam. Within group 5 the *P. nicotianae* isolates had a mean of 96.02% inhibition that was significantly more than the *P. citrophthora* isolates in the same group (92.35% inhibition) (Table 2). Both these means of group 5 were significantly less than those of group 6. Also, within group 4 the mean inhibition of *P. nicotianae* isolates was 80.94% and was statistically greater than the mean inhibition (70.29%) of the *P. citrophthora* isolates in this group (Table 2). These two means were less than that observed for the two species in groups 5 and 6. Only *P. citrophthora* isolates were grouped into group 2 and 3. In these groups the mean inhibition of isolates was 54.88% (group 3) and 44.30% (group 2), significantly less than the mean inhibition percentages for isolates in the other groups (Table 2). Isolates of both

species were placed in group 1 that had the least sensitivity to mefenoxam. In this group, mean inhibition of the *P. citrophthora* isolates was 39.26% compared to 30.74% for the *P. nicotianae* isolates. The mean percentage inhibition for both species in group 1 were also the least observed in any of the groups for either pathogen species (Table 2).

The rate of inhibition results for the group × species interaction showed similar trends to those for the PInhbCon100 results. The groups with the greater PInhbCon100 means, also had the greatest rates of inhibition (Table 2). Group 6 had the greatest mean rate of inhibition of all the groups. The mean rate of inhibition of *P. citrophthora* isolates was 17.75, significantly greater than the rate for the *P. nicotianae* isolates (15.82) in this group. The mean inhibition rate of the *P. citrophthora* isolates in group 5 was 2.62 which was significantly greater again than the rate of the *P. nicotianae* isolates (1.86). This mean rate for *P. nicotianae* isolates was comparable to the rates observed for the two species in group 4 (Table 2). In this group, the mean rate of inhibition for *P. citrophthora* was 1.81 compared to 1.31 for *P. nicotianae*. In groups 1, 2 or 3, the mean rates for the two species (0.002 to 0.068) were statistically similar (Table 2).

For the isolates of both species sensitivity group 1, at 100 ppm mefenoxam, the maximum inhibition only reached a mean of 39.36% and the rate of inhibition was only 0.068 (Table 2). Consequently, no EC_{50}, EC_{80} or EC_{90} values could be determined for this group (Table 3). For group 2, the mean EC_{50} value was 123.69 ppm, which was statistically greater than the mean EC_{50} of any other group. Similarly, the mean EC_{80} (214.12 ppm) and EC_{90} (250.25 ppm) values of this group were the greatest of all the groups (Table 3). For group 3, the calculated mean EC_{50} was 76.12 ppm, which was the second greatest of all the groups. Again, no EC_{80} or EC_{90} means could be determined, possibly also due to the slow rate of inhibition of this group (Tables 2 and 3). Group 4 had mean EC_{50} (0.82 ppm) and EC_{90} (2.86 ppm) values that were statistically similar to those for groups 5 and 6. However, an EC_{90} could also not be calculated for this group (Table 2). Groups 5 and 6 had mean EC_{50} (0.45 ppm), EC_{80} (0.10 ppm) and EC_{90} (1.68 ppm) values that were statistically similar to those of group 6. For group 6, the mean EC_{50} (0.04 ppm), EC_{80} (0.10 ppm) and EC_{90} (1.15 ppm) values were the least of all the groups (Table 3). This indicated that the isolates in this group were the most sensitive to mefenoxam.

Table 3. Mean EC_{50}, EC_{80} and EC_{90} values for different mefenoxam sensitivity groups identified after *in vitro* exposure of *Phytophthora citrophthora* and *Phytophthora nicotianae* isolates to different mefenoxam concentrations.

Sensitivity group	EC_{50}	EC_{80}	EC_{90}
1	---	---	---
2	123.69 a[1]	214.12 a	250.25 a
3	76.11 b	---	---
4	0.82 c	2.86 b	---
5	0.45 c	1.11 b	1.68 b
6	0.04 c	0.10 b	0.15 b
LSD	5.101	8.545	12.608
P	< 0.0001	< 0.0001	< 0.0001

[1] Means followed by the same letter are not statistically different at a 95% confidence level.

Distribution of isolates in sensitivity groups

Within the different sensitivity groups, the number of isolates of *P. citrophthora* and *P. nicotianae* varied greatly. For *P. citrophthora*, most of the isolates (90%) were in groups 1, 2, 3 or 4, where the mean percentage inhibition at 100 ppm mefenoxam ranged from 39.36% to 70.29% (Table 4). Based on the classification of Hu *et al*. (2008), these isolates were intermediately insensitive or sensitive to mefenoxam. However, for *P. nicotianae* only 5% of isolates were in the groups 1 to 4, while 95% were in groups 5 and 6. These isolates would be classified as mefenoxam sensitive (Table 4).

Table 4. Numbers (and proportions) of *Phytophthora citrophthora* and *Phytophthora nicotianae* isolates occurring in the different mefenoxam sensitivity groups.

Species	Mefenoxam sensitivity group	No. of isolates in group
Phytophthora citrophthora (n= 60)	1	5 (8%)
	2	2 (3%)
	3	4 (7%)
	4	43 (72%)
	5	4 (7%)
	6	2 (3%)
Phytophthora nicotianae (n = 61)	1	1 (2%)
	2	0 (0%)
	3	0 (0%)
	4	2 (3%)
	5	36 (59%)
	6	22 (36%)

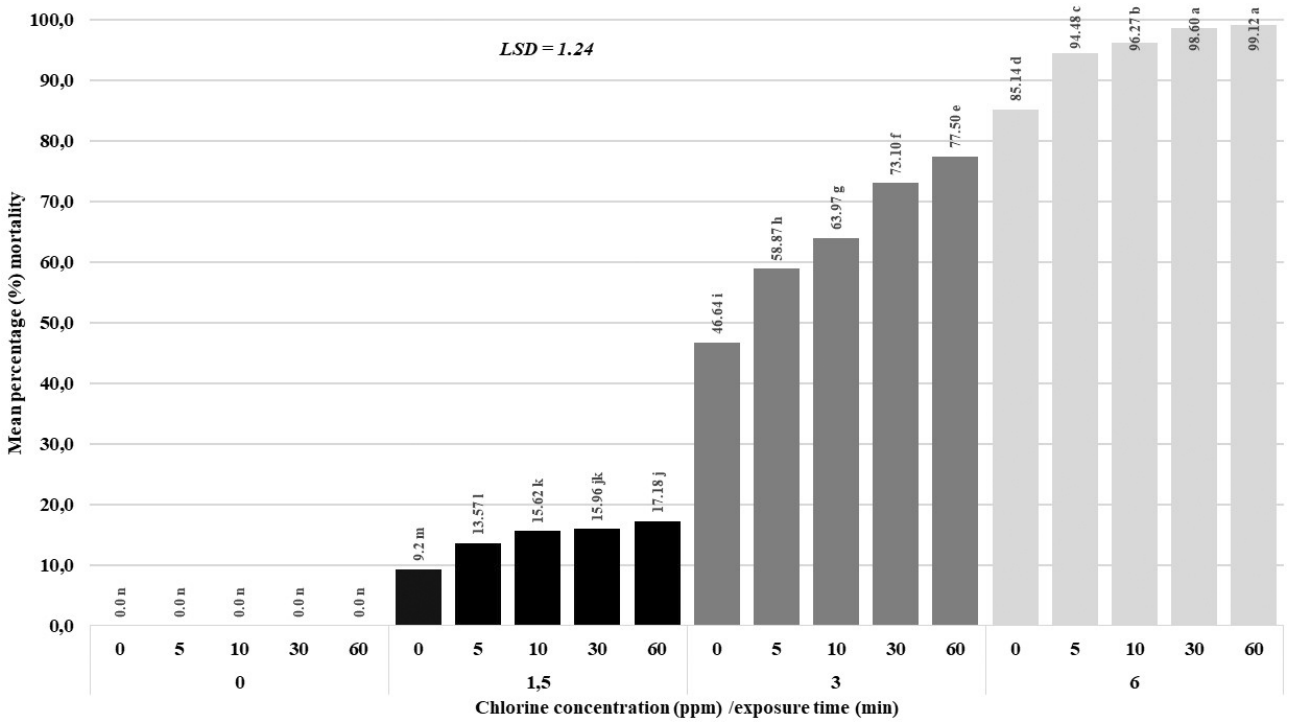

Figure 2. Mean percentage (%) mortality of *Phytophthora* spp. propagules exposed to 0, 1.5, 3 or 6 ppm active chlorine for exposure times of 0, 5, 10, 30 or 60 min.

Chlorine sensitivity testing

The analysis of variance (ANOVA) of the percentage mortality data indicated a highly significant ($P < 0.0001$) experimental repetition × species × isolate × chlorine concentration interaction, and an experimental repetition × chlorine concentration × exposure time interaction. These multifactor interactions are attributed to the significant ($P < 0.0001$) variation seen in the percentage inhibition data between the two experimental repetitions, which could be due to different mycelium suspensions used for each repetition, combined with the significant ($P < 0.0001$) variation seen in mean percentage inhibition between the different chlorine concentrations. Between isolates in the two *Phytophthora* spp., the ANOVA also indicated that there were statistical ($P < 0.0001$) differences between percentage mortalities obtained. Mean mortality of *P. citrophthora* isolates due to chlorine ranged from 27.09 to 73.47%, whereas those for *P. nicotianae* isolates were from 19.69 to 62.30%.

The results from the significant ($P < 0.0001$) chlorine concentration × exposure time interaction indicated no mortality of the *Phytophthora* spp. for 0 ppm chlorine. With chlorine concentration of 1.5 ppm, the mean percentage mortality at no exposure (chlorine deactivated immediately) was 9.20%. This then increased with each increase in exposure time to reach a maximum mean of 17.18% after 60 min exposure to 1.5 ppm active chlorine (Figure 2). When the 3 ppm chlorine treatment was deactivated immediately, the mean mortality was 46.64% and was significantly greater to be 77.50% after 60 min exposure. At 6 ppm chlorine the initial mean mortality was 85.14% with immediate deactivation. Increase in exposure time led to greater mortality at 99.12% after 60 min exposure (Figure 2).

DISCUSSION

Production of citrus trees in nurseries that are free from the soilborne pathogens *Phytophthora nicotianae* and *P. citrophthora* is regarded as essential for establishment of new orchards. This has been concluded from previous studies indicating that infected nursery trees can be sources of infection of new citrus orchards (Ippolito *et al*., 2004). As a result, nurseries need to treat irrigation water with chlorine, because the water is a potential source of infection (Ghimire *et al*., 2011). As a curative measure, infected plants are often treated in these nurseries with drenches of metalaxyl or mefenoxam (Hu *et al*., 2008).

In the present study a total of 121 *P. nicotianae* and *P. citrophthora* isolates were subjected to mefenoxam sensitivity testing at eight different mefenoxam concentrations. Results indicated that the isolates were divided in six sensitivity groups. The mean percentage inhibition calculated at 100 ppm mefenoxam for the different groups varied from 30.74 to 100.00% (Table 2). In the groups with the least inhibition (groups 1, 2 and 3), the rate of increase in inhibition was also very low and could explain the low maximum inhibition achieved at 100 ppm mefenoxam (Table 2). Hwang and Benson (2005) demonstrated that isolates of *P. cryptogea, P. nicotianae* and *P. palmivora*, occurring on floriculture crops in North Carolina, were also divided into different mefenoxam sensitivity groups.

In groups 1, 2 and 3 the mean percentage inhibition at 100 ppm mefenoxam was less than 60%, indicating (Hu *et al*., 2008), that the isolates of *P. citrophthora* and *P. nicotianae* in these groups were insensitive to mefenoxam. Compared to this, the isolates of these two species in groups 4 and 5 were intermediately sensitive, while the isolates in group 6 were sensitive to the compound. There were more *P. citrophthora* isolates than *P. nicotianae* isolates in the groups with the least sensitivity to mefenoxam. Within the groups, *P. citrophthora* isolates often had lower percentages of inhibition compared to *P. nicotianae*, indicating less mefenoxam sensitivity. This is similar to the results of Farih *et al.* (1981) and Coffey and Bower (1984), who found *P. citrophthora* isolates from citrus were less sensitive to mefenoxam than *P. nicotianae* isolates from citrus.

Mean $EC_{50,}$ EC_{80} and EC_{90} values for the isolates in groups 1 to 4 could not be calculated (Table 3). This was probably because at 100 ppm mefenoxam, the highest concentration used in this study, the calculated percentage inhibition for *P. citrophthora* and *P. nicotianae* isolates in these groups was less than 50–90%. For these isolates, 100% inhibition would only be achieved at mefenoxam concentrations much greater than 100 ppm. This was shown by the results for the group 2 isolates, where the mean mefenoxam EC_{90} value was 250.25 ppm.

Timmer *et al.* (1998) published similar results, where they found that some isolates of *P. nicotianae* from citrus had EC_{50} values greater than 100 ppm. Similarly, Farih *et al.* (1981) reported that some isolates of *P. citrophthora* and *P. nicotianae* from citrus had 100% inhibition of mycelium growth at mefenoxam concentrations greater than 100 ppm. High levels of insensitivity to mefenoxam or metalaxyl among isolates of these two citrus pathogens are therefore not unknown. Even among *P. nicotianae* isolates from ornamental crops, Ferrin and Kabashima (1991) showed that highly insensitive isolates had EC_{50} values greater than 100 ppm. In groups 5 and 6 in the present study, the isolate EC_{50} values were well below 2 ppm, and in the case of group 6 below 0.2 ppm. These values were similar to those for isolates described in the reports cited above. These levels of mefenoxam insensitivity have also been determined for *P. infestans* from potato (Goodwin *et al*., 1996) and *P. cinnamomi* from avocado (Coffey and Bower, 1984) and ornamental crops (Hu *et al*., 2010).

For *P. infestans* isolates, the basis of mefenoxam or metalaxyl insensitivity or resistance, and differences in sensitivity between isolates within a species, and between species, were found to be due to genotypic differences between the isolates and species (Goodwin *et al*., 1996). The particular level of sensitivity within a genotype was determined by insensitivity loci present (Fabritius *et al*., 1997). Childers *et al*. (2015) also discovered that sensitive isolates of *P. infestans* can acquire mefenoxam resistance after repeated *in vitro* exposure to the fungicide. However, isolates developing resistance *in vitro* lost some resistance when they were repeatedly plated onto media not amended with mefenoxam.

It is therefore possible that sensitive isolates of *P. nicotianae* and *P. citrophthora* could acquire resistance to mefenoxam when repeatedly exposed to this fungicide in nurseries or orchards. Careful use of this fungicide in citrus nurseries is therefore important, to prevent development of highly insensitive isolates in nurseries that may reach newly established orchards. This was emphasized by the results of Timmer *et al*. (1998), who showed that insensitive isolates from citrus nurseries competed with sensitive isolates as causes of root rot. Furthermore, these insensitive isolates maintained their insensitivity after use of the fungicide ceased.

No differences in sensitivity to chlorine were observed between isolates of *P. citrophthora* and *P. nicotianae*. Increasing exposure time at particular chlorine concentrations increased percentage mortality of both pathogens. However, mean percentage mortality only came close to 100% when the isolates (32 *P. nicotianae* and 30 *P. citrophthora*) were exposed to 6 ppm chlorine for 60 min (Figure 2). Hong *et al*. (2003) tested limited numbers of isolates of *P. nicotianae, P. capsici, P. cinnamomi, P. citricola, P. citrophthora, P. cryptogea* and *P. megasperma*, from irrigation water from ornamental nurseries, for their sensitivity to chlorine. They also observed that with increasing active chlorine concentrations, mean percentage mortality of these pathogens increased. However, they did not detect a chlorine concentration and exposure time interaction. This interaction recorded in the present study could have been due to the higher numbers of isolates of the two

species tested. Chlorination was introduced in South African citrus nurseries based on the study of Grech and Rijkenberg (1992). They indicated that chlorination eliminates soilborne pathogen propagules from irrigation water, consequently reducing the level of *Phytophthora* infection in roots of citrus rootstock seedlings irrigated with treated water. This treatment was also shown to not cause phytotoxic effects on the irrigated seedlings.

The present study is the first to focus on chlorine sensitivity of multiple *P. citrophthora* and *P. nicotianae* isolates subjected to a range of chlorine concentrations and exposure times. Practically, the results indicate that for complete elimination of *Phytophthora* spp. propagules from citrus nursery irrigation water, treatment with 6 ppm active chlorine for 60 min or longer, is required. Mefenoxam as a curative soil drench treatment should also be used with care, as low numbers of highly resistant *P. citrophthora* and *P. nicotianae* isolates were found in this study. For the elimination of these pesticide-resistant pathogens from nurseries, it is therefore important to make use of alternative fungicides (e.g. captan) for curative soil drenches.

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Research Paper

Identification and pathogenicity of lignicolous fungi associated with grapevine trunk diseases in southern Italy

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Summary. Over the last 10 years, several fungi were isolated from grapevines with grapevine trunk disease (GTD) symptoms, in the Apulia and Molise regions of Italy. Morphological and molecular analyses allowed the identification of species belonging to Botryosphaeriaceae, *Phaeoacremonium* species, *Phaeomoniella chlamydospora*, *Pleurostoma richardsiae* and less-common fungi associated with grapevine trunk diseases, such as *Cadophora*, *Colletotrichum*, *Seimatosporium* and *Truncatella*. These last genera were isolated at significant frequencies, so they were investigated for possible involvement in GTDs. To screen the large numbers of isolates collected, microsatellite-PCR analysis was carried out with the M13 primer, and 29 strains were further studied by amplification of different genes, for multi-locus analyses. Phylogenies and morphological analyses allowed identification, for first time in Italy, of fungi associated with GTDs, including *Cadophora luteo-olivacaea*, *Colletotrichum fioriniae*, *Seimatosporium vitis-vinifera* and *Truncatella angustata*. Pathogenicity assays with these fungi and other fungi known to be pathogens for grapevines (*Lasiodiplodia citricola*, *Phaeoacremonium italicum*, *Pleurostoma richardsiae*) showed that they caused disease symptoms on two Italian grapevine cultivars ('Bombino bianco', 'Nero di Troia'), although with different degrees of severity. Among the fungi isolated for the first time in Italy, *Sei. vitis-vinifera* was the most aggressive, while *C. fioriniae* the least pathogenic. All of these fungi were re-isolated from grapevine, and thus fulfilled Koch's postulates, confirming their pathogenicity on grapevine.

Keywords. *Cadophora luteo-olivacea*, *Colletotrichum fioriniae*, *Seimatosporium vitis-vinifera*, *Truncatella angustata*, phylogenies, artificial inoculation.

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INTRODUCTION

Several diseases caused by fungi that have been associated with grapevines over the last 20 years have caused severe yield losses in other grape producing countries (Gramaje *et al.*, 2018; Guerin-Dubrana *et al.*, 2019). Grapevine trunk diseases (GTDs) are considered to be the most destruc-

tive and severe diseases of grapevine in Mediterranean countries, including Spain, France, Portugal and Italy, and also in the United States of America, Australia, and Asia (Gubler *et al*., 2005; Gramaje *et al*., 2018; Guerin-Dubrana *et al.,* 2019). The main fungi that cause GTDs are species involved in different diseases, which include Esca and Petri disease (Larignon and Dubos, 1997; Mugnai *et al.,* 1999; Gramaje *et al.,* 2011; Navarrete *et al.,* 2011; Bertsch *et al*., 2013; Carlucci *et al.,* 2015a; Travadon *et al.,* 2015), Botryosphaeria dieback (Urbez-Torres, 2011), Diaporthe and Eutypa diebacks (Larignon and Dubos, 1997; Fourie and Halleen, 2004; Urbez-Torres *et al.,* 2013), and black foot disease (Halleen *et al*., 2004; Agusti-Brisach and Armengol, 2013; Carlucci *et al*., 2017).

The main external symptom of GTDs is general decline of affected plants. The specific external symptoms consist of tiger-stripe leaves, stunted shoots and chlorotic leaves which are sometimes cupped and with necrotic margins, flattened areas of the wood without bark, cankered wood and wedge-shaped perennial cankers, black and sunken necrotic lesions on roots, and reddish brown discolouration at the bases of trunks (Gramaje *et al.,* 2018). Internal symptoms include dark-coloured xylem vessels of the grapevine trunks, with exudate from the vessels when the trunks are cut in cross-section, and dark streaks in longitudinal sections (*Phaeomoniella chlamydospora, Phaeoacremonium* spp., *Cadophora* spp.). There can also be black subcortical streaking (*Pleurostoma richardsiae*) and necrosis of the wood tissues. Other symptoms are of rootstock browning in young grapevines (due to black foot fungi). Cordon dieback can also occur, with loss of spurs and internal necrotic wedge-shaped staining in stem cross-sections (*Eutypa lata*, *Botryosphaeria* spp.), and wood white rot (caused by Basidiomycete fungi) (Gramaje *et al.,* 2018).

Grapevines can be affected by one or more GTDs at the same time, as individual plants can be infected by different pathogens, due to co-occurrence of multiple infections throughout a season, and over years. This produces overlapping of the symptoms described above, which makes their association with the specific responsible fungi particularly difficult to define, and detection of causal pathogens challenging (Gramaje *et al*., 2018).

High isolation frequency of particular fungal species involved in GTDs from mature, young and nursery grapevines in different countries can be different, due to climatic and geographic conditions, to specific pathogen aggressiveness, and to host cultivar susceptibility (Guerin-Dubrana *et al.,* 2019). Petri and black-foot diseases are mostly detected on planting material and young vines (Rego *et al.,* 2000; Agusti-Brisach and Armengol, 2013; Carlucci *et al.,* 2017). Conversely, apoplexy, Esca and grapevine leaf symptoms, and Phomopsis, Eutypa and Botryosphaeria diebacks are most frequently observed on mature grapevines (Guerin-Dubrana *et al*., 2019).

To date, up to 138 fungal species belonging to 35 genera have been reported as responsible for GTDs. However, pathogenicity towards grapevine wood has not been tested and/or confirmed for all of these fungi (Gramaje *et al.,* 2018; Berlanas *et al*., 2020; Brown *et al*., 2020). For instance, 'Pestalotioides fungi' have been frequently associated with symptomatic and asymptomatic vineyards (Farr and Rossman, 2018; Liu *et al.,* 2019), although no detailed information is available about their involvement in GTD symptoms. In Italy, incidence of Esca, grapevine leaf symptoms and apoplexy is significant and increasing in all grapevine production regions (Guerin-Dubrana *et al.,* 2019). In Apulia, Molise and Sicily, Botryosphaeria dieback has also been reported (Cristinzio, 1978; Burruano *et al.,* 2008; Carlucci *et al.,* 2009; 2015b). Eutypa and Phomopsis diebacks are known to occur, if not frequently, in Italian vineyards (Guerin-Dubrana *et al*., 2019). *Pleurostoma richardsiae*, *Dactylonectria torresensis*, *Ilyonectria liriodendri* and *Thelonectria blackeriella* were reported for the first time in Italy by Carlucci *et al*. (2015a; 2017).

A collection of fungi from a decennial survey carried out in symptomatic vineyards in the Apulia and Molise regions of Italy was subjected to identification and characterisation by morphological and molecular approaches, and pathogenicity testing of representative isolates was carried out to determine their putative involvement in GTDs. The present paper describes results from this research.

MATERIALS AND METHODS

Fungal isolates

Symptomatic grapevine samples were collected and analysed during the years 2009 to 2018. The samples were from many vineyards in the Foggia, Barletta-Trani-Andria and Campobasso provinces in Italy, and were taken from different grapevine cultivars, including 'Sangiovese', 'Montepulciano', 'Nero di Troia', 'Pinot grigio', 'Trebbiano toscano', 'Moscato bianco' and 'Chardonnay' (Table 1).

External symptoms observed on affected grapevine plants included stunting, reduced grapevine vigour, shoot dieback, and leaf discolouration with interveinal chlorosis and necrosis. Internal symptoms included black discolouration of wood under the bark, and necro-

Table 1. Information on vineyards surveyed and sampled in the Apulia and Molise regions (southern Italy).

Survey Year	Location	Vineyard			
		Cultivar	Age (year)	N. samples	GTD Incidence* (%)
2009	Cerignola (FG)	'Sangiovese'	27	8	13.4
	Lucera (FG)	'Nero di Troia'	15	3	11.5
	Lucera (FG)	'Moscato bianco'	10	3	9.8
2011	Canosa di Puglia (BT)	'Montepulciano'	21	4	16.2
	Canosa di Puglia (BT)	'Sangiovese'	12	3	11.3
	Foggia (FG)	'Nero di Troia'	19	5	13.5
	Foggia (FG)	'Pinot grigio'	13	3	9.2
2012	Foggia (FG)	'Moscato bianco'	17	4	6.7
	Campobasso (CB)	'Pinot grigio'	2	3	11.2
	Barletta (BT)	'Sangiovese'	14	3	17.3
	Barletta (BT)	'Trebbiano toscano'	11	3	16.4
2013	San Severo (FG)	'Trebbiano toscano'	29	10	21.4
	San Severo (FG)	'Nero di Troia'	21	6	18.3
	San Severo (FG)	'Pinot grigio'	12	3	16.9
	Termoli (CB)	'Pinot grigio'	5	4	8.5
	Termoli (CB)	'Chardonnay'	5	3	9.2
	Cerignola (FG)	'Sangiovese'	14	3	12.6
	Cerignola (FG)	'Trebbiano toscano'	8	6	9.3
2015	Campomarino (CB)	'Chardonnay'	2	6	11.5
	Campomarino (CB)	'Pinot grigio'	2	6	14.2
	Stornara (FG)	'Sangiovese'	31	8	19.8
	Torremaggiore (FG)	'Trebbiano toscano'	25	6	19.2
	Torremaggiore (FG)	'Nero di Troia'	15	4	14.8
2017	Foggia (FG)	'Sangiovese'	9	3	10.5
	Foggia (FG)	'Trebbiano toscano'	17	4	13.7
	Canosa di Puglia (BT)	'Montepulciano'	21	6	19.4
	Canosa di Puglia (BT)	'Sangiovese'	23	3	18.9
2018	Cerignola (FG)	'Trebbiano toscano'	19	3	13.6
	Barletta (BT)	'Chardonnay'	14	6	11.8
	Barletta (BT)	'Montepulciano'	17	6	10.3

* GTD Incidence was calculated on the basis of vines showing symptoms on 2,500 plants for each surveyed vineyard.

sis of xylem tissues. The samples included grapevine trunks, cordons and woody shoots. These were transported to the laboratory for analyses, where they initially underwent surface sterilization (Fisher *et al.*, 1992). The bark of each sample was removed with a sterile scalpel, and thin wood sections were cut (1 to 3 mm thick). From each section of each sample, five small wood tissue samples were cut and placed onto potato dextrose agar (PDA; 3.9% potato dextrose agar; Oxoid Ltd), and onto malt extract agar (MEA; 2% malt extract, 2% agar; Oxoid Ltd), both of which were supplemented with 500 mg L^{-1} streptomycin sulphate (Oxoid Ltd). After 7 to 10 d of incubation at 22±3°C in the dark, all of the fungal cultures obtained were purified by transferring single germinated conidia or small pieces of hyphae to Petri dishes containing fresh PDA.

Morphological and culture characteristics of isolated fungi were initially used to distinguish genera and species that were isolated from these symptomatic tissues (Crous and Gams, 2000; Mostert *et al.*, 2006; Essakhi *et al.*, 2008; Agustì-Brisach *et al.*, 2013; Phillips *et al.*, 2013; Raimondo *et al.*, 2014; Carlucci *et al.*, 2015a). The isolation frequency (IF; %) for each species was calculated as the number of tissue segments infected by each fungus, divided by the total number of tissue segments incubated.

DNA extraction and microsatellite PCR profiles

Genomic DNA was extracted from the 420 isolates obtained, from 15-d-old cultures grown on PDA (Carlucci *et al.*, 2013). Many of the isolates (339) belonged to Botryosphaeriaceae and *Phaeoacremonium*, *Phaeomoniella*, *Pleurostoma*, *Cadophora*, *Colletotrichum*, *Seimatosporium* and *Truncatella*, so preliminary screening was carried out for each genus based on the M13 minisatellite primers (5'-GAGGGTGGCGGTTCT-3') (Meyer *et al.,* 1993). Microsatellite (MSP)-PCR profiles were generated according to Santos and Phillips (2009). The DNA banding patterns were analysed using the Bionumerics v. 5.1 software (Applied Maths), with calculation of Pearson's correlation coefficients according to the unweighted pair group method with arithmetic means. The reproducibility levels were calculated by comparisons of the banding profiles obtained for the M13 primer. For this purpose, from any cluster, 10% of the strains were chosen at random, and their profiles were repeat.

Molecular characterisation

The MSP dendrogram generated for each genus produced different clades from which representative isolates were chosen for phylogenetic analysis data not shown). Eighty-four representative isolates of Botryosphaeriaceae, *Phaeoacremonium* spp., *Phaeomoniella* spp. and *Pleurostoma* spp. were identified using the keys, descriptions and sequence data from Phillips *et al.* (2013), Mostert *et al.* (2006), Essakhi *et al.* (2008), Raimondo *et al.* (2014), Crous and Gams (2000) and Carlucci *et al.* (2015a). For 41 Botryosphaeriaceae strains, ITS1-5.8S-ITS2 were amplified using the universal primers ITS1 and ITS4 (White *et al.*, 1990), and part of EF1-a was amplified using the primers EF1-688F and EF1-1251R (Alves *et al.*, 2008), according to Carlucci *et al.* (2015b). For 27 *Phaeoacremonium* strains, partial β-tubulin and partial actin genes were amplified using the universal primers T1 (O'Donnell and Cigelnik, 1997), Bt2b (Glass and Donaldson, 1995) and ACT-512F/ACT-783R (Carbone and Kohn, 1999), according to Raimondo *et al.* (2014). For seven *Phaeomoniella* and *Pleurostoma* strains, ITS1-5.8S-ITS2 were amplified using the universal primers ITS1 and ITS4 (White *et al.* 1990), according to Damm *et al.* (2010) and Carlucci *et al.* (2015a).

The other 29 representative strains that belonged to the *Seimatosporium*, *Truncatella*, *Cadophora* or *Colletotrichum* were further studied using molecular and morphological tools. Five loci were amplified for seven *Seimatosporium* and 11 *Truncatella* strains that were representative of the MSP-PCR groups. For large subunit RNA (LSU; ca. 500 bp) were used NL1/NL4 primer pairs (O'Donnell and Gray, 1993); for internal transcribed spacers (ITS) 1 and 2 (including 5.8S of nuclear ribosomal DNA; ca. 500 bp) were used ITS5/ITS4 (White *et al.*, 1990); for the partial β-tubulin gene (*tub*; ca. 680 bp) were used T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995); for the partial translation elongation factor 1-alpha (*tef-1α*; ca. 300 bp) were used EF1-688F and EF1-1251R (Alves *et al.,* 2008); and for the second-largest subunit of DNA-directed RNA polymerase II (*rpb2*; ca. 500 bp) were used RPB2-5f2/RPB2-7cr (Liu *et al.,* 1999; Sung *et al.*, 2007).

The LSU and ITS PCR reactions and conditions were performed according to Carlucci *et al.* (2012), with those for β-tubulin, *tef-1α* and *rpb2* according to Liu *et al.* (2019).

Three loci including ITS (ca. 550 bp), the partial translation elongation factor 1-alpha (*tef-1α*; ca. 420bp) and the partial β-tubulin gene (*tub*; ca. 500 bp) were amplified from six *Cadophora* strains, as representative of the MSP-PCR groups. These amplifications used the following primer pairs: ITS5/ITS4 (White *et al.*, 1990) for internal transcribed spacers (ITS) 1 and 2; EF1-728F and EF1-986R (Carbone and Kohn, 1999) for the partial translation elongation factor 1-alpha; and BTCadF 5' and BTCadR 5' (Travadon *et al.*, 2015) for the partial β-tubulin gene. The ITS PCR reactions and conditions were performed as described above, while those for *tef-1α* and β-tubulin according to Travadon *et al.* (2015).

Six loci were amplified for five *Colletotrichum* strains, as representative of the MSP-PCR groups. These included: the 5.8S nuclear ribosomal gene with the two flanking ITS (ca. 538 bp); β-tubulin (*tub*; ca. 500 bp); partial actin (*act*; ca. 250 bp); the intron of glyceraldehyde-3-phosphate dehydrogenase (*gapdh*; ca. 250 bp), and chitin synthase (*chs-1*; ca. 280 bp). The primer pairs used were ITS5/ITS4 (White *et al.*, 1990) for internal transcribed spacers (ITS) 1 and 2; T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995)) for β-tubulin; ACT-512F/ACT-783R (Carbone and Kohn, 1999) for partial actin; GDF1/GDR1 (Guerber *et al.*, 2003) for the intron of glyceraldehyde-3-phosphate dehydrogenase; and CHS-79F/CHS-345R (Carbone and Kohn, 1999) for chitin synthase. The PCR amplifications and conditions were performed according to Fu *et al.* (2019).

Five microlitres of each amplicon was analysed by electrophoresis, using 1.5% (w/v) agarose gels in 1× TAE buffer (40 mM Tris, 40 mM acetate, 2 mM EDTA, pH 8.0) at 100 V for 30 min. The gels were stained with ethidium bromide and visualised under ultraviolet light (Gel Doc EZ System; BioRad). The PCR products were

purified before DNA sequencing (Nucleo Spin Extract II purification kits; Macherey-Nagel), according to the manufacturer instructions. Both strands of the PCR products were sequenced by Eurofins Genomics Service (Milan, Italy).

Phylogenetic analyses

The nucleotide sequences obtained were manually edited using BioEdit version 7.0.9 (http://www.mbio.ncsu.edu/BioEdit). Consensus sequences were compared with those available in the GenBank database, using the Basic Local Alignment Search Tool (BLAST) to confirm the preliminary morphological identification, and to select and download closely related sequences for phylogenetic analyses. GenBank sequences from different species of *Seimatosporium*, *Truncatella*, *Cadophora* and *Colletotrichum* were then selected and added to the sequences dataset obtained (Tables 2, 3).

The sequences were manually concatenated and aligned using the online multiple alignment programme MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/) (Katoh and Standley, 2013). The alignments were visually checked and manually improved where necessary. Multilocus analyses according to maximum parsimony and maximum likelihood were carried out for the LSU, ITS, β-tubulin, *tef-1α* and *rpb2* genes of the *Seimatosporium* and *Truncatella* sequence data.

The maximum parsimony analyses were performed using PAUP, version 4.0b10 (Swofford, 2003), with the heuristic search option with 100 random taxa additions, and tree bisection and reconstruction as the branch swapping algorithm. Branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Bootstrap support values were calculated from 1,000 heuristic search replicates and ten random taxon additions. The tree length (TL), consistency index (CI), retention index (RI), homoplasy index (HI), and rescaled consistency index (RC) were calculated for each, and the resulting trees were visualised with TreeView, version 1.6.6 (Page, 1996). Alignment gaps were treated as missing data for *Seimatosporium* strains, and as fifth characters for *Truncatella* strains.

The maximum likelihood analysis was carried out using RAxML-HPC v.8.2.12 (Stamatakis, 2006; Stamatakis *et al*., 2008) on the XSEDE Teragrid of the CIPRES Science Gateway (https://www.phylo.org) (Miller *et al*., 2010), with rapid bootstrap analysis, followed by 1,000 bootstrap replicates. The final trees were selected among the suboptimal trees from each run by comparing the likelihood and bootstrap scores. The outgroups in the *Seimatosporium* multigenic analysis were *Synnemapestaloides juniperi* (CBS 447.77) and *Discosia artocreas* (CBS 124848), and those for *Truncatella* were *Phlogicylindrium eucalypti* (CBS 120080) and *Beltrania pseudorhombica* (CBS 138003).

Multilocus alignment of the *Cadophora* (ITS, *tef-1α*, β-tubulin genes) and *Colletotrichum* (ITS, β-tubulin, *act*, *gapdh*, *chs-1* genes) strains was performed as described above with alignment gaps treated as missing data. *Hyaloscypha finlandica* (CBS 444.86) was used as outgroup in the *Cadophora* analysis, and *Colletotrichum gloeosporioides* (ICMP 17821) for the *Colletotrichum* analysis.

Morphological analyses

For each species identified using molecular tools (as described above), three isolates were used for morphological studies. To enhance sexual sporulation or conidiation, these fungi were grown on MEA in Petri dishes for 10 to 21 d under UV light at 23±2°C. Fungal structures were observed and measured from 100% lactic acid microscope slide mounts by making 30 measurements (at ×400 or ×1,000 magnification), using a measurement module (Leica Application Suite; Leica Microsystems GmbH). Photomicrographs were recorded using a digital camera (DFC320; Leica) on a microscope fitted with Nomarski differential interference contrast optics (DMR; Leica). The morphological features of conidiogenous cells and conidia were also determined in distilled water, by picking mycelium plugs from 30-d-old cultures grown on MEA, with images captured using a microscope (DM5500; Leica) at ×40 magnification.

Pathogenicity tests

Three isolates of each species were used in pathogenicity tests, to determine the infection of grapevine wood tissues by the less-known GTD fungi, and to compare their aggressiveness with the most common and previously determined GTD fungi. The previously determined GTD fungi used were: *Lasiodiplodia citricola* (Carlucci *et al*., 2015b), *Phaeoacremonium italicum* (Raimondo *et al*., 2014), and *Pleurostoma richardsiae* (Carlucci *et al*., 2015a).

Inoculations were carried out in June 2018, on 1-year-old canes (diam. 1.0-2.5 cm) from 10-y-old grapevines of the cultivars 'Nero di Troia' and 'Bombino bianco' in vineyards in an open field. The canes were inoculated at the internodes by wounding, as described by Carlucci *et al*. (2013). The wounds (each 1.0-2.0 cm long) were made on the cane surfaces with a sterile scalpel.

Table 2. Isolate identification numbers, locations, hosts and GenBank accession numbers of the strains of *Seimatosporium* spp., *Truncatella* spp. and *Cadophora* spp. used in the multigenic analyses.

Species	Isolate number[a]	Location	Host	GenBank accession number				
				LSU	ITS	*tub*	*tef-1α*	*rpb2*
Discosia artocreas	CBS 124848 ET[b]	Germany	*Fagus sylvatica*	MH554213	MH553994	MH554662	MH554420	MH554903
Seimatosporium botan	NBRC 104200 HT	Japan	*Paeonia suffruticosa*	AB593731	AB594799	LC047770	–	–
Sei. germanicum	CBS 437.87 HT	Germany	Unknown	MH554259	MH554047	MH554723	MH554482	MH554957
Sei. luteosporum	CBS 142599 HT	USA	*Vitis vinifera*	KY706309	KY706284	KY706259	KY706334	–
Sei. physocarpi	CBS 139968 HT	Russia	*Physocarpus opulifolius*	KT198723	KT198722	MH554676	MH554434	MH554917
	CBS 789.68	The Netherlands	*Physocarpus amurensis*	MH554278	MH554066	MH554742	MH554502	MH554979
Sei. pistaciae	CBS 138865 HT	Iran	*Pistacia vera*	KP004491	KP004463	MH554674	MH554432	MH554915
	CPC 24457	Iran	*Pistacia vera*	MH554331	MH554126	MH554799	MH554561	MH555035
Sei. rosae	CBS 139823 ET	Russia	*Rosa kalmiussica*	KT198727	LT853105	LT853253	LT853203	LT853153
Sei. vitifusiforme	CBS 142600 HT	USA	*Vitis vinifera*	KY706321	KY706296	KY706271	KY706346	–
Sei. vitis-viniferae	CBS 123004 HT	Spain	*Vitis vinifera*	MH554211	MH553992	MH554660	MH554418	MH554901
	CBS 116499	Iran	*Vitis vinifera*	MH554201	MH553984	MH554643	MH554402	MH554884
	CRCC 212[c]	Italy	*Vitis vinifera*	**MN862466**	**MN862459**	**MN862452**	**MN862445**	**MN862473**
	CRCC 213	Italy	*Vitis vinifera*	**MN862467**	**MN862460**	**MN862453**	**MN862446**	**MN862474**
	CRCC 214	Italy	*Vitis vinifera*	**MN862468**	**MN862461**	**MN862454**	**MN862447**	**MN862475**
	CRCC 229	Italy	*Vitis vinifera*	**MN862472**	**MN862465**	**MN862458**	**MN862451**	**MN862479**
	CRCC 247	Italy	*Vitis vinifera*	**MN862469**	**MN862462**	**MN862455**	**MN862448**	**MN862476**
	CRCC 248	Italy	*Vitis vinifera*	**MN862470**	**MN862463**	**MN862456**	**MN862449**	**MN862477**
	CRCC 251	Italy	*Vitis vinifera*	**MN862471**	**MN862464**	**MN862457**	**MN862450**	**MN862478**
Sei. vitis	MFLUCC 14-0051	Italy	*Vitis vinifera*	KR920362	KR920363	–	–	–
	Napa774	Napa County, USA	*Vitis vinifera*	KY706276	KY706301	KY706251	KY706326	–
	Napa772	Napa County, USA	*Vitis vinifera*	KY706275	KY706300	KY706250	KY706325	–
	Napa782	Napa County, USA	*Vitis vinifera*	KY706278	KY706303	KY706253	KY706328	–
	Napa764	Napa County, USA	*Vitis vinifera*	KY706273	KY706298	KY706248	KY706323	–
	Napa759	Napa County, USA	*Vitis vinifera*	KY706282	KY706307	KY706257	KY706332	–
	VMT2_1	Italy	*Vitis vinifera*	–	LS991528	LS997596	LS999502	–
Sporocadus biseptatus	CBS 110324 HT	Unknown	Unknown	MH554179	MH553956	MH554615	MH554374	MH554853
Spo. cornicola	CBS 143889	Germany	*Cornus sanguinea*	MH554326	MH554121	MH554794	MH554555	MH555029
Spo. incanus	CBS 123003 HT	Spain	*Prunus dulcis*	MH554210	MH553991	MH554659	MH554417	MH554900
Spo. lichenicola	CBS 354.90	Germany	*Fagus sylvatica*	MH554252	MH554035	MH554711	MH554470	MH554948
	CPC 24528	Germany	*Juniperus communis*	MH554332	MH554127	MH554800	MH554562	MH555036
	NBRC 32625; IMI 079706 ET	UK	*Rosa canina*	MH883646	MH883643	MH883645	MH883644	MH883647
Spo. mali	CBS 446.70 HT	The Netherlands	*Malus sylvestris*	MH554261	MH554049	MH554725	MH554484	MH554960
Spo. microcyclus	CBS 424.95 HT	Germany	*Sorbus aria*	MH554258	MH554045	MH554721	MH554480	MH554956

(Continued)

Table 2. (Continued).

Species	Isolate number[a]	Location	Host	GenBank accession number				
				LSU	ITS	*tub*	*tef-1α*	*rpb2*
	CBS 887.68	The Netherlands	*Ribes* sp.	MH554280	MH554068	MH554744	MH554504	MH554981
Spo. multiseptatus	CBS 143899 HT	Serbia	*Viburnum* sp.	MH554343	MH554141	MH554814	MH554576	MH555047
Spo. rosarum	CBS 113832	Sweden	*Rosa canina*	MH554189	MH553970	MH554629	MH554388	MH554864
Spo. rosigena	CBS 116498	Iran	*Vitis vinifera*	MH554200	MH553983	MH554642	MH554401	MH554883
	CBS 129166	Latvia	*Rhododendron*	MH554215	MH553996	MH554665	MH554423	MH554905
	CBS 182.50	The Netherlands	*Pyrus communis*	MH554233	MH554013	MH554689	MH554447	MH554926
	CBS 250.49	The Netherlands	*Rubus fruticosus*	MH554245	MH554023	MH554699	MH554457	MH554934
	CBS 466.96	The Netherlands	*Rubus* sp.	MH554265	MH554052	MH554728	MH554487	MH554965
Spo. rotundatus	CBS 616.83 HT	Canada	*Arceuthobium pussilum*	MH554273	MH554060	MH554737	MH554496	MH554974
Spo. sorbi	CBS 160.25	Unknown	Unknown	MH554229	MH554008	MH554684	MH554442	MH554924
Sporocadus sp. 1	CBS 506.71	Italy	*Euphorbia* sp.	MH554268	MH554055	MH554731	MH554490	MH554968
Spo. trimorphus	CBS 114203 HT	Sweden	*Rosa canina*	MH554196	MH553977	MH554636	MH554395	MH554876
Synnemapestaloides juniperi	CBS 477.77 HT	France	*Juniperus phoenicea*	MH554266	MH554053	MH554729	MH554488	MH554966
Bartalinia bella	CBS 464.61 HT	Brazil	Air	MH554264	MH554051	MH554727	MH554486	MH554964
Bar. robillardoides	CBS 122615	South Africa	*Cupressus lusitanica*	MH554207	MH553989	MH554657	MH554415	MH554897
	CBS 122705 ET	Italy	*Leptoglossus occidentalis*	KJ710438	LT853104	LT853252	LT853202	LT853152
Bar. pini	CBS 143891 HT	Uganda	*Pinus patula*	MH554330	MH554125	MH554797	MH554559	MH555033
	CBS 144141	USA	*Acacia koa*	MH554364	MH554170	MH554843	MH554605	MH555067
Beltrania pseudorhombica	CBS 138003	China	*Pinus tabulaeformis*	KJ869215	MH554124	–	MH554558	MH555032
Broomella vitalbae	HPC 1154	Unknown	Unknown	MH554367	MH554173	MH554846	MH554608	MH555069
Diversimediispora humicola	CBS 302.86 HT	USA	Soil	MH554247	MH554028	MH554705	MH554463	MH554941
Heterotruncatella proteicola	CBS 144020 HT	South Africa	*Protea acaulos*	MH554288	MH554077	MH554751	MH554512	MH554989
Het. quercicola	CBS 143895 HT	USA	*Quercus walshii*	MH554337	MH554135	MH554808	MH554570	MH555041
Het. restionacearum	CBS 118150	South Africa	*Restio filiformis*	MH554203	DQ278914	MH554649	MH554407	MH554889
	CBS 119210 HT	South Africa	*Ischyrolepis cf. gaudichaudiana*	DQ278929	DQ278915	MH554653	MH554411	MH554892
Het. spadicea	CBS 118144	South Africa	*Ischyrolepis* sp.	DQ278926	DQ278921	MH554646	MH554404	MH554886
	CBS 118145 ET	South Africa	*Cannomois virgata*	DQ278927	DQ278912	MH554647	MH554405	MH554887
	CBS 118148	South Africa	*Rhodocoma capensis*	DQ278928	DQ278913	MH554648	MH554406	MH554888
	CPC 17911; CMW 22206	South Africa	*Elegia filacea*	MH554308	MH554098	MH554771	MH554532	MH555012
	CPC 28956	Australia	*Sorghum halepense*	MH554353	MH554157	MH554830	MH554592	MH555056
Hymenopleella austroafricana	CBS 143886 HT	South Africa	*Gleditsia triacanthos*	MH554320	MH554115	MH554788	MH554549	MH555023
	CBS 144026	South Africa	*Bridelia mollis*	MH554322	MH554117	MH554790	MH554551	MH555025
	CBS 144027	Zambia	*Combretum hereroense*	MH554324	MH554119	MH554792	MH554553	MH555027

(Continued)

Table 2. (Continued).

Species	Isolate number[a]	Location	Host	GenBank accession number				
				LSU	ITS	*tub*	*tef-1α*	*rpb2*
Hym. polyseptata	CBS 143887 HT	South Africa	*Combretum* sp.	MH554321	MH554116	MH554789	MH554550	MH555024
Hym. hippophaeicola	CBS 113687	Sweden	*Hippophae rhamnoides*	MH554188	MH553969	MH554628	MH554387	MH554863
	CBS 140410 ET	Austria	*Hippophae rhamnoides*	MH554224	KT949901	MH554678	MH554436	MH554919
Hym. subcylindrica	CBS 164.77	India	*Cocos nucifera*	MH554230	MH554009	MH554685	MH554443	MH554925
	CBS 647.74 HT	India	*Gypsophilla* seeds	MH554275	MH554062	MH554739	MH554498	MH554976
Morinia acaciae	CBS 100230	New Zealand	*Prunus salicina* Omega	MH554174	MH553950	MH554609	MH554368	MH554847
	CBS 137994 HT	France	*Acacia melanoxylon*	MH554221	MH554002	MH554673	MH554431	MH554914
Mor. crini	CBS 143888 HT	South Africa	*Crinum bulbispermum*	MH554323	MH554118	MH554791	MH554552	MH555026
Mor. longiappendiculata	CBS 117603 HT	Spain	*Calluna vulgaris*	MH554202	AY929324	MH554644	AY929316	MH554885
Parabartalinia lateralis	CBS 399.71 HT	South Africa	*Acacia karroo*	MH554256	MH554043	MH554719	MH554478	MH554954
Pseudosarcostroma osyridicola	CBS 103.76 HT	France	*Osyris alba*	MH554177	MH553954	MH554613	MH554372	MH554851
Truncatella angustata	CBS 113.11	Germany	*Picea abies*	MH554185	MH553966	MH554625	MH554384	MH554860
	CBS 135.97	Spain	Decaying bark	MH554220	MH554001	MH554671	MH554429	MH554912
	CBS 165.25	Unknown	*Prunus armeniaca*	MH554231	MH554010	MH554686	MH554444	–
	CBS 231.77 = CBS 296.77	Turkey	*Gossypium* sp.	MH554243	MH554021	MH554697	MH554455	MH554932
	CBS 338.32	The Netherlands	*Lupinus* sp.	MH554250	MH554033	MH554709	MH554467	MH554945
	CBS 398.71	Turkey	Soil	MH554255	MH554042	MH554718	MH554477	MH554953
	CBS 144025 NT	France	*Vitis vinifera* Prunelard	MH554318	MH554112	MH554785	MH554546	MH555021
	CBS 449.51	Unknown	*Salix* sp. or *Thuja* sp.	MH554262	MH554050	MH554726	MH554485	MH554961
	CBS 938.70	The Netherlands	*Prunus laurocerasus*	MH554281	MH554070	MH554746	MH554506	MH554982
	CPC 21366	France	*Vitis vinifera* Prunelard	MH554319	MH554113	MH554786	MH554547	MH555022
	CBS 208.80	The Netherlands	Food	MH554239	MH554020	MH554696	MH554454	–
	CBS 443.54	UK	*Picea abies*	MH554260	MH554048	MH554724	MH554483	MH554959
	CPC 21354	France	*Vitis vinifera* Prunelard	MH554317	MH554111	MH554784	MH554545	MH555020
	CBS 642.97	Switzerland	*Heterodera carotae* cyst egg mass, on *Daucus carota*	MH554274	MH554061	MH554738	MH554497	MH554975
	CBS 564.76	Switzerland	*Pyrus malus*	MH554271	MH554057	MH554733	MH554492	MH554970
	CRCC 147	Italy	*Vitis vinifera*	–	–	–	–	–
	CRCC 165	Italy	*Vitis vinifera*	–	–	–	–	–
	CRCC 188	Italy	*Vitis vinifera*	**MN862441**	**MN862439**	**MN862437**	**MN862435**	**MN862443**
	CRCC 189	Italy	*Vitis vinifera*	–	–	–	–	–
	CRCC 195	Italy	*Vitis vinifera*	–	–	–	–	–
	CRCC 199	Italy	*Vitis vinifera*	–	–	–	–	–
	CRCC 201	Italy	*Vitis vinifera*	–	–	–	–	–

(Continued)

Table 2. (Continued).

Species	Isolate number[a]	Location	Host	GenBank accession number				
				LSU	ITS	*tub*	*tef-1α*	*rpb2*
	CRCC 240	Italy	*Vitis vinifera*	–	–	–	–	–
	CRCC 241	Italy	*Vitis vinifera*	–	–	–	–	–
	CRCC 243	Italy	*Vitis vinifera*	–	–	–	–	–
	CRCC 245	Italy	*Vitis vinifera*	**MN862442**	**MN862440**	**MN862438**	**MN862436**	**MN862444**
Phlogicylindrium eucalypti	CBS 120080 HT	*Australia*	*Eucalyptus globulus*	DQ923534	NR_132813	MH704633	MH704607	MH554893
Robillarda africana	CBS 122.75 HT	South Africa	Unknown	KR873281	KR873253	MH554656	MH554414	MH554896
Rob. australiana	CBS 143882 HT	Australia	Unknown	MH554301	MH554091	MH554764	MH554525	MH555005
Rob. terrae	CBS 587.71 HT	India	Soil	KJ710459	KJ710484	MH554734	MH554493	MH554971
Rob. roystoneae	CBS 115445 HT	Hong Kong	*Roystonea regia*	KR873282	KR873254	KR873317	KR873310	MH554880
Strickeria kochii	CBS 140411 ET	Austria	*Robinia pseudoacacia*	KT949918	NR_154423	MH554679	MH554437	MH554920
Cad. gregata	ATCC11073 HT	Unknown	Soybean root	–	U66731	Mf677920	Mf979586	–
Cad. helianthii	CBS 144752 HT	Ukraine	*Helianthus annuus*	–	MF962601	MH733391	MH719029	–
Cad. interclivum	BAP37	Banff, Canada	*Picea glauca,* root	–	MF677930	MF677919	MF979585	–
	BAP33	Banff, Canada	*Picea glauca*, root	–	MF677929	MF677918	MF979584	–
	CBS143323 HT	Banff, Canada	*Carex sprengelii*, root	–	MF677928	MF677917	MF979583	–
Cad. luteo-olivacea	CBS 141.41 HT	Sweden	Unknown	–	AY249066	KM497133	KM497089	–
	A19	California, USA	*Vitis viniferae*	–	KM497038	KM497119	KM497075	–
	A41	California, USA	*Vitis vinifera* 'Chardonnay'	–	KM497039	KM497120	KM497076	–
	A42	California, USA	*Vitis vinifera* 'Chardonnay'	–	KM497040	KM497121	KM497077	–
	U5	California, USA	*Vitis vinifera* 'Sangiovese'	–	KM497041	KM497122	KM497078	–
	U7	California, USA	*Olea europa*	–	KM497044	KM497125	KM497081	–
	U8	California, USA	*Vitis vinifera* 'Semillon'	–	KM497042	KM497123	KM497079	–
	U17	California, USA	*Vitis vinifera* 'Chardonnay'	–	KM497043	KM497124	KM497080	–
	U21	California, USA	*Vitis vinifera*	–	KM497045	KM497126	KM497082	–
	U22	California, USA	*Vitis vinifera* 'Chardonnay'	–	KM497046	KM497127	KM497083	–
	U53	California, USA	*Vitis vinifera* 'Chardonnay'	–	KM497047	KM497128	KM497084	–
	U56	California, USA	*Vitis vinifera* 'Syrah'	–	KM497048	KM497129	KM497085	–
	CRCC 11B	Italy	*Vitis vinifera*	–	–	–	–	–
	CRCC 113A	Italy	*Vitis vinifera*	–	**MN871929**	**MN871925**	**MN871927**	–
	CRCC 122	Italy	*Vitis vinifera*	–	**MN871930**	**MN871926**	**MN871928**	–
	CRCC 131	Italy	*Vitis vinifera*	–	–	–	–	–
Cad. malorum	CBS 165.42 HT	The Netherlands	*Amblystoma mexicanum*	–	AY249059	KM497134	KM497090	–
Cad. melinii	CBS 268.33 HT	Unknown	Unknown	–	AY249072	KM497132	KM497088	–
	U11	California, USA	*Vitis vinifera* 'Sangiovese'	–	KM497032	KM497113	KM497069	–

(Continued)

Table 2. (Continued).

Species	Isolate number[a]	Location	Host	GenBank accession number				
				LSU	ITS	*tub*	*tef-1α*	*rpb2*
	ONC1	Ontario, Canada	*Vitis vinifera* 'Cabernet Franc'	–	KM497033	KM497114	KM497070	–
Cad. meredithiae	CBS143322 HT	Banff, Canada	*Carexsprengelii,* root	–	MF677925	MF677914	MF979580	–
	BAP6	Banff, Canada	*Picea glauca*, root	–	MF677926	MF677915	MF979581	–
	BAP13	Banff, Canada	*Picea glauca*, root	–	MF677927	MF677916	MF979582	–
Cad. novi-eboraci	NYC14 HT	New York, USA	*Vitis labruscana* 'Concord'	–	KM497037	KM497118	KM497074	–
	NYC2	New York, USA	*Vitis labruscana* 'Concord'	–	KM497034	KM497115	KM497071	–
	NYC13	New York, USA	*Vitis vulpina*	–	KM497036	KM497117	KM497073	–
	NYC1	New York, USA	*Vitis vinifera* 'Cabernet' Sauvignon	–	KM497035	KM497116	KM497072	–
Cad. orchidicola	UAMH8152	Alberta, Canada	Northern green orchid, root	–	AF214576	MF677921	MF979587	–
Cad. orientoamericana	NHC1 HT	New Hampshire, USA	*Vitis* hybrid 'Niagara'	–	KM497018	KM497099	KM497055	–
	CTC1	Connecticut, USA	*Vitis vinifera* 'Chardonnay'	–	KM497012	KM497093	KM497049	–
Cad. spadicis	RIC1	Rhode Island, USA	*Vitis vinifera* 'Cabernet' Sauvignon	–	KM497029	KM497110	KM497066	–
	RIC3	Rhode Island, USA	*Vitis hybrid* 'Vidal'	–	KM497030	KM497111	KM497067	–
	QCC1	Quebec, Canada	*Vitis vinifera* 'Gamay'	–	KM497031	KM497112	KM497068	–
	CBS 111743 HT	Italy	*Actinidia chinensis*	–	DQ404351	KM497136	KM497091	–
Cad. viticola	CBS 139517 HT	Spain	*Vitis vinifera* 'Syrah'	–	HQ661096	–	HQ661081	–
	Cme-1	Spain	*Vitis vinifera* 'Syrah'	–	HQ661096	–	HQ661081	–
	Cme-3	Spain	*Vitis vinifera* 'Syrah'	–	HQ661098	–	HQ661083	–
Hyaloscypha finlandica	CBS 444.86 HT	Finland	Unknown	–	AF486119	KM497130	KM497086	–

[a] **ATCC**: American Type Culture Collection, Virginia, USA; BCC: BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology (BIOTEC), Khlong Luang, Pathumthani, Thailand. **CBS**: Culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands. **CMW**: Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. **CPC**: Culture collection of Pedro Crous, housed at the Westerdijk Institute. **CRCC**: Carlucci and Raimondo Culture Collection, housed at Dept. SAFE of University of Foggia. **HPC**: Herbarium of Pedro Crous, housed at the Westerdijk Institute. **IMI**: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, United Kingdom. **MFLU(CC)**: Mae Fah Luang University Culture Collection. **NBRC**: Biological Resource Center.
[b] Status: status of the strains. ET: ex-epitype. NT: ex-neotype. HT: ex-Holotype.
[c] Strain numbers and newly generated sequences are indicated in bold font.

Table 3. Isolate identification numbers, locations, hosts and GenBank accession numbers of the strains of *Colletotrichum* spp. used in the multigenic analyses.

Species	Isolate number[a]	Location	Host	GenBank accession number				
				ITS	*gapdh*	*chs-1*	*act*	*tub*
C. acerbum	CBS 128530 HT[b]	New Zealand	*Malus domestica*, bitter rot of fruit	JQ948459	JQ948790	JQ949120	JQ949780	JQ950110
C. acutatum	CBS 112996 HT	Australia	*Carica papaya*	JQ005776	JQ948677	JQ005797	JQ005839	JQ005860
C. australe	CBS 116478 HT	South Africa	*Trachycarpus fortunei*	JQ948455	JQ948786	JQ949116	JQ949776	JQ950106
C. brisbanense	CBS 292.67 HT	Australia	*Capsicum annuum*	JQ948291	JQ948621	JQ948952	JQ949612	JQ949942
C. chrysanthemi	IMI 364540, CPC 18930	China	*Chrysanthemum coronarium,* leaf spot	JQ948273	JQ948603	JQ948934	JQ949594	JQ949924
C. cosmi	CBS 853.73 HT	The Netherlands	*Cosmos* sp., seed	JQ948274	JQ948604	JQ948935	JQ949595	JQ949925
C. costaricense	CBS 330.75 HT	Costa Rica	*Coffea arabica,* cv. 'Typica', berry	JQ948180	JQ948510	JQ948841	JQ949501	JQ949831
C. cuscutae	IMI 304802, CPC 18873 HT	Dominica	*Cuscuta* sp.	JQ948195	JQ948525	JQ948856	JQ949516	JQ949846
C. fioriniae	CBS 128517 HT	USA	*Fiorinia externa* (elongate hemlock scale, insect)	JQ948292	JQ948622	JQ948953	JQ949613	JQ949943
	CBS 125396	USA	*Malus domestica*, fruit lesion	JQ948299	JQ948629	JQ948960	JQ949620	JQ949950
	CBS 124958	USA	*Pyrus* sp., fruit rot	JQ948306	JQ948636	JQ948967	JQ949627	JQ949957
	CBS 126526	The Netherlands	*Primula* sp., leaf spots	JQ948323	JQ948653	JQ948984	JQ949644	JQ949974
	IMI 324996, CPC 18880	USA	*Malus pumila*	JQ948301	JQ948631	JQ948962	JQ949622	JQ949952
	CRCC 104[c]	Italy	*Vitis vinifera*	**MN871933**	**MN871939**	**MN871937**	**MN871931**	**MN871935**
	CRCC 140	Italy	*Vitis vinifera*	**MN871934**	**MN871940**	**MN871938**	**MN871932**	**MN871936**
	CRCC 144	Italy	*Vitis vinifera*	–	–	–	–	–
	CRCC 154	Italy	*Vitis vinifera*	–	–	–	–	–
	CRCC 160	Italy	*Vitis vinifera*	–	–	–	–	–
C. godetiae	CBS 133.44 HT	Denmark	*Clarkia hybrida*, cv. 'Kelvon Glory', seed	JQ948407	JQ948738	JQ949068	JQ949728	JQ950058
C. guajavae	IMI 350839, CPC 18893 HT	India	*Psidium guajava*, fruit	JQ948270	JQ948600	JQ948931	JQ949591	JQ949921
C. indonesiense	CBS 127551 HT	Indonesia	*Eucalyptus* sp.	JQ948288	JQ948618	JQ948949	JQ949609	JQ949939
C. johnstonii	CBS 128532 HT	New Zealand	*Solanum lycopersicum*, fruit rot	JQ948444	JQ948775	JQ949105	JQ949765	JQ950095
C. kinghornii	CBS 198.35 HT	UK	*Phormium* sp.	JQ948454	JQ948785	JQ949115	JQ949775	JQ950105
C. laticiphilum	CBS 112989 HT	India	*Hevea brasiliensis*	JQ948289	JQ948619	JQ948950	JQ949610	JQ949940
C. lauri	MFLUCC 17-0205 HT	Italy	*Laurus nobilis*	KY514347	KY514344	KY514341	KY514338	KY514350
C. limetticola	CBS 114.14 HT	USA, Florida	*Citrus aurantifolia*, young twig	JQ948193	JQ948523	JQ948854	JQ949514	JQ949844
C. lupini	CBS 109225 HT	Ukraine	*Lupinus albus*	JQ948155	JQ948485	JQ948816	JQ949476	JQ949806
C. melonis	CBS 159.84 HT	Brazil	*Cucumis melo*, peel of fruit	JQ948194	JQ948524	JQ948855	JQ949515	JQ949845
C. nymphaeae	CBS 515.78 HT	The Netherlands	*Nymphaea alba*, leaf spot	JQ948197	JQ948527	JQ948858	JQ949518	JQ949848
C. orchidophilum	CBS 632.80 HT	USA	*Ascocenda* sp.	JQ948152	JQ948482	JQ948813	JQ949473	JQ949803
C. paxtonii	IMI 165753, CPC 18868 HT	Saint Lucia	*Musa* sp.	JQ948285	JQ948615	JQ948946	JQ949606	JQ949936
C. phormii	CBS 118194 HT	Germany	*Phormium* sp.	JQ948446	JQ948777	JQ949107	JQ949767	JQ950097
C. pyricola	CBS 128531 HT	New Zealand	*Pyrus communis*, fruit rot	JQ948445	JQ948776	JQ949106	JQ949766	JQ950096

(Continued)

Table 3. (Continued).

Species	Isolate number[a]	Location	Host	GenBank accession number				
				ITS	*gapdh*	*chs-1*	*act*	*tub*
C. rhombiforme	CBS 129953 HT	Portugal	*Olea europaea*	JQ948457	JQ948788	JQ949118	JQ949778	JQ950108
C. salicis	CBS 607.94 HT	The Netherlands	*Salix* sp., leaf, spot	JQ948460	JQ948791	JQ949121	JQ949781	JQ950111
C. scovillei	CBS 126529 HT	Indonesia	*Capsicum* sp.	JQ948267	JQ948597	JQ948928	JQ949588	JQ949918
C. simmondsii	CBS 122122 HT	Australia	*Carica papaya*, fruit	JQ948276	JQ948606	JQ948937	JQ949597	JQ949927
C. sloanei	IMI 364297, CPC 18929 HT	Malaysia	*Theobroma cacao*, leaf	JQ948287	JQ948617	JQ948948	JQ949608	JQ949938
C. tamarilloi	CBS 129814 HT	Colombia	*Solanum betaceum*, fruit, anthracnose	JQ948184	JQ948514	JQ948845	JQ949505	JQ949835
C. walleri	CBS 125472 HT	Vietnam	*Coffea* sp., leaf tissue	JQ948275	JQ948605	JQ948936	JQ949596	JQ949926
C. paranaense	CBS 134729 HT	–	–	KC204992	KC205026	KC205043	KC205077	KC205060

[a] **CBS**: Culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands. **CPC**: Culture collection of Pedro Crous, housed at the Westerdijk Institute. **CRCC**: Carlucci and Raimondo Culture Collection, housed at Dept. SAFE of University of Foggia. **IMI**: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, United Kingdom. **MFLU(CC)**: Mae Fah Luang University Culture Collection.

[b] Status: status of the strains. ET: ex-epitype. NT: ex-neotype. HT: ex-Holotype.

[c] Strain numbers and newly generated sequences are indicated in bold font.

Agar plugs (diam. 0.5 cm) were taken from 7-d-old fungal cultures grown on water agar at 23±2°C, and the plugs were placed under the cane bark. Wounds were then wrapped with wet sterile cotton wool and sealed with Parafilm. The experimental control canes were inoculated with sterile agar plugs. Each experiment included 18 replicates per treatment.

The canes were examined at 240 d after inoculation, and the lengths of any visible necrotic wood lesions, after removal of the bark, were measured and subjected to mycological analyses. Ten tissue pieces from each inoculated cane were placed on MEA supplemented with streptomycin sulphate at 300 mg L^{-1}, and incubated at 23±2°C in the dark. Resulting fungal colonies were identified to fulfil the Koch's postulates, and the proportions of re-isolation (%) were calculated.

Shapiro-Wilk (W) tests were used to determine whether the data obtained followed normal distributions. Homogeneity of the variances of the dataset was assessed using Levene tests. Statistical analyses were performed using Statistica version 6 (StatSoft). Factorial ANOVA analyses were performed to define the significance of any differences in mean lesion lengths caused by the isolates of each fungal species and the different fungal species, and to detect any interactions between these factors (i.e., isolate × fungal species). One-way ANOVA analyses were performed to evaluate statistically significant differences in the mean brown wood streaking lengths caused by each fungal species inoculated. Fischer's tests were used for the comparisons of the treatment means, at $P < 0.01$.

RESULTS

Fungal isolates

The data related to grapevine trunk disease incidence, recorded during surveys carried out through 10 y in vineyards of different cultivars, in the Apulia and Molise regions, are summarized in Table 1. Isolation frequencies of the fungal taxa isolated from symptomatic grapevine samples affected by GTDs and collected during the 10 y are shown in Figure 1.

The Botryosphaeriaceae (IF = 29.3%) and *Phaeoacremonium* spp. (IF = 19.3%) were the most frequently isolated fungi. *Phaeomoniella chlamydospora* (IF = 5.0%) and *Pleurostoma richardsiae* (IF = 6.4%) were responsible for vascular and subcortical streaking discolouration. The fungal taxa considered as less-known, including *Seimatosporium vitis-vinifera*,

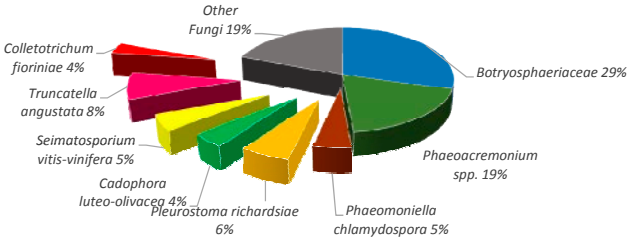

Figure 1. Isolation frequencies of fungal species obtained from symptomatic grapevines during a 10 year survey in the Apulia and Molise regions of Italy.

Truncatella angustata, *Cadophora luteo-olivacea* and *Colletotrichum fioriniae*, were isolated at IFs of 3.6% to 7.9%. The other group denoted here as 'other fungi' had IF of 19.0%, and included several fungal species, including *Alternaria* spp., *Aspergillus* spp., *Epicoccum nigrum*, *Fusarium* spp., *Penicillium* spp. and *Phoma*-like. These were not considered to be the causes of the disease symptoms observed, because these fungi are known common saprophytes.

Molecular identification of representative isolated fungi

Based on the keys, descriptions and sequence of Phillips *et al.* (2013), Mostert *et al.* (2006), Essakhi *et al.* (2008), Raimondo *et al.* (2014), Crous and Gams (2000) and Carlucci *et al.* (2015b), the 84 isolates selected as representative MSP-PCR clades were identified as follows (number of isolates): *Botryosphaeria dothidea* (four); *Diplodia corticola* (one); *D. mutila* (three); *D. seriata* (15); *Lasiodiplodia citricola* (eight); *L. theobromae* (five); *Neofusicoccum parvum* (five); *Phaeoacremonium iranium* (five); *P. italicum* (11); *P. minimum* (six); *P. scolyti* (three); *P. sicilianum* (two); *Phaeomoniella chlamydospora* (seven); and *Pleurostoma richardsiae* (nine) (data not shown).

The data obtained from the phylogenetic studies carried out on the 29 strains that were considered less well-known pathogens, and were representative of MSP-PCR clades related to the *Seimatosporium*, *Truncatella*, *Cadophora* and *Colletotrichum*, are summarized below.

The LSU, ITS, β-tub*ulin, tef-1α* and *rpb2* sequences were generated for seven *Seimatosporium* strains selected from the MSP-PCR profiles, and were aligned with 41 sequences retrieved from GenBank (Table 2). The dataset consisted of 48 taxa, which included the outgroup taxa *Synnemapestaloides juniperi* and *Discosia artocreas*. After alignment and exclusion of incomplete portions at either end, the dataset consisted of 3,344 characters (including alignment gaps), of which 2,276 were constant, while 279 were variable and parsimony uninformative. Maximum parsimony analysis of the remaining 789 parsimony-informative characters resulted in the 100 most-parsimonious trees (TL = 2,274; CI = 0.576; RI = 0.826; RC = 0.476; HI = 0.424). The maximum likelihood analysis produced a tree with similar topology (TreeBASE S25531; Figure 2). All of the *Seimatosporium* strains obtained clustered as a single clade with the type sequences of *Sei. vitis-viniferae* (CBS 123004) and *Sei. vitis* (MFLUCC 14-0051) (Figure 2). For the type strain of *Sei. vitis*, only the LSU and ITS sequences were available in GenBank, which were identical to those of *Sei. vitis-viniferae*. However, the isolates analysed here showed β-tubulin, *tef-1α* and *rpb2* sequences identical to those of *Sei. vitis-vinifera*, and therefore the morphological features (conidium dimensions and basal appendages) were used to discriminate between these two species, according to Liu *et al.* (2019).

The LSU, ITS, β-tub*ulin, tef-1α* and *rpb2* sequences were generated for 11 *Truncatella* isolates selected from the MSP-PCR profiles, which were aligned with 53 sequences retrieved from GenBank (Table 2). The dataset consisted of 64 taxa, which included two outgroup taxa, *Beltrania pseudorhombica* and *Phlogicylindrium eucalypti*. After alignment and exclusion of incomplete portions at either end, the dataset consisted of 3,983 characters (including alignment gaps), of which 1,124 were constant and 511 were variable and parsimony uninformative. Maximum parsimony analysis of the remaining 2,348 parsimony-informative characters resulted in 35 most-parsimonious trees (TL = 10,415; CI = 0.522; RI = 0.798; RC = 0.417; HI = 0.478). Maximum likelihood analysis produced a tree with similar topology (TreeBASE S25532; Figure 3). All of the *Truncatella* strains obtained in this study clustered with the *ex-neotype* sequences of *T. angustata* (*Stilbospora angustata* CBS 114025) (Figure 3).

The ITS, *tef-1α* and β-tubulin sequences generated for six *Cadophora* strains selected from the MSP-PCR profiles were aligned with 44 sequences retrieved from GenBank (Table 2). The dataset consisted of 60 taxa, which included the outgroup taxon, *Hyaloscypha finlandica*. After alignment and exclusion of incomplete portions at either end, the dataset consisted of 1,613 characters (including alignment gaps), of which 952 were constant, while 167 were variable and parsimony uninformative. Maximum parsimony analysis of the remaining 494 parsimony-informative characters resulted in 100 most-parsimonious trees (TL = 1,255; CI = 0.735; RI = 0.932; RC = 0.686; HI = 0.265). Maximum likelihood analysis produced a tree with similar topology (TreeBASE S25533; Figure 4). All of the *Cadophora* isolates obtained in this study clustered with the type sequences of *Cadophora luteo-olivacea* (CBS 141.41) (Figure 4).

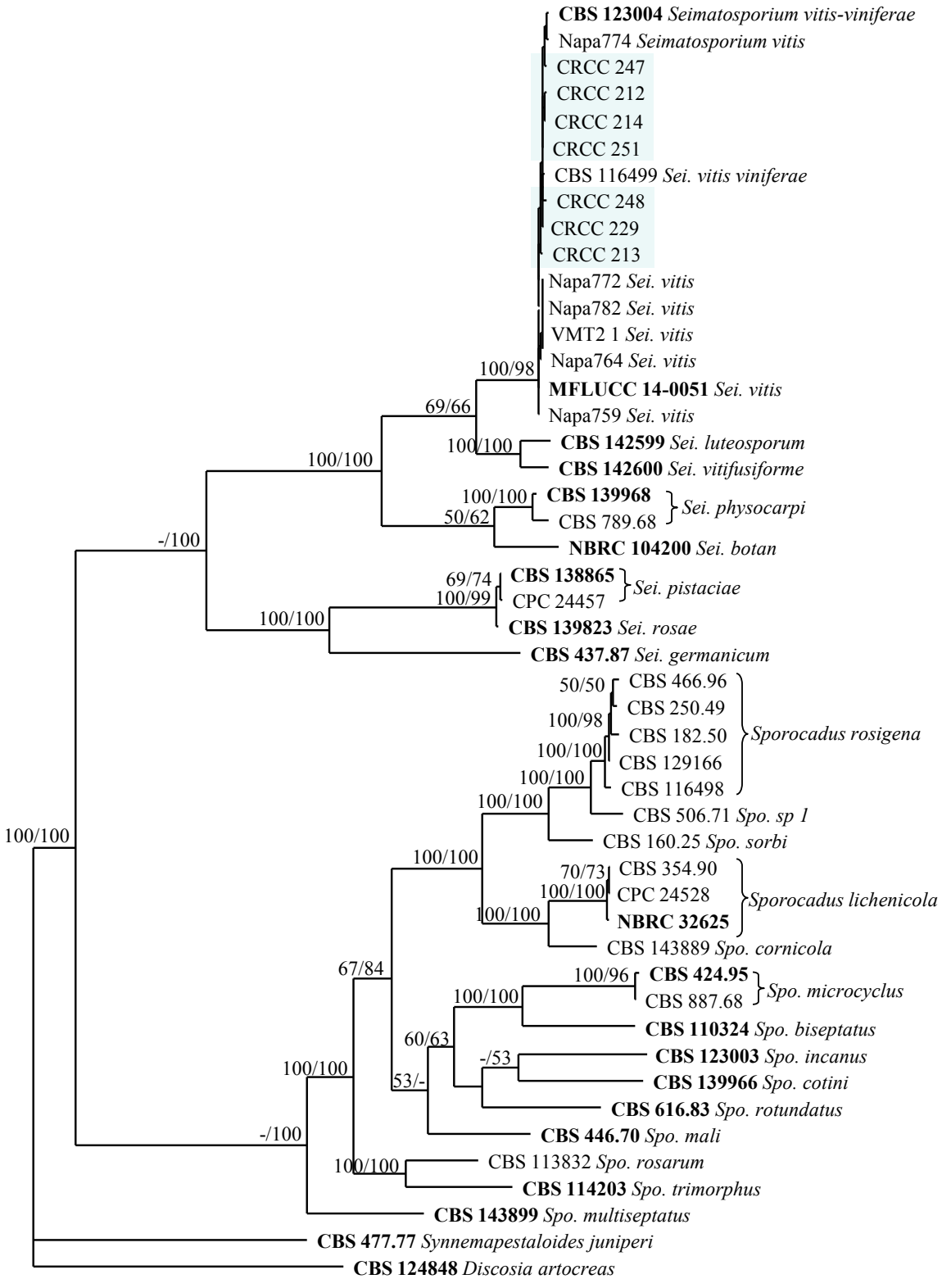

Figure 2. One of the most parsimonious trees obtained from the combined alignment of the LSU, ITS, *tub*, *tef-1*α and *rpb2* sequence datasets of *Seimatosporium* isolates, with bootstrap support values from maximum parsimony/maximum likelihood analyses. Isolates obtained in this study are indicated by blue rectangles. *Ex-type* sequences are given in bold. *Synnemapestaloides juniperi* and *Discosia artocreas* were used as outgroups.

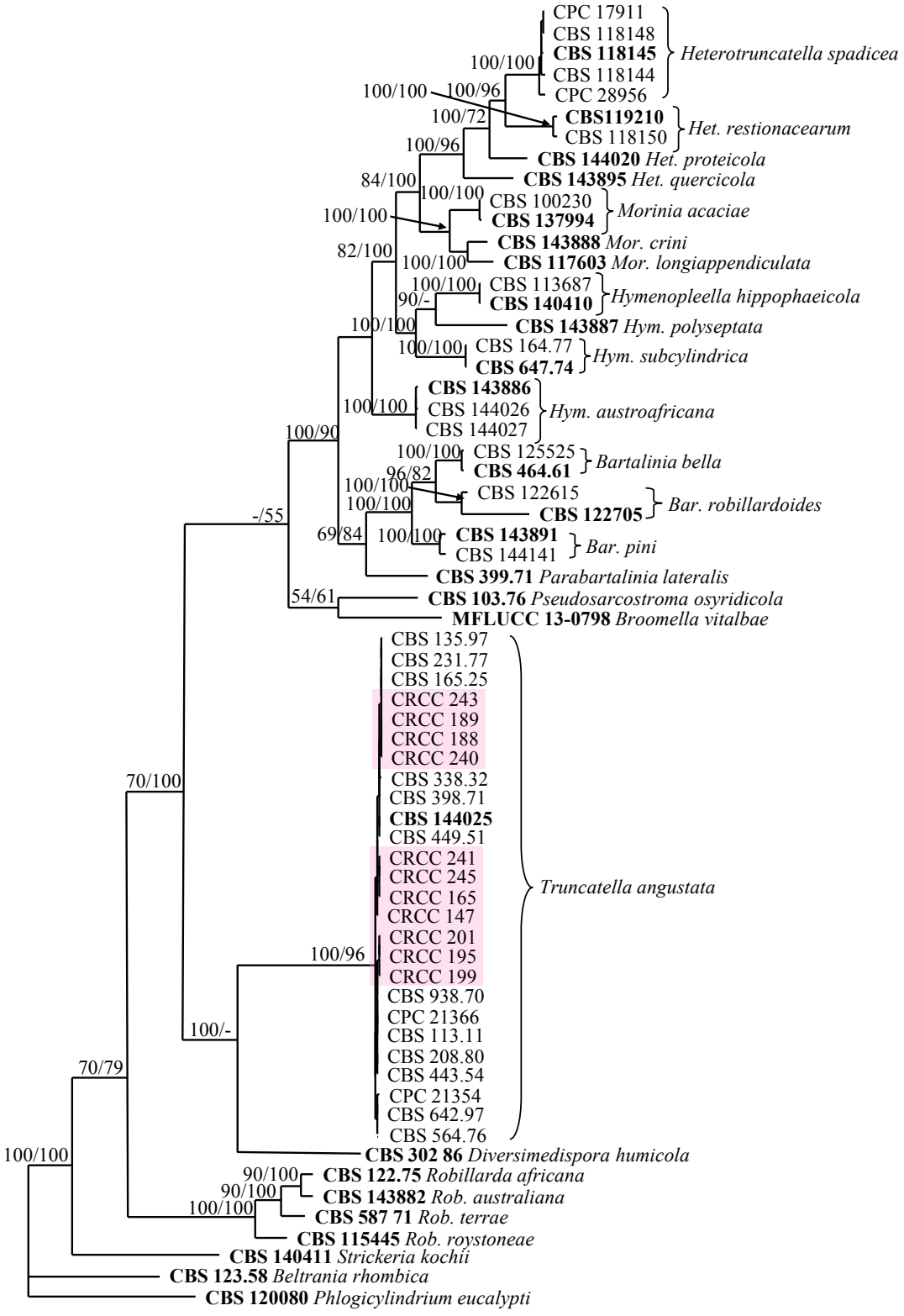

Figure 3. One of the most parsimonious trees obtained from combined alignment of the LSU, ITS, *tub*, *tef-1*α and *rpb2* sequence datasets of *Truncatella* isolates, with bootstrap support values from maximum parsimony/maximum likelihood analyses. Isolates obtained in this study are indicated by pink rectangles. *Ex-type* sequences are indicated in bold. *Beltrania pseudorhombica* and *Phlogicylindrium eucalypti* were used as outgroups.

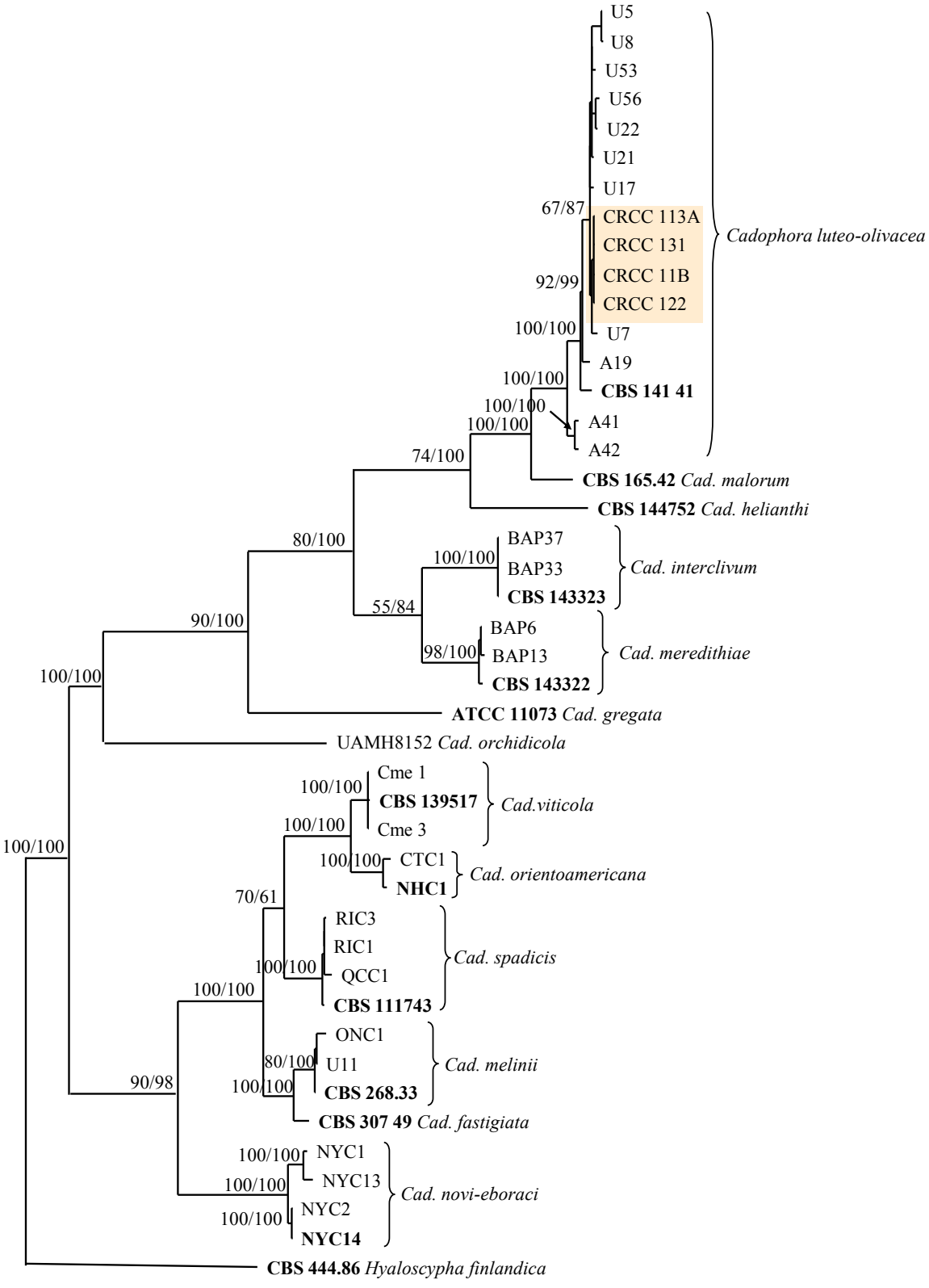

Figure 4. One of the most parsimonious trees obtained from combined ITS, *tef-1*α and *tub* sequence datasets of *Cadophora* isolates, with bootstrap support values from maximum parsimony/maximum likelihood analyses. Isolates obtained in this study are indicated by an orange rectangle. *Ex-type* sequences are indicated in bold. *Hyaloscypha finlandica* was used as outgroup.

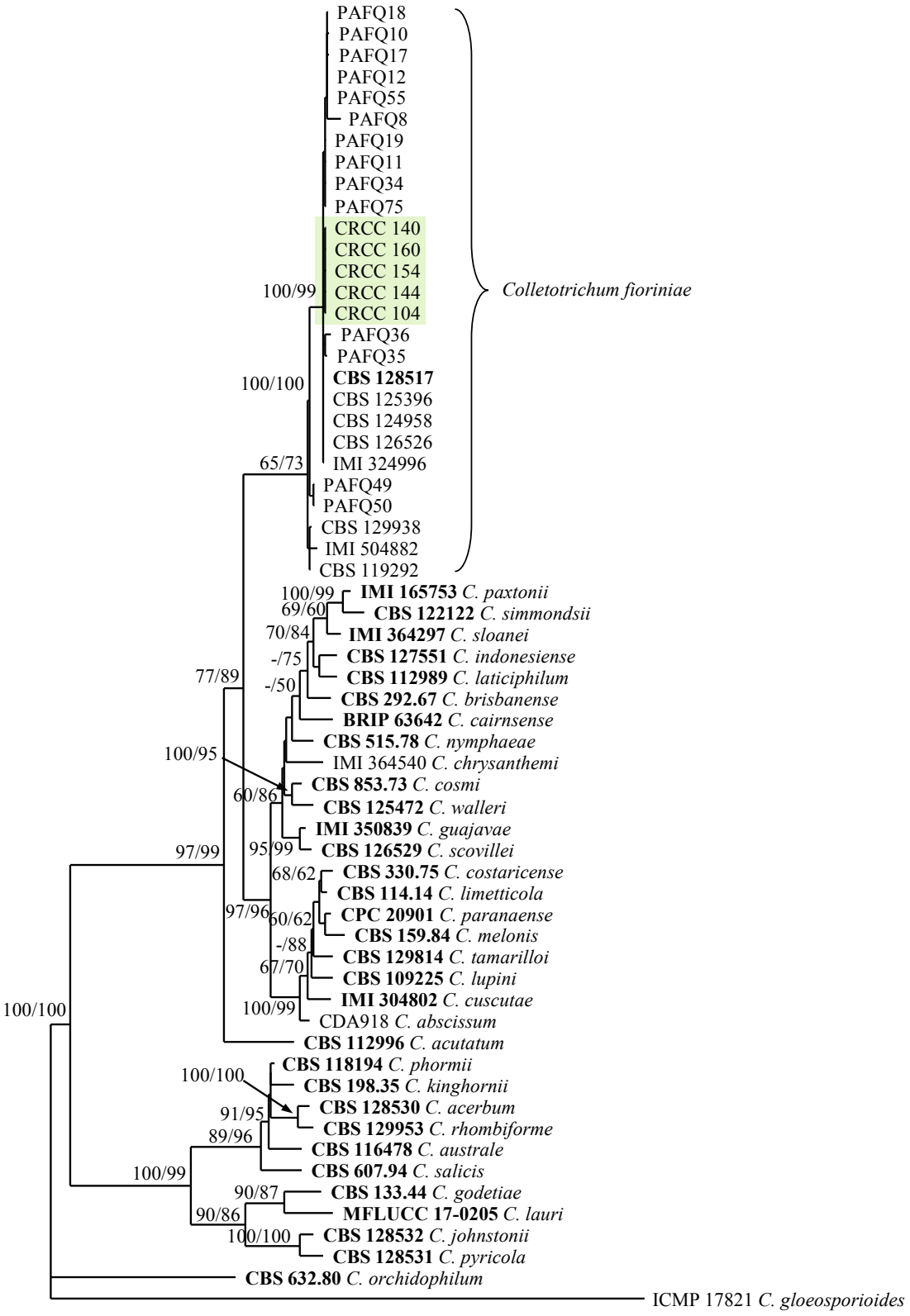

Figure 5. One of the most parsimonious trees obtained from the combined alignment of the ITS, *gapdh*, *chs-1*, *act* and *tub* sequence datasets of *Colletotrichum* isolates, with bootstrap support values from maximum parsimony/maximum likelihood analyses. Isolates obtained in this study are indicated by green rectangles. *Ex-type* sequences are indicated in bold. *Colletotrichum orchidophilum* and *C. gloeosporioides* were used as outgroups.

The ITS, β-tubulin, *act*, *gapdh* and *chs*-1 sequences generated for five *Colletotrichum* strains selected from the MSP-PCR profiles were aligned with 44 sequences retrieved from GenBank (Table 3). The dataset consisted of 61 taxa, which included the two outgroup taxa, *Colletotrichum gloeosporioides* and *C. orchidophilum*). After alignment and exclusion of incomplete portions at either end, the dataset consisted of 1,879 characters (including alignment gaps), of which 1,304 were constant, while 339 were variable and parsimony uninformative. Maximum parsimony analysis of the remaining 236 parsimony-informative characters resulted in 100 most-parsimonious trees (TL = 977; CI = 0.736; RI = 0.872; RC = 0.642; HI = 0.264). Maximum likelihood analysis produced a tree with similar topology (TreeBASE S25534; Figure 5). The *Colletotrichum* isolates obtained in this study clustered in the clade of *Colletotrichum fioriniae* with the *holotype* sequences of *C. fioriniae* (CBS 128517) (Figure 5).

Morphological characterisation of representative isolates

Colonies of the *Seimatosporium* isolates on MEA had entire edges, with brown to purplish grey mycelia, and reached mean diameter of 6.9 cm after 21 d at 23°C. The conidiomata were black and immersed. Conidia were fusoid, 3(-6)-septate, with measurements of 13.8-24.0 × 4.1-5.9 µm. They had truncated basal cells 2.3-3.8 µm long, similar to that of median cells. The median cells (2 -4) were each 3.3-5.1 µm long, and the conidium apical cells were 1.3-4.2 µm long. The majority of conidia each had a single unbranched appendage at both ends (apical appendage, 3.9-11.5 µm long; basal appendage, 3.6-10.3 µm long). On the basis of these culture and morphological features, all of the *Seimatosporium* strains had characteristics similar to those reported by Liu *et al.* (2019) for *Sei. vitis-viniferae*.

Colonies of the *Truncatella* isolates on MEA had entire edges, with white to pale grey mycelia, and reached mean diameter of 7.1 cm after 21 d at 23°C. Conidiomata were black, gregarious, semi-immersed and stromatic. Conidia were fusoid, occasionally slightly curved, mostly 3-septate, and not constricted at the septa (mean, 18.3 ± 1.69 × 6.8 ± 0.50 µm). The basal cells of the conidia had truncate bases, were hyaline to pale brown, 1.3-3.6 µm long, each with two pale to mid-brown doliiform median cells which were pale to mid-brown, each 5.3-7.7 µm long, and the apical cells were conic, hyaline, and 1.9-4.9 µm long. Each conidium had 2 to 4 apical appendages, which were centric, flexuous and branched, 0.6-22 µm long, and were without basal appendages. On the basis of these culture and morphological features, all of the *Truncatella* isolates studied had characteristics similar to those reported by Liu *et al.* (2019) for *Truncatella angustata*, which confirmed the data obtained in the molecular analysis.

Colonies of the *Cadophora* isolates on MEA had entire edges, and the mycelia were white to olivaceous green to grey. Mean colony diameter reached 4.5 cm after 21 d at 23°C. The conidiophores were mostly short, usually unbranched, up to 7-septate and measuring (-11.5) 26-63.90 × 1.78-1.94 (-2.5) µm. The conidiogenous cells were monophialidic, hyaline, terminal or lateral, mostly cylindrical, sometimes elongated ampulliform and attenuated at the base or navicular and tapering towards the apex. These cells measured 7.9-27.3 × 1.4-3.1 µm. The conidia were hyaline, mostly biguttulate, ovoid and aseptate, and measured 3.7-7.3 × 2.1-3.6 µm. On the basis of these culture and morphological features, all of the *Cadophora* strains studied had characteristics similar to those reported by Gramaje *et al.* (2011) and Travadon *et al.* (2015) for *Cadophora luteo-olivacea*, which confirmed the data obtained in the molecular analysis.

Colonies of the *Colletotrichum* isolates on MEA had entire edges, with aerial cottony pink to vinaceous mycelia. Mean colony diameter reached 4.5 cm after 21 d at 23°C. The conidiomata were sparse, with masses of orange conidia. Conidiophores were hyaline to pale brown, septate, branched, and up to 33 µm long. Conidiogenous cells were hyaline to pale brown, cylindrical to elongate ampulliform, monophialic and measured 3.8-11.9 × 2.2-3.9 µm. Conidia were elliptical, hyaline, with both ends acute, and measured 8.0-15.3 × 3.2-4.6 µm. On the basis of these culture and morphological features, all of the *Colletotrichum* isolates studied had characteristics similar to those reported by Damm *et al.* (2012) for *C. fioriniae*, which confirmed the data obtained in the molecular analysis.

Pathogenicity tests

According to Shapiro-Wilk tests, the data from the pathogenicity tests carried out on the grapevine cultivars 'Nero di Troia' and 'Bombino bianco' 240 d after, inoculations followed a normal distribution, with W values, respectively for the cultivars, of 0.96 ($P < 0.01$) and 0.97 ($P < 0.01$). The Levene tests determined for the two cultivars showed that the homogeneity of the variance was significant for 'Nero di Troia' ($F = 7.04$; $P < 0.01$) and 'Bombino bianco' ($F = 4.93$; $P < 0.01$). Factorial ANOVA demonstrated that significant differences in pathogenicity were detected among the fungal species inoculated on both 'Nero di Troia' ($F = 44.5$; $P < 0.01$) and 'Bombino bianco' ($F = 83.40$; $P < 0.01$). There were no significant

Table 4. Mean lesion lengths from the pathogenicity assays carried out for isolates of seven fungal species on two grapevine cultivars (one-way ANOVA).

Cultivar	Fungal species	Length of brown wood discolouration (cm)			Re-isolation (%)
		Mean	SD	Min–Max[a]	
'Nero di Troia'	Control	0.63 A	0.24	0.30–1.10	0.00
	Colletotrichum fiorinae	8.83 B	2.57	5.00–16.00	73.33
	Cadophora luteo-olivacaea	12.97 C	5.01	11.00–34.00	88.33
	Seimatosporium vitis-vinifera	16.98 D	3.13	13.00–24.70	80.00
	Truncatella angustata	18.01 D	7.31	3.40–29.50	91.67
	Pleurostoma richardsiae	18.53 DE	3.87	6.00–19.90	76.67
	Phaeoacremonium italicum	19.25 DE	3.41	13.60–27.00	93.33
	Lasiodiplodia citricola	21.77 E	4.59	12.00–27.00	86.67
'Bombino bianco'	Control	0.62 A	0.29	0.30–1.10	0.00
	Colletotrichum fiorinae	8.43 B	2.34	4.40–12.40	78.33
	Cadophora luteo-olivacaea	14.13 C	7.08	3.30–38.20	91.67
	Truncatella angustata	15.71 CD	3.20	9.50–19.50	80.00
	Phaeoacremonium italicum	18.41 DE	4.47	14.00–29.60	86.67
	Pleurostoma richardsiae	20.11 E	5.47	9.20–30.50	78.33
	Seimatosporium vitis-vinifera	23.63 F	3.23	13.00–23.00	91.67
	Lasiodiplodia citricola	29.20 G	2.00	24.40–32.00	95.00

[a] Minimum and maximum values detected (18 observations).
Data within each cultivar followed by different capital letters within the column are significantly different ($P < 0.01$; Fischer's tests).

differences in aggressiveness among the isolates of each fungal species used in the artificial inoculations of 'Nero di Troia' (F = 0.12; $P = 0.89$) or 'Bombino bianco' (F = 0.99; $P = 0.37$).

The mean lengths of vascular discolouration caused by the inoculated, fungal species used in the pathogenicity tests, and examined for one-way analysis of variance, are reported in the Table 4. All of the fungi produced brown wood discolourations on canes of both grapevine cultivars. The most aggressive species was *Lasiodiplodia citricola* towards 'Nero di Troia' and 'Bombino bianco', which produced the longest brown wood discolourations (respective mean lengths = 21.77 and 29.20 cm). *Phaeoacremonium italicum* and *Pleurostoma richardsiae* were pathogenic for both grapevine cultivars, which confirmed their aggressiveness reported by Carlucci *et al.* (2015a) and Raimondo *et al.* (2014). These fungi produced discolourations with mean lengths from 18.41 to 20.11 cm. Among the reference grapevine pathogens used, *Cadophora luteo-olivacea* was less pathogenic than *P. italicum* and *L. citricola*, as it produced mean discolouration lengths of 14.13 and 12.97 cm, respectively, on 'Nero di Troia' and 'Bombino bianco'. *Seimatosporium vitis-vinifera* and *Truncatella angustata* produced variable significant discolouration lengths on both grapevine cultivars, similar to those produced by *Cad. luteo-olivacea*. *Sei. vitis-vinifera* was less aggressive on 'Nero di Troia' (mean discolouration length = 16.98 cm) than on 'Bombino bianco' (mean length = 23.63 cm). *Truncatella angustata* produced different and variable discolouration lengths on 'Nero di Troia' and 'Bombino bianco' of 18.01 and 15.71 cm, respectively. *Colletotrichum fioriniae* was less aggressive, as it produced the least mean discolouration lengths on 'Nero di Troia' and 'Bombino bianco', which were, respectively, 8.83 and 8.43 cm. All of these fungi were re-isolated from the inoculated grapevines, which fulfilled Koch postulates (Table 4).

DISCUSSION

The data obtained in the present study show that vineyards in southern Italy were affected by different fungal species, some of which are known to be responsible for GTDs, such as Esca and Petri disease, and Botryosphaeria dieback. During the survey carried out on symptomatic vineyards over a 10 year period, different fungal species were among the samples collected, including *Botryosphaeria* spp., *Phaeoacremonium* spp. *Phaeomoniella chlamydospora* and *Pleurostoma richardsiae* as the most frequently isolated, and less frequently isolated taxa included *Seimatosporium*, *Truncatella*, *Cadophora* and *Colletotrichum*. The fungi of the first group are spread in most world grape-growing regions, and their

pathogenicities and involvement in diseases associated with grapevines are known (Raimondo *et al*., 2014; Carlucci *et al*., 2015a; 2015b).

The molecular analysis used in the present study allowed identification of the second group of fungi as *Seimatosporium vitis-viniferae*, *Truncatella angustata*, *Cadophora luteo-olivacea* and *Colletotrichum fioriniae*. The morphological characterisation confirmed the molecular data, and helped in the identification of isolates of *Sei. vitis-viniferae*, for which molecular identification was not discriminatory.

To date, many studies have reported the isolation of "pestalotioides fungi", such as *Seimatosporium* species, from symptomatic grapevines or from dead stems in different countries, initially including Australia (Shivas**,** 1989), England and France (Sutton**,** 1980), England and Germany (Nag Raj, 1993) and Pakistan (Ahmad, 1969; Ahmad *et al*., 1997). More recent reports also include Chile, Hungary, Iran, Italy, Spain and the USA (Castillo-Pando *et al*., 2001; Sergeeva *et al.,* 2005; Dìaz *et al.,* 2012; Senanayake *et al.,* 2015; Mehrabi *et al*., 2017; Vàczy, 2017; Lawrence *et al.,* 2018, Camele and Mang, 2019, Liu *et al.,* 2019). However, little information has been provided about their involvement in specific grapevine diseases.

Nine *Seimatosporium* species have been associated with grapevines, including *Sei. botan*, *Sei. hysterioides*, *Sei. lonicerae*, *Sei. luteosporum*, *Sei. macrospermum*, *Sei. parasiticum*, *Sei. vitifusiforme*, *Sei. vitis* and *Sei. vitis-viniferae* (Farr and Rossman, 2018; Liu *et al.,* 2019). However, only four of these have been assessed in standard pathogenicity trials on trunks and canes of vineyard grapevines, to confirm their pathogenicity roles and involvement in GTDs. *Seimatosporium botan* was isolated from symptomatic grapevines in Chile and was reported to be pathogenic on woody canes and trunks of potted grapevines (Dìaz *et al.,* 2012). *Seimatosporium vitis* strains were isolated from symptomatic grapevines in Hungary (Vàczy, 2017), North Carolina, USA (Lawrence *et al*., 2018) and Italy (Camele and Mang, 2019), and were demonstrated to be pathogenic on green shoots and woody stems of potted grapevines. *Seimatosporium luteosporum* and *Sei. vitifusiforme* were reported as pathogens on woody stems of grapevines in North Carolina, USA (Lawrence *et al*. 2018).

In the present study, the pathogenicity of *Sei. vitis-viniferae* was tested for the first time, which increased the number of *Seimatosporium* species that have been confirmed to be associated with GTDs to five. Based on molecular and morphological studies on the pestalotioides fungi reported by Liu *et al*. (2019), the identification of *Sei. vitis* in some studies appears to have been incorrect. The multilocus analyses performed with LSU, ITS, *tef-1α*, β-tubulin and *rpb2* sequences in the present study demonstrated that the strains of *Sei. vitis* reported by Lawrence *et al*. (2018) and Camele and Mang (2019) all clustered in the clade of *Sei. vitis-viniferae*. The morphological description provided by Lawrence *et al*. (2018) for *Sei. vitis* strains, including conidium dimensions and the presence of appendages at both ends of conidia does not agree with the description of *Sei. vitis* by Senanayake *et al.* (2015), although it does agree with that of Liu *et al*. (2019) for *Sei. vitis-viniferae*. Although the *tef-1α* and β-tubulin sequences of *Sei. vitis* reported by Camele and Mang (2019) were identical to those of *ex-type Sei. vitis-viniferae* described by Liu *et al*. (2019), no detailed morphological information was reported. Therefore, to the best of our knowledge, the present study provides the first report of *Sei. vitis-viniferae* associated with GTD symptoms in Italy.

The genus *Truncatella* is closely related to *Seimatosporium*, which belongs to the pestalotioides fungi, and it has wide distribution and occurs in many hosts, including grapevines (Sutton, 1980). Few reports are available about the association of *Truncatella* with grapevine, and its involvement in GTDs. Nag Raj (1993) reported *T. angustata* and *T. pitospora* (now *Pestalotia pitospora*) on grapevine, but did not include any information on their pathogenicity. Some years later, Casieri *et al*. (2009), in Switzerland, and Gonzalez and Tello (2011), in Spain, reported *T. angustata* as endophytes that were collected from different grapevine cultivars. Urbez-Torrez *et al*. (2009) also isolated *T. angustata* from cankers on grapevines in Texas, and performed pathogenicity tests to demonstrate that this fungus can be a weak and/or opportunistic pathogen on lignified grapevine canes. The pathogenicity of *T. angustata* and its involvement in GTD symptoms were also confirmed by Arzanlou *et al*. (2013) in Iran. Maharachchikumbura *et al*. (2016) and Pintos *et al*. (2018) reported *T. angustata* associated with GTD symptoms on grapevines in France, but no pathogenicity trials were performed. Based on a recent taxonomic revision of the genus *Truncatella* by Liu *et al*. (2019), there is now just one accepted species, as *T. angustata*, while other *Truncatella* species were transferred to different genera, including *Bartalinia*, *Heterotruncatella* and *Morinia*, due to the polyphyletic nature of this genus or to synonymy with *T. angustata*. The pathogenicity tests performed in the present study confirmed the pathogenic behaviour of *T. angustata* and its involvement in GTDs (Arzanlou *et al*. 2013). This is the first report of *T. angustata* associated with GTD symptoms on grapevines in Italy.

To date, seven *Cadophora* species have been reported from grapevines, including *Cad. fastigiata*, *Cad. luteo-*

olivacea, *Cad. melinii*, *Cad. novi-eboraci*, *Cad. orientoamericana*, *Cad. spadicis* and *Cad. viticola* (Overton *et al*., 2005; Halleen *et al.* 2007; Crous *et al*., 2015; Travadon *et al.,* 2015). Halleen *et al.* (2007) reported *Cad. luteo-olivacea* from grapevines showing decline symptoms, and from apparently healthy plants in commercial nurseries in South Africa. Pathogenicity tests demonstrated that *Cad. luteo-olivacea* caused significant lesions on the trunks and pruned wood of 15-year-old grapevines.

Casieri *et al.* (2009), in Switzerland, and Fischer *et al*. (2016), in Germany, reported *Cad. fastigiata* and *Cad. luteo-olivacea* as fungal species that can cause grapevine diseases. Gramaje *et al.* (2011) reported *Cad. luteo-olivacea* and *Cad. melinii* from nursery grapevines, although pathogenicity tests demonstrated that only *Cad. luteo-olivacea* caused grapevine disease on 1-year-old grapevine cutting rootstock. Travadon *et al.* (2015) confirmed the involvement of *Cad. luteo-olivacea* in GTDs, and associated four other *Cadophora* species with wood decay of grapevines in North America (*Cad. melinii*, and three new species, *Cad. orientoamericana*, *Cad. novi-eboraci* and *Cad. spadicis*). In 2015, Crous *et al.* (2015) described a new species of *Cad. viticola* (previously identified as *Cad. melinii* by Gramaje *et al*., 2011), which was isolated from grapevine shoots that showed black streaks. *Cad. luteo-olivacea* is the most frequently isolated *Cadophora* species associated with GTD symptoms in different countries, including the USA, France, Germany, New Zealand, South Africa, Spain, Switzerland and Uruguay (Casieri *et al.,* 2009; Manning and Munday, 2009, Gramaje *et al.,* 2011; Travadon *et al*., 2015; Fischer *et al*., 2016; Pintos *et al*., 2018). Isolation of *Cad. luteo-olivacea* in the present study confirms the wide distribution of this species, while the pathogenicity tests performed here confirm the pathogenic behaviour of *Cad. luteo olivacea* and its involvement in GTDs. This is the first report of *Cadophora luteo-olivacea* associated with GTD symptoms in Italy.

Colletotrichum fioriniae was also less frequently isolated that other fungi, and this species is in the *C. acutatum* species complex. The role of *Colletrotrichum* on grapevines is not clear; there have been few reports of species of the *C. acutatum* complex that have described their behaviour on grapevines. *Colletotrichum fioriniae* (Kepner and Swett 2018) and *C. godetiae* (Zapparata *et al.,* 2017) have been associated with grape berry rot, respectively, in the USA and Italy. *Colletotrichum godetiae* has also been reported as a leaf anthracnose agent in the United Kingdom (Baroncelli *et al.,* 2014), and as a saprophyte in China, Italy, Russia and Thailand (Jayawardena *et al*., 2018). In 2016, Liu *et al.* (2016) reported the first association of species in the *C. acutatum* complex with the wood of grapevines when they described *C. nymphaeae* from twig anthracnose in China.

To date, there has only been one report of *Colletotrichum* spp. associated with GTDs, from a grapevine nursery in France (Pintos *et al*., 2018), although no specific identification was carried out. In the present study, as *C. fioriniae* produced wood discolouration on both of the grapevine cultivars included, and although this was less severe (shorter discolouration) than for the other fungi inoculated. This fungus can now be considered as a weak pathogen on grapevine wood. This is, therefore, the first report of *C. fioriniae* associated with GTD symptoms.

The study reported in the present paper has demonstrated the presence of *Cadophora luteo-olivacea*, and *Truncatella angustata*, as well as their virulence, also on grapevine in Italy. *Seimatosporium vitis-vinifera*, isolated from grapevine for first time in Italy, when artificially inoculated, was the most aggressive fungus among the less-common fungi assayed here, indicating its involvement in GTDs. *Colletotrichum fioriniae*, although less aggressive among the fungi assayed, was also shown to be another fungus involved in GTDs. These results add to knowledge on the expanding group of fungi involved in the GTD complex.

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Short Notes

Complete genome sequence of *Alfalfa mosaic virus*, infecting *Mentha haplocalyx* in China

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Summary. *Mentha haplocalyx* (*Lamiaceae*) is a herbaceous perennial economic plant which is widely cultivated in China. Plants of *M. haplocalyx* with mosaic symptoms were collected from Zhaotong, Yunnan Province. *Alfalfa mosaic virus* (AMV) was detected from symptomatic leaf samples using small RNA sequencing and RT-PCR. The complete genome sequence of AMV-Mint was determined. AMV-Mint RNA1 was 3,644 nt, RNA2 was 2,594 nt and RNA3 was 2,040 nt, encoding P1 of 1,126 amino acids (aa), P2 of 794 aa, MP of 300 aa and CP of 218 aa. The genome structure of AMV-Mint was similar to those reported previously, except for an insertion of one adenine nucleotide at 2,389 nt of the RNA2, which results in reading frame shift mutation of the P2. Phylogenetic analysis based on RNA3 sequences grouped AMV-Mint into the Group I clade, with closest relationship to AMV-Lst. No recombination event was detected in the genome of AMV-Mint. This is the first report of the complete genome sequence of AMV from *M. haplocalyx*.

Keywords. AMV, mint, small RNA sequencing, insertion mutation, phylogenetic analysis.

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INTRODUCTION

Mentha haplocalyx (*Lamiaceae*), commonly known as mint, is a widely cultivated herbaceous perennial plant (Dorman *et al.*, 2003; She *et al.*, 2010). It is an important vegetable crop, and a traditional Chinese medicinal plant with functions including spasmolytic, analgesic, antibacterial and promotion of gas secretion (Dorman *et al.*, 2003; She *et al.*, 2010). In addition, it is one of the most important sources of essentials, which can be used in food, pharmaceutical, flavour and fragrance industries (Zheljazkov *et al.*, 2013). Because cultivation of mint depends mainly on vegetative propagation, infections by viruses are serious threat to mint production. Several viruses have been

reported on cultivated *Mentha* species, including *Tomato spotted wilt virus* (Sether *et al.*, 1991), *Tobacco mosaic virus* (Samad *et al.*, 2000), *Strawberry latent ringspot virus* (Postman *et al.*, 2004) and *Tomato leaf curl Pakistan virus* (Samad *et al.*, 2009).

Alfalfa mosaic virus (AMV) belongs to *Alfamovirus* in the *Bromoviridae*, having a tripartite positive single-stranded RNA genome. The RNA1 encodes the replicase protein P1 and RNA2 the protein P2 (Bergua *et al.*, 2014). The RNA3 encodes the movement protein (MP) in 5'-proximal half and the coat protein (CP) in 3'-proximal half (Bergua *et al.*, 2014). Based on phylogenetic analyses of the CP gene or RNA3 genome sequences, AMV isolates have been clustered into two groups (Group I and Group II) (Parrella *et al.*, 2011; Song *et al.*, 2019). AMV occurs widely with a broad host range, infecting more than 430 plant species, including many important crops such as potato, tomato, pepper and soybean (Fleysh *et al.*, 2001; Abdalla *et al.*, 2015).

Mentha haplocalyx plants exhibiting obvious virus-like symptoms were recently found in Zhaotong, in the Yunnan Province of China. This paper describes identification of AMV associated with this disease. Furthermore, the complete genome sequence of the AMV *M. haplocalyx* isolate (AMV-Mint) was determined and analyzed.

MATERIALS AND METHODS

Sample collection and RNA extraction

In August 2018, *M. haplocalyx* plants with disease symptoms of mosaic (Figure 1) were collected in Zhaotong, Yunnan Province of China. All of the samples were immediately frozen using liquid nitrogen and stored at -80'. Total RNA was extracted from symptomatic leaf tissues each of approx. around 200 mg, using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The quality of the extracted RNA was estimated using Bio-Analyzer 2100 (Agilent Technologies) and quantity of RNA was determined using NanoDrop ND-100 (NanoDrop Technologies).

Sequencing of small RNA, and data analysis

The qualified RAN was sent to Biomarker Technologies (Beijing, China) for the construction of a library of small RNAs, as described by Mi *et al.* (2008), followed by sequencing using an IIIumina HiSeq200 platform. The raw data obtained from original image data by base-calling were firstly processed by trimming adapter sequences and removing reads shorter than 18 nt or longer than 35 nt. The obtained clean reads were aligned to the databases Silva (Pruesse *et al.*, 2007), GtRNAdb (Chan *et al.*, 2009), Rfam (Griffiths-Jones *et al.*, 2003) and Repbase (Jurka *et al.*, 2005), using Bowtie software (Langmead *et al.*, 2009) to eliminate non-coding RNA and repeated sequences. The remaining clean reads were assembled using the Velvet program with a minimal overlapping length (*k*-mer) of 17 (Wu *et al.*, 2010). To identify potential viral sequences, the obtained contigs were compared against the nucleotide (nt) sequence databases in NCBI using BLASTn, and nonredundant protein (nr) using BLASTx, with e-value of 10^{-5} (Wu *et al.*, 2010).

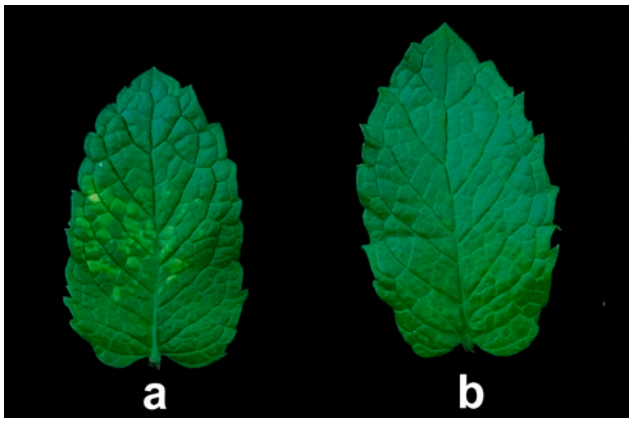

Figure 1. *Mentha haplocalyx* leaf with mosaic symptoms (a), and symptomless leaf (b).

Validation of candidate viruses

In order to confirm the presence of viruses identified by small RNA sequencing, primers were designed based on the obtained virus contigs (Table S1). Single-stranded cDNA was synthesized from total RNA using random hexamer primers. PCR was carried out in 25 µL reaction mixtures containing 12.5 µL Premix *LA Taq* DNA polymerase (TaKaRa), 1.0 µL 10 µM primers and 1.0 µL cDNA. The PCR products were examined by 1% agarose gel electrophoresis.

Determination of full-length genome and sequence analysis

To determine the full-length genome sequence of the AMV *M. haplocalyx* isolate (AMV-Mint), four primers (Table S1) were designed based on the sequences of contigs mapped to AMV genome, and were used in RT-PCR assays. The 5' and 3' terminal sequences of genomic

RNA were obtained using the SMARTer™ RACE cDNA Amplification Kit (Clontech) with the primers described previously (Song *et al.*, 2019). The amplicons were cloned into the pMD18-T simple vector (TaKaRa) and sequenced by Sangon Biotech (Shanghai) Co., Ltd. Three independent clones were sequenced for each amplicon.

The full-length genome sequences of RNA1-3 were assembled using Vector NTI (Invitrogen) based on overlapping fragments, and were used for BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All the genome sequences of AMV available in GenBank database (Table 1) were downloaded and analyzed. The SDT software (Muhire *et al.*, 2014) was used to determine pairwise nucleotide and amino acid sequence similarities.

Phylogenetic and recombination analyses

Multiple sequence alignments were performed by Clustal W algorithm of MEGA 5.0 software (Tamura *et al.*, 2011) with default settings. Phylogenetic neighbour-joining (NJ) and maximum likelihood (ML) trees were constructed based on RNA3 sequences with 1,000 bootstrap replicates, and Tamura three-parameter was used as the best-fitting model of substitution determined by MEGA 5.0.

Recombination analysis was carried out using seven different algorithms of the Recombination Detection Program v.3.44 (RDP3) (Heath *et al.*, 2006) including RDP, GENECONV, CHIMERA, BOOTSCAN, MAXCHI, SISCAN and 3SEQ. Only recombination events predicted by at least five algorithms with P value <0.01 were accepted.

RESULTS AND DISCUSSION

Analysis of small RNA sequencing data

From small RNA sequencing, a total of 22,918,853 good quality clean reads of 18-35 nt were obtained from 28,510,612 raw reads. RNAs of lengths 21-24 nt were the most abundant. After removing the non-coding RNA and repeated sequences, a total of 9,096,901 clean reads were used for further analyses. Using the Velvet program, we assembled 2,314 contigs from these clean reads with contigs N50 of 63. More than half of these contigs were 30 to 60 nt in length. BLAST analysis against the NCBI database identified 112 contigs mapped to viral genomes, including 46 contigs mapped to AMV, 39 mapped to *Watermelon mosaic virus* (WMV), 19 to *Blackcurrant reversion virus* (BRV), four to *Soybean mosaic virus* (SMV), two to *Cherry leaf roll virus* (CLRV), and two mapped to *Grapevine bulgarian latent virus* (GBLV).

Validation of candidate viruses

To validate the presence of the above-mentioned candidate viruses, a total of eight primer pairs were designed based on the candidate viral contigs (Table S1). However, only AMV was detected by RT-PCR using the primer pair AMV-F1/R1. None of WMV, BRV, SMV, CLRV or GBLV was RT-PCR positive, even when three primer pairs were used for WMV. This strongly indicated that AMV infection was associated with the disease of *M. haplocalyx*.

Next-generation sequencing (NGS) of small RNAs in plant tissues has been widely used for identification of plant viruses, because these virus-derived small RNAs generated by plant RNA-silencing machinery can be sequenced by NGS, assembled *in silico* and used for searching in databases (Seguin *et al.*, 2014; *Liang et al.*, 2015). However, the results in the present study and in previous reports showed that false positive results are common using this method, and further confirmation by PCR or other methods is necessary (Song *et al.*, 2019).

Determination and characterization of the genome sequence of AMV-Mint

The nearly full-length and terminal fragments of the genomic RNAs of AMV-Mint were amplified, respectively, using RT-PCR and RACE technology. The complete genomic sequences were assembled and submitted to the GenBank under accession numbers MK883819, MK88320 and MK883821. The RNA1 of AMV-Mint was 3,644 nt, RNA2 was 2,594 nt, and RNA3 was 2,040 nt, having a similar genome organization to those reported previously (Trucco *et al.*, 2014; Song *et al.*, 2019). The RNA1 encodes P1 of 1,126 amino acids (aa) from 100 to 3,480 nt. The RNA2 encodes P2 of 794 aa from 55 to 2,439 nt. The RNA3 encodes MP of 300 aa from 241 to 1,143 nt, and CP of 218 aa from 1,193 to 1849 nt.

Compared to the other AMV genome sequences available in GenBank, AMV-Mint shared nucleotide sequence similarities of 96.0-98.0% for RNA1, 94.9-97.9% for RNA2, and 94.7-98.5% for RNA3 (Table 1). P1, P2, MP and CP of AMV-Mint shared nucleotide sequence similarities of 96.3-97.9%, 94.9-98.1%, 93.4-98.4% and 94.2-98.3%, respectively, compared with other isolates. Amino acid sequence similarities were 98.1-99.6% for P1, 91.9-97.2% for P2, 93.3-97.7% for MP and 94.5-99.1% for CP (Table 1). An insertion of one adenine nucleotide was

Table 1. Nucleotide and amino acid sequence similarities between AMV-Mint and other AMV isolates.

GenBank No.	Isolate	Host	Country	Length (nt)	Nucleotide/Amino acid similarities (%)		
RNA1					Genome	P1	
KC881008	Manfredi	*Medicago sativa*	Argentina	3,643	98.0	97.9 / 99.4	
MH332897	Gyn	*Gynostemma pentaphyllum*	China	3,643	97.9	97.8 / 99.5	
HQ316635	HZ	*Nicotiana glutinosa*	China	3,643	97.8	97.7 / 99.0	
MF990284	175	*Solanum tuberosum*	Canada	3,631	97.6	97.6 / 99.6	
FN667965	Lst	*Lavandula stoechas*	Italy	3,543	97.2	97.2 / 98.2	
L00163	425 Leiden	-	-	3,644	97.2	97.3 / 98.5	
FR715040	Tec1	*Tecomaria capensis*	Spain	3,643	96.5	96.3 / 98.2	
KY810767	FERA160224	*Nicotiana tabacum*	England	3,643	96.0	96.3 / 98.1	
RNA2					Genome	P2	
FN667966	Lst	*Lavandula stoechas*	Italy	2,593	97.9	98.1 / 97.2	
KY810768	FERA160224	*Nicotiana tabacum*	England	2,593	97.7	97.9 / 96.5	
KC881009	Manfredi	*Medicago sativa*	Argentina	2,593	97.7	97.9 / 96.7	
MH332898	Gyn	*Gynostemma pentaphyllum*	China	2,598	97.0	97.1 / 96.1	
FR715041	Tec1	*Tecomaria capensis*	Spain	2,594	96.5	96.5 / 95.8	
X01572	A1M4	-	-	2,593	95.4	95.5 / 91.9	
HQ316636	HZ	*Nicotiana glutinosa*	China	2,595	94.9	94.9 / 92.9	
RNA3					Genome	MP	CP
FN667967	Lst	*Lavandula stoechas*	Italy	2,038	98.5	98.4 / 97.3	98.3 / 98.2
KC881010	Manfredi	*Medicago sativa*	Argentina	2,038	98.0	97.6 / 97.3	98.0 / 97.7
M59241	-	alfalfa	America	2,188	98.0	98.1 / 97.3	98.0 / 97.7
HQ316637	HZ	*Nicotiana glutinosa*	China	2,041	97.8	97.1 / 97.0	98.2 / 98.2
X00819	S	-	-	2,055	97.6	97.6 / 96.3	97.4 / 97.7
K02703	425 Madison	-	-	2,037	97.5	97.3 / 97.7	97.3 / 97.2
K03542	-	clover	America	2,142	97.5	96.9 / 96.0	97.6 / 96.8
MH332899	Gyn	*Gynostemma pentaphyllum*	China	2,040	97.5	97.1 / 97.3	97.1 / 97.7
MF990286	175	*Solanum tuberosum*	Canada	2,041	97.5	97.2 / 97.0	97.0 / 97.7
AF332998	N20	-	Australia	2,257	97.3	96.7 / 96.0	97.6 / 96.3
AB126031	AZ	-	-	2,037	97.1	96.3 / 96.0	97.9 / 99.1
AB126032	Kr	-	-	2,037	96.8	96.9 / 96.0	96.2 / 96.3
FR715042	Tec1	*Tecomaria capensis*	Spain	2,037	95.5	94.2 / 95.3	94.4 / 94.5
KC767662	178	*Actinidia fortunatii*	New Zealand	1,986	95.3	94.6 / 94.3	95.0 / 95.0
KC767661	176	*Actinidia glaucophylla*	New Zealand	1,976	95.2	94.7 / 93.3	96.0 / 95.0
KY810769	FERA160224	*Nicotiana tabacum*	England	2,039	95.1	94.0 / 95.3	94.5 / 94.5
AF015716	VRU	garden lupin	England	2,038	95.1	93.4 / 95.7	95.1 / 95.0
KC767660	175	*Actinidia guilinensis*	New Zealand	1,977	94.9	93.9 / 94.3	95.0 / 94.5
AF015717	15/64	garden lupin	England	2,038	94.7	93.4 / 94.0	94.2 / 94.5

found at 2,389 nt of the AMV-Mint RNA2, resulting in a reading frame shift mutation in the P2 protein from 779 aa to the C-terminal (Figure 2). The AMV-Gyn isolate reported previously (Song *et al.*, 2019) had an insertion of three adenine nucleotides at the same position, resulting in an extra lysine at 779 aa of AMV-Gyn P2 protein. This indicated that the 12 amino acids in the C-terminal region of AMV P2 may be unnecessary for successful AMV infection of host plants.

Phylogenetic and recombination analyses

The RNA3 sequences of AMV-Mint and the other 20 isolates available in GenBank were used to construct the NJ (Figure 3) and ML (Figure S1) phylogenetic trees, and similar topological structures were observed. All of the 20 AMV isolates clustered into two groups. AMV-Mint was located in the Group I, together with 12 of the other available isolates. The closest evolutionary relation-

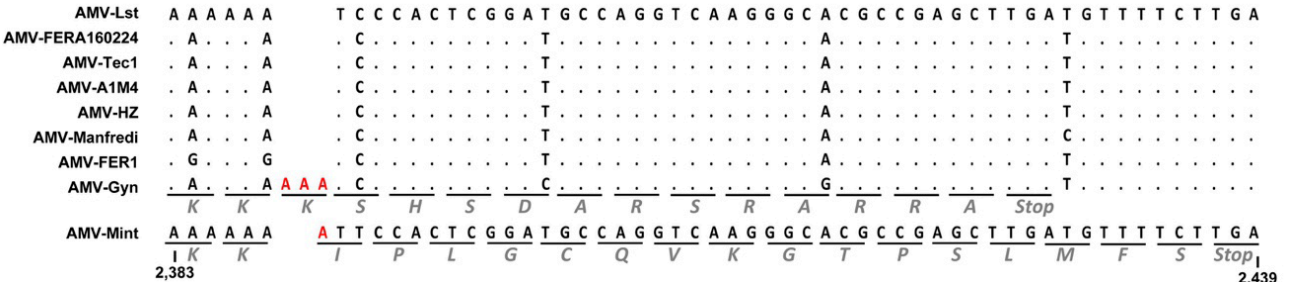

Figure 2. Alignments of partial RNA3 sequences (2,383-2,439 nt for AMV-Mint) showing an insertion of one adenine nucleotide at 2,389 nt of AMV-Mint RNA2, resulting in a reading frame shift mutation in the C-terminal of the AMV-Mint P2 protein. The inserted nucleotides in RNA2 of AMV-Mint and AMV-Gyn are in red. The codons are underlined, and the corresponding amino acids are indicated in italics below the lines.

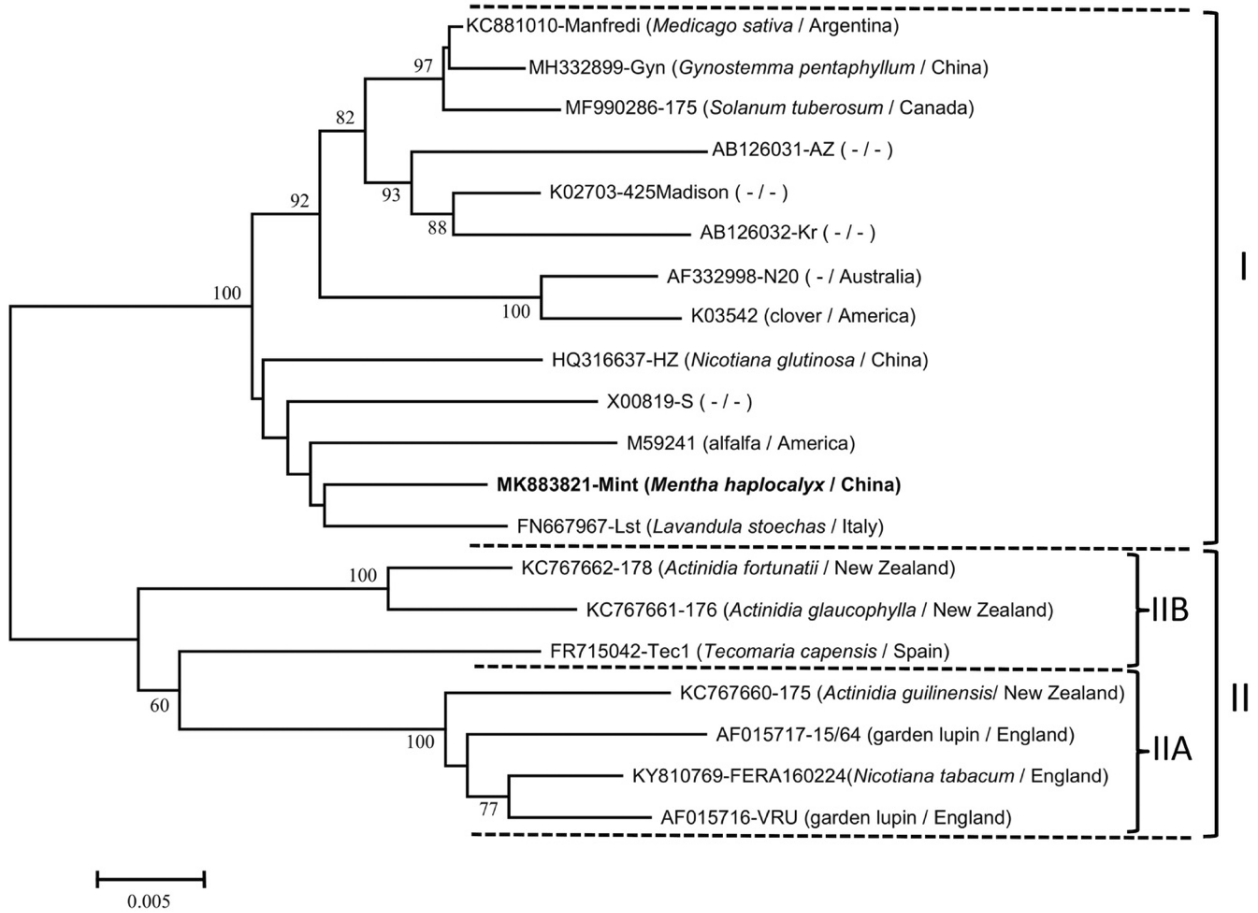

Figure 3. Phylogenetic analysis using the neighbour-joining method, based on RNA3 sequences of AMV isolates. Different groups are indicated and separated by dotted lines. AMV-Mint from the present study is indicated in bold font.

ship of AMV-Mint was with AMV-Lst, an isolate from *Lavandula stoechas* from Italy. The other seven isolates in Group II formed two subgroups including IIA (AMV-175, AMV-15/64, AMV-FERA160224 and AMV-VRU) and IIB (AMV-178, AMV-176 and AMV-Tec1), as reported previously (Parrella *et al.*, 2011; Song *et al.*, 2019). These results showed no phylogenetic correlation to hosts or geographical regions. In addition, no recombination events were detected in the genome of AMV-Mint.

This was the first report of the occurrence, and the complete genome sequence, of AMV from *M. haplocalyx*.

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COMPLIANCE WITH ETHICAL STANDARDS

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Conflict of Interest: The authors declare that they have no conflicts of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Short Notes

First report of *Diplodia africana* on *Grevillea robusta*

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Summary. Branch cankers and dieback were observed on silky-oak trees (*Grevillea robusta*) along some streets of Palermo (Sicily, Italy). Fungi isolated from symptomatic branches were identified as *Diplodia africana* and *D. seriata* by morphological characters and phylogenetic analyses of combined ITS and translation elongation factor 1-α sequences. Pathogenicity was verified by inoculating twigs of 3-y-old silky-oak plants. This is the first report of *D. africana* on *G. robusta* and the first record of *D. seriata* on this host in the northern hemisphere.

Keywords. *Botryosphaeriaceae*, silky-oak, *D. seriata*, dieback, canker.

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INTRODUCTION

Grevillea robusta A. Cunn. ex R.Br. (Proteaceae), commonly known as silky-oak, is an evergreen tree native to the subtropical coastal regions of northern New South Wales and southern Queensland, Australia. The plant was introduced by the British to the Ligurian Riviera of Italy, and some shrubby species of *Grevillea* are common in Italian gardens. *G. robusta* is widely used as an ornamental tree along streets and in public and private gardens. This tree is well suited to the climates of southern Italy and the major islands of Sicily and Sardinia, as it prefers high atmospheric humidity (Raimondo *et al*., 1995; Venturella *et al*., 1990). In the USDA fungus-host database, 110 taxa are listed as associated with *G. robusta* (Farr & Rossman, 2019). Among these fungi there are three Botryosphaeriaceae species associated with branch cankers and dieback of *G. robusta* in Africa. and all three were shown to be pathogenic (Toljander *et al*., 2007; Njuguna *et al*., 2011). Members of the Botryosphaeriaceae have cosmopolitan distribution, occur on a very wide range of hosts, and several are known to cause dieback of woody hosts (Úrbez-Torres, 2011).

Symptoms of branch cankers and dieback similar to those previously reported in Africa (Toljander *et al*., 2007; Njuguna *et al*., 2011) were recently observed on silky-oak trees along some urban streets in Palermo, Sicily. The aims of the research reported here was to identify the Botryosphaeriaceae

species associated with diseased silky-oak trees, using a combination of morphological and DNA sequence data, and to evaluate the pathogenicity of the identified species to silky-oak plants.

MATERIALS AND METHODS

In September 2016 and in March 2017, 96 trees of *G. robusta* of approximate age 13 y, and cultivated as ornamental plants in Roccazzo Street in Palermo (38°07'18.1"N, 13°18'28.1"E) were examined for disease symptoms. The symptomatic plants showed branch dieback, lesions and cankers on branches and stems, dead shoots and presence of orange to red-brown resin exudation on the stems and branches. After preliminary observation, the trees with cankers were counted to determine disease incidence. In both surveys, samples of symptomatic branches were collected from nine randomly selected trees and transferred to the laboratory for analyses. Wood portions were flame sterilized, and 70 small fragments, cut from the margins of necrotic lesions, were transferred to plates of potato dextrose agar (PDA Oxoid Ltd) supplemented with 0.5 g L^{-1} of streptomycin sulphate (Sigma-Aldrich). After incubation at 25±1°C in the dark for 4 d, the percentage isolation frequency (IF) of fungi was determined, as the number of tissue portions infected by a given fungus divided by the total number of tissue segments incubated. Hyphal tips taken from colony margins were sub-cultured on PDA. Sporulation was induced by culturing the isolates on quarter-strength PDA (¼ PDA) supplemented with double-autoclaved pine needles for 2 months at 23°C. After sporulation, pycnidia were mounted in water, and 50 conidia were measured using a light microscope (Axioskop; Zeiss) at ×400 magnification, and images were captured using the Axio-Vision 4.6 software (Zeiss). Conidium dimensions are presented here as 5th and 95th percentiles with extreme values in parentheses, L/W ratios, means and standard deviations (S.D.).

One isolate of each species was used for determination of the optimum temperature for growth and for pathogenicity tests, and morphology of colonies grown on PDA at 25°C in the dark for 4 d was recorded. Cardinal temperatures for growth of one selected isolate for each species was determined by incubating the culture on PDA in the dark at temperatures from 5 to 40°C at 5°C intervals, with three replicates per temperature. Colony diameters were measured along two perpendicular axes when the colony reached at least two-thirds of the plate diameters, and the data were converted to daily radial growth rates)mm d^{-1}). Plates incubated at temperatures in which there was no growth were then incubated at 25°C to determine if these temperatures were fungistatic or fungicidal.

Genomic DNA was isolated from 1-week-old fungal cultures grown on PDA at 25°C in the dark, using a standard CTAB-based protocol (O'Donnell *et al*., 1998). The internal transcribed spacer (ITS) region of the ribosomal DNA and part of the translation elongation factor 1 alpha gene (tef1-α) were amplified and sequenced, respectively, with primers ITS1/ITS4 (White *et al*., 1990) and EF1-728F/EF1-986R (Carbone and Kohn, 1999). PCR amplification and sequencing of amplicons was carried out as described by Phillips *et al*. (2013). Sequences were edited with Sequencher v 4.7 (Gene Codes Corporation), and compared with sequences deposited in GenBank through BLASTn searches. New sequences were deposited in GenBank (Table 1). Phylogenetic analyses was performed as described by Giambra *et al*. (2016). Alignments were made using ClustalX v. 1.83 (Thompson *et al*. 1997), and when necessary these were manually edited using MEGA6 (Tamura *et al*. 2013). Maximum likelihood (ML) analyses were performed on a Neighbour-Joining starting tree automatically generated by MEGA6. Nearest-Neighbour-Interchange (NNI) was used as the heuristic method for tree inference, and 1,000 bootstrap replicates were performed.

Pathogenicity was tested by twig inoculation of one isolate for each species in July 2018, on 3-year-old silky-oak seedlings. For each inoculation, an agar plug, cut from the margin of a 6-d-old colony grown on PDA at 25°C in the dark, was placed in a wound (≅4 mm^2) made with a scalpel on the twig of each seedling, after bark disinfection with 70% ethanol. The plug was then covered with Parafilm®. Non-colonized agar plugs were applied to similar wounds on control plants. Five seedlings were inoculated with each isolate and five seedlings for controls were used in a randomized block experimental design. The inoculated plants were kept outdoors under natural environmental conditions. The lengths of vascular discoloration were measured 3 months after inoculation, and these data were checked for normality using Shapiro-Wilk test and were subjected to ANOVA.

RESULTS AND DISCUSSION

Surveys carried out on silky-oaks in an urban street revealed branch dieback, lesions and cankers on branches and stems, dead shoots, and the presence of orange to red-brown resin exudation on stems and branches (Figure 1a, b, c). In some cases, the disease caused the death of the affected trees. Disease incidence increased from

Table 1. Isolates included in the phylogenetic study. The newly generated sequences are indicated in italics and ex-type strains are indicated in bold font.

Species	Isolate number	Host	Country	GenBank	
				ITS	*tef1*-α
Diplodia africana	**CBS 120835**	*Prunus persica*	South Africa	EF445343	EF445382
	CBS 121104	*Prunus persica*	South Africa	EF445344	EF445383
	DA1	*Juniperus phoenicea*	Italy	JF302648	JN157807
	GB34	*Grevillea robusta*	Italy	KY486864	KY486895
	GB35	*Grevillea robusta*	Italy	*KY486865*	*KY486896*
	GB36	*Grevillea robusta*	Italy	*KY486866*	*KY486897*
	GB37	*Grevillea robusta*	Italy	*KY486867*	*KY486898*
	GB38	*Grevillea robusta*	Italy	*KY486869*	*KY486899*
	GB39	*Grevillea robusta*	Italy	*KY486870*	*KY486900*
	GB40	*Grevillea robusta*	Italy	*KY486871*	*KY486901*
	GB41	*Grevillea robusta*	Italy	*KY486872*	*KY486902*
	GB42	*Grevillea robusta*	Italy	*KY486873*	*KY486903*
	GB43	*Grevillea robusta*	Italy	*KY486874*	*KY486904*
	GB44	*Grevillea robusta*	Italy	KY486875	KY486905
	GB45	*Grevillea robusta*	Italy	*KY486876*	*KY486906*
	GB46	*Grevillea robusta*	Italy	*KY486877*	*KY486907*
D. agrifolia	**CBS 132777**	*Quercus agrifolia*	California	JN693507	JQ517317
	UCROK 1429	*Quercus agrifolia*	California	JQ411412	JQ512121
D. alatafructa	**CBS 124931**	*Pterocarpus angolensis*	South Africa	FJ888460	FJ888444
D. allocellula	**CBS 130408**	*Acacia karroo*	South Africa	JQ239397	JQ239384
	CBS 130410	*Acacia karroo*	South Africa	JQ239399	JQ239386
D. bulgarica	**CBS 124254**	*Malus sylvestris*	Bulgaria	GQ923853	GQ923821
	CBS 124135	*Malus sylvestris*	Bulgaria	GQ923852	GQ923820
D. corticola	**CBS 112549**	*Quercus suber*	Portugal	AY259100	AY573227
	BL10	*Quercus ilex*	Italy	JX894191	JX894210
D. cupressi	**CBS 168.87**	*Cupressus sempervirens*	Israel	DQ458893	DQ458878
	BL102	*Cupressus sempervirens*	Tunisia	DQ458894	DQ458879
D. fraxini	**CBS 136010**	*Fraxinus angustifolia*	Portugal	KF307700	KF318747
	CBS 136013	*Fraxinus angustifolia*	Italy	KF307710	KF318757
D. intermedia	**CBS 124462**	*Malus sylvestris*	Portugal	GQ923858	GQ923826
	CBS 112556	*Malus sylvestris*	Portugal	AY259096	GQ923851
D. malorum	**CBS 124130**	*Malus sylvestris*	Portugal	GQ923865	GQ923833
	BL127	*Populus alba*	Italy	KF307717	KF318764
D. mutila	**CBS 136014**	*Populus alba*	Portugal	KJ361837	KJ361829
	CBS 112553	*Vitis vinifera*	Portugal	AY259093	AY573219
D. neojuniperi	**CBS 138652**	*Juniperus chinensis*	Thailand	KM006431	KM006462
	CPC 22754	*Juniperus chinensis*	Thailand	KM006432	KM006463
D. olivarum	**CBS 121887**	*Olea europaea*	Italy	EU392302	EU392279
	CAP 257	*Olea europaea*	Italy	GQ923874	GQ923842
D. pseudoseriata	**CBS 124906**	*Blepharocalyx salicifolius*	Uruguay	EU080927	EU863181
D. quercivora	**CBS 133852**	*Quercus canariensis*	Tunisia	JX894205	JX894229
	CBS 133853	*Quercus canariensis*	Tunisia	JX894206	JX894230
D. rosacearum	**CBS 141915**	*Eriobotrya japonica*	Italy	KT956270	KU378605
	NB8	*Eriobotrya japonica*	Italy	KT956271	KU378606
D. rosulata	**CBS 116470**	*Prunus africana*	Ethiopia	EU430265	EU430267
	CBS 116472	*Prunus africana*	Ethiopia	EU430266	EU430268

(Continued)

Table 1. (Continued).

Species	Isolate number	Host	Country	GenBank	
				ITS	*tef1*-α
D. sapinea	**CBS 393.84**	*Pinus nigra*	Netherlands	DQ458895	DQ458880
	CBS 109725	*Pinus patula*	Indonesia	DQ458896	DQ458881
D. scrobiculata	**CBS 118110**	*Pinus banksiana*	Wisconsin	KF766160	KF766399
	CBS 109944	*Pinus greggii*	Mexico	DQ458899	DQ458884
	CBS 113423	*Pinus greggii*	Mexico	DQ458900	DQ458885
D. seriata	**CBS 112555**	*Vitis vinifera*	Portugal	AY259094	AY573220
	CBS 119049	*Vitis vinifera*	Italy	DQ458889	DQ458874
	NB4	*Eriobotrya japonica*	Italy	KT956267	KU310680
	GB1	*Grevillea robusta*	Italy	*KY486863*	*KY486893*
	GB2	*Grevillea robusta*	Italy	*KY486868*	*KY486894*
D. tsugae	**CBS 418.64**	*Tsuga heterophylla*	Canada	DQ458888	DQ458873

Acronyms of culture collections: BL: B.T. Linaldeddu culture collection housed at Dipartimento di Agraria, Università di Sassari, Italy; CAP, A.J.L. Phillips, Universidade Nova de Lisboa, Portugal; CBS: Centraalbureau voor Schimmelcultures, The Netherlands; CPC: Collection of Pedro Crous housed at CBS; UCROK, Department of Plant Pathology and Microbiology, University of California, Riverside.

Figure 1. a, b: Symptoms of branch dieback and stem cankers, on *Grevillea robusta*. c: resin exudation on trunk.

61% in September 2016 to 83% in March 2017, indicating rapid development of the disease. Samples collected from diseased trees showed brown to black vascular longitudinal discoloration under the bark, and wedge-shaped necrotic sectors visible in cross sections.

Fifteen *Diplodia* isolates were obtained from the collected samples (Table 1). Identification based on morphological characters revealed two distinct species: 13 isolates of *Diplodia africana* Damm & Crous and two isolates of *Diplodia seriata* De Not., showing different isolation frequency (26% for *D. africana* and 4.0% for *D. seriata*).

Colonies of *D. africana* isolates on PDA at 25°C were initially white and became dark grey-olivaceous after 6 d, with moderate amounts of aerial mycelium, and the colonies reached the edges of Petri dishes within 5 d (Figure 2a). Colonies produced conidia that were hyaline, thick-walled, aseptate, smooth, cylindrical to

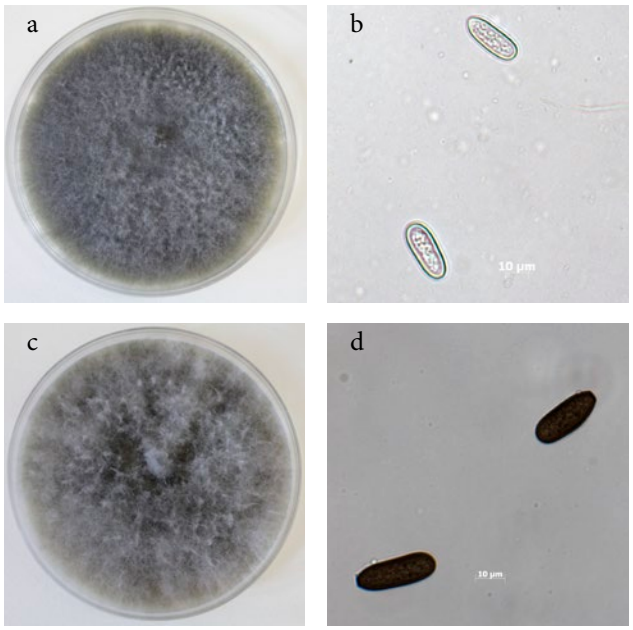

Figure 2. Colonies and conidia on PDA after 7 d at 25°C: (a, b) *Diplodia africana;* (c, d) *Diplodia seriata*.

oblong elliptical, with rounded apices. The conidium dimensions were (25.3–)26.3–32.3(–34.3) × (9.5–)11.9–15.6(–16.3) µm, 29.7 ± 1.9 × 14.1 ± 1.1 µm, with L/W ratio = 2.1 (Figure 2b). The optimum temperature for colony growth of *D. africana* was 25°C with a temperature range of 5–30°C. None of the tested temperatures were fungicidal. Conidium morphology and cultural features of the isolates agreed with the description of *D. africana* by Damm *et al*. (2007). Nevertheless, they reported a maximum temperature for growth of 35°C and optimum of 20°C (Damm *et al*., 2007).

Colonies of *D. seriata* on PDA at 25°C filled Petri dishes before 6 d, and had compact aerial mycelium, which was initially white, and becoming dark gray after 5 d (Figure 2c). Conidia were initially hyaline, becoming dark brown, aseptate, moderately thick-walled, wall externally smooth, and were ovoid, with obtuse apices. They measured (20.0–)21.3– 27.7(–28.3) × (8.7–)9.3–11.0(–11.8) µm, 24.9 ± 1.9 × 10.2 ± 0.6 µm, with L/W = 2.4 (Figure 2d). Isolates of *D. seriata* had optimum growth temperature of 26°C, with a temperature range of 5–35°C. Cultures incubated at 40°C did not grow when returned to 25°C. Optimum temperature, conidium dimensions and morphology determined in this study corresponded to those reported by Phillips *et al*. (2013).

Identification was confirmed by analysis of the ITS and *tef1*-α sequences. The phylogenetic tree (Figure 3) differentiated the *Diplodia* species, and bootstrap values showed strong support for many branches. Thirteen isolates were included in a clade with the ex-type isolate of *D. africana*, and two isolates clustered in a clade with the ex-epitype of *D. seriata*.

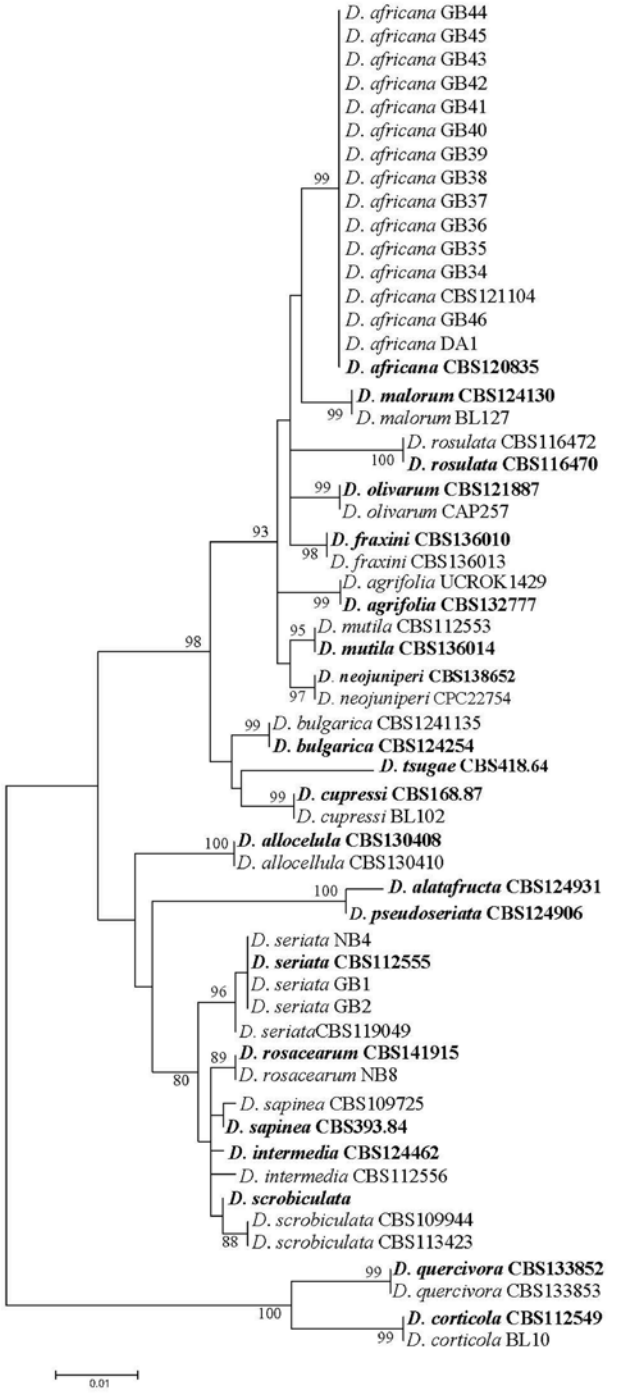

Figure 3. Maximum Likelihood tree of combined ITS and *tef1-α* sequence data for *Diplodia* species. The tree was drawn to scale, with branch lengths measured for the number of substitutions per site. Bootstrap support values ≥ 70% (1,000 replicates) are given at the nodes. The type species are in bold.

Figure 4. Subcortical dark-brown discolorations spreading from the inoculation points on *Grevillea robusta* twigs, 3 months after artificial inoculation with *Diplodia africana* (a), *Diplodia seriata* (b) and agar plugs not inoculated with a fungus (c).

Three months after inoculation, twigs of test plants displayed necrotic bark lesions spreading upward and downward from the inoculation points (Figure 4a, b). Slight sub-cortical discoloration was observed in the lesions caused by *D. seriata* while infections caused by *D. africana* developed from the bark tissues toward the wood where they penetrated a few millimeters, and the bark surface was longitudinally fissured (Figure 4a, b). No discoloration was observed in the stems of control plants (Figure 4c). *Diplodia africana* induced lesions with a mean length of 18.2±1.3 mm, while mean lesion lengths caused by *D. seriata* were 15.5±1.4 mm. Differences between the two species were not statistically significant ($F_{1, 6}$ = 1.98; P = 0.21). Both fungus species tested were successfully re-isolated from inoculated plants, thus fulfilling Koch's postulates. No fungal pathogens were isolated from the control plants.

The results reported here indicate that *D. africana* and *D. seriata* were the etiological agents for branch cankers and dieback of *G. robusta* trees in Sicily. This is the first report of *D. africana* on *G. robusta*, and also the first record of *D. seriata* on *G. robusta*, in the northern hemisphere. Similar disease symptoms were observed in Africa on *G. robusta*, and the causal agents were shown to be *Neofusicoccum parvum* (Pennycook and Samuels) Crous, Slippers and Phillips, *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl. and, *D. seriata* (Toljander *et al*., 2007; Njuguna *et al*., 2011). Pathogenicity tests showed that *N. parvum* and *L. theobromae* were highly pathogenic on *G. robusta*, whereas *D. seriata* was moderately or weakly pathogenic (Toljander *et al*., 2007; Njuguna *et al*., 2011). *Diplodia seriata* is a cosmopolitan botryosphaeriaceous fungus and is known to be polyphagous, occurring on many native or introduced plants (Phillips *et al*. 2013), while *D. africana* has a limited reported host range geographical occurrence. *Diplodia africana* was associated for the first time with disease symptoms on *Prunus persica* (L.) Batsch in South Africa (Damm *et al*., 2007). The occurrence of *D. africana* on *Juniperus phoenicea* L. and *J. oxycedrus* L. in Italy (Linaldeddu *et al*., 2011) shows the ability of this species to colonize different hosts.

Many authors have shown that diseases caused by fungi in the Botryosphaeriaceae are usually linked to environmental stress factors acting on the host (Úrbez-Torres, 2011). Desprez-Loustau *et al*. (2006) highlighted that environmental stresses predispose plants to infection by endophytes or opportunistic pathogens, indicating that host stress may trigger shifts from latent to pathogenic phases of some endophytic fungi, including the Botryosphaeriaceae. In the present case, environmental stresses caused by poor soil aeration and low water content, due to the road pavement, could have introduced sufficient stress to predispose *G. robusta* to infection by the *Diplodia* pathogens.

Further studies should be undertaken to assess the distribution of the disease, and to determine the roles of biotic and abiotic factors in the development of dieback. It would also be of interest to study the possible interaction between *D. africana* and *D. seriata* in the disease.

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Short Notes

Different inoculation methods affect components of Fusarium head blight resistance in wheat

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Summary. Fusarium head blight (FHB) is one of the most important fungal diseases of cereals, and *Fusarium graminearum* is the most damaging FHB pathogen. infection is linked to host anthesis, and symptoms include necrosis, bleaching of heads and shrivelled kernels. No fully effective fungicides are available for FHB control, so utilization of other mitigation measures, such as the use of resistant cultivars, is necessary for FHB management. Resistance to FHB is quantitative and multigenic and five components of resistance (Type I, II, III, IV and V) have been described. The main problem in testing for FHB resistance is reproducibility, so necessary tools for breeding resistant cultivars are reliable inoculation methods and the testing for different FHB-associated characteristics. We screened three Italian wheat genotypes, 'Palesio', 'Claudio' and 'Marco Aurelio', for Type I, Type II, and, in part, for Type V resistances, with both phenotypic (% of disease incidence and severity, thousand kernel weight (TKW) and molecular (quantification of fungal biomass with Real-Time *q*PCR) approaches, using spray and point inoculation protocols. Results underlined that 'Palesio' bread wheat showed Type I tolerance to initial infection, and 'Marco Aurelio' durum wheat showed an important Type II resistance to disease spread when spray-inoculated (27% disease severity). Quantification of fungal biomass showed that differentiation among the three wheat cultivars was best visualized when spray inoculation was used. TKW data showed that % yield loss was greater after point inoculations, except in 'Marco Aurelio', which was not affected by inoculation method. This study has highlighted the complexity of testing for FHB resistance, and demonstrated the necessity to use as many resistance screening protocols as possible.

Keywords. *Fusarium graminearum*, *Triticum aestivum*, *Triticum durum*, spray inoculation, point inoculation.

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INTRODUCTION

Wheat is one the most cultivated crops, followed by rice and maize. Modern wheat cultivars include two species: hexaploid bread wheat, *Triticum aestivum* L. (AABBDD), and tetraploid, durum-type wheat, *T. turgidum* subsp. *durum* (Desfontaines) Husnache (AABB) used for pasta and low-rising bread

(Doebley *et al*., 2006; Dubcovsky and Dvorak, 2007; Charmet, 2011; Feldman and Levy, 2012). Bread wheat accounts for 95% of world wheat production, while durum wheat is the remaining 5%. Wheat accounts for more than 20% of total human food calories. Wheat crops are extensively grown, on 17% of all crop areas, and is the staple food for 40% of the world's population, mainly in Europe, North America and the western and northern parts of Asia (Peng *et al*., 2011).

Fusarium head blight (FHB) is one of the most important fungal diseases of grain crops, including wheat, barley and maize (Goswami and Kistler, 2004; Osborne and Stein, 2007; van der Lee *et al*., 2015). FHB is caused by the *Fusarium graminearum* Species Complex (FGSC), which comprises 16 different species. These produce various mycotoxins, including deoxynivalenol (DON) and zearalenone (ZEA), which are toxic to humans and animals (Desjardins and Proctor, 2007; Foroud and Eudes, 2009;, Walter *et al*., 2010; Darwish *et al*., 2014).

During the past decade numerous FHB epidemics have been reported worldwide, causing significant economic losses (millions to billions of $US) (McMullen *et al*., 2012; Wegulo *et al*., 2015). The spectrum of *Fusarium* spp. causing FHB on wheat varies at the regional level depending on weather conditions, especially during host anthesis. Fungal growth is favoured by high temperatures and humidity, and abundant rain, during the growing season which favour pathogen infection, and can lead to significant yield losses. Given the current global warming associated with increased temperatures, major epidemics of the *Fusarium* diseases are likely (Vaughan *et al*., 2016; Khaledi *et al*., 2017).

Fusarium graminearum Schwabe is the predominant FHB pathogen, but its infection biology is yet to be fully understood. Airborne spores are transported by rain and wind to host floral tissues, where, at anthesis, they proliferate and spread rapidly intracellularly throughout the host spikelets, down into the rachial nodes and ultimately up and down the rachides until FHB symptoms are clear, involving necrosis and bleaching of heads causing shrivelled kernels (Nelson *et al*., 1994; Dweba *et al*., 2017).

In Italy, FHB on wheat has occurred each year since 1995, at varying levels of incidence and severity depending on the year, the region and the wheat genotype involved. (Pancaldi *et al.,* 2010). The disease has been reported mostly in the Northern-Central regions of Italy, and there is evidence indicating that the prevalent FHB species have shifted from *F. culmorum* (W. G. Smith) Saccardo to *F. graminearum* and *F. poae* (Peck) Wollenweber (Shah *et al*., 2005). FHB incidence and severity increase from the South to the North of Italy, and is closely related to the amounts of precipitation during wheat anthesis (Covarelli *et al*., 2015). Regarding mycotoxin production, DON is the most frequently found in Italy, and, as for disease incidence, occurrence of this mycotoxin increases from Southern to Northern Italian regions (Aureli *et al*., 2015). Since durum wheat is grown more widely than bread wheat in Italy, but also is more susceptible to FHB than bead wheat, mycotoxin accumulation in kernels is of particular concern as a food safety issue (Boutigny *et al*., 2008).

Chemical control of FHB using appropriate effective fungicides and correct application methods and timing are feasible for reducing disease severity (Blandino *et al.,* 2012). However, no fully effective FHB fungicide is available (Haidukowski *et al*., 2012), and the application window is very narrow, spanning just a few days around host anthesis (Mesterházy *et al*., 2003). Therefore, while new and eco-sustainable plant protection strategies are being developed (Fortunati *et al.,* 2019), the utilization of resistant genotypes remains important, and is possibly the most effective strategy for FHB control (D'Mello *et al*., 1999).

Resistance to FHB in wheat and other cereals has quantitative and multigenic characteristics (Zhu *et al*., 1999; Gervais *et al*., 2003; Massman *et al*., 2011). It is a non-trivial task for plant breeders to develop FHB-resistant and productive wheat cultivars, since plant breeding requires two essential pre-conditions: availability of genetic resources carrying positive alleles for the trait of interest and reliable testing methods that allow breeders to identify the desired genotypes (Buerstmayr *et al*., 2014; Steiner *et al*., 2017). Two main components of resistance have been described: Type I resistance operates against initial infections and Type II against the spread of symptoms induced by pathogens within their hosts (Schroeder and Christensen, 1963). Furthermore, Type I and Type II resistances vary independently among genotypes (Schroeder and Christensen, 1963). Three other types of FHB resistances have been described, but these are still not well understood. Type III resistance is the host plant's ability to degrade DON (Miller and Arnison, 1986), Type IV is the host's ability to tolerate high DON concentrations (Wang and Miller, 1988), and Type V is resistance to kernel infection, evaluated by analysing grain samples post-harvest for incidence of diseased kernels (Mesterházy, 1995). Gilbert and Tekauz (2000) distinguished between resistance (host ability to prevent infection) and tolerance (host ability to mitigate the infection, with low impacts on yield), and attributed Type IV and V resistances as FHB tolerance.

The most important goal in FHB resistance breeding is that resistant varieties should develop low symptom severity and simultaneously low mycotoxin contamination (Bai *et al*., 2001; Snijders, 2004; Wilde *et al*., 2007). In the second half of the 20th Century, large numbers of varieties, breeding lines and germplasm accessions were evaluated for FHB resistance. Quantitative variation in FHB susceptibility was detected, but no genotype was immune (Miller and Arnison, 1986; Wang and Miller, 1988; Buerstmayr *et al*., 1999). Durum wheat was also more susceptible than bread wheat, where almost no variation in resistance to FHB has been found within historic and current *T. durum,* with most lines being susceptible, even among large germplasm collections of several thousand lines (Otto *et al*., 2002; Stack *et al*., 2002; Ghavami *et al*., 2011; Prat *et al*., 2014).

One of the main problems in testing for FHB resistance is the lack of reproducibility of results (Groth *et al*., 1999; McCallum and Tekauz, 2002; Geddes *et al*., 2008). The chief goal is to measure differences in genetic resistance, taking into account non-genetic factors, which can lead to errors in the results. Under natural conditions, infection pressure is usually not uniform in time and space, while in FHB resistance screenings, infection is achieved by applying uniform inoculum pressure over time (at flowering) and space (in greenhouses) (Campbell and Lipps, 1998). A necessary tool for breeding resistant lines is a reliable inoculation method enabling accurate quantitative disease assessment. Further, since FHB resistance is a complex quantitative trait, a single and simple method for measuring FHB resistance is sometimes insufficient (Buerstmayr *et al*., 2014).

The objectives of the present study were to screen for Type I and Type II resistances in three prominent Italian wheat cultivars, whose FHB responses were unclear, by using phenotyping and molecular tools to assess FHB incidence and severity. Real-Time *q*PCR (FHB Type II resistance) and measurement of thousand kernel weight (TKW) (FHB Type V resistance) were carried out to quantify the fungal biomass in wheat chaff and rachides, and to assess the impacts of *F. graminearum* infection on yield loss.

MATERIALS AND METHODS

Plant material and growth conditions

Italian wheat genotypes 'Palesio' (bread wheat), and 'Marco Aurelio' and 'Claudio' (durum wheat) were grown in a greenhouse, following the protocol developed by Watson *et al.* (2018), with modifications. Seeds were surface sterilized with sodium hypochlorite (0.5% v/v) for 20 min and then rinsed twice for 5 min. in sterile distilled water. Seeds were then germinated in the dark on paper imbibed with sterile distilled water for 15 d at 4°C to break dormancy, followed by 2 d at room temperature. Subsequently, seedlings were transferred to 40 × 20 cm pots (20 plants for each pot), filled with TYPical Brill soil, and were grown at 16–20°C until boot stage, 20–24°C during anthesis, and 24–29°C until maturity. The plants were fertilised to avoid nitrogen deficit, by providing ammonium nitrate at the following proportions and plant stages: 20% at sowing, 40% at tillering and 40% at heading.

Fungal material, inoculum preparation and infection techniques

The highly virulent and DON-producing isolate of *F. graminearum,* wild type 3824 (Mandalà *et al*., 2019), was cultured at 21°C on potato dextrose agar (PDA) and on synthetic nutrient poor agar (SNA) (Urban *et al*., 2002) to obtain macroconidia for inoculum preparation. To prepare inocula, after a minimum of 10 d on SNA, conidia were scraped with a glass rod after pipetting 1 mL of sterile distilled water onto the surface of each Petri dish. The resulting conidium suspension was recovered, and the concentration measured using a Thoma Chamber (0.100 mm depth and 0.0025 mm^2). Inocula were prepared in sterile distilled water supplemented with 0.05% (v/v) of Tween-20. Two inoculum methods and several conidium concentrations were tested: spray inoculation to evaluate Type I FHB resistance, and point inoculation to evaluate Type II resistance, and 500, 1,500 or 2,500 conidia per spike (c/s), to assess dependent disease pressure responses in symptom development. Conidium concentrations were prepared following the protocol of Stein *et al.* (2009). 10 µL of conidium suspension was applied to the central spike floret of each plant for point inoculations (using a laboratory pipette), or 100 µL of conidium suspension was applied to plants (using a manual nasal sprayer) for spray inoculations. Thus, 5×10^4, 15×10^4 and 25×10^4 conidia mL^{-1} concentrations were prepared for point inoculations, while 5×10^3, 15×10^3 and 25×10^3 conidia mL^{-1} were prepared for spray inoculation, in order to inoculate each test plant with 500, 1,500 or 2,500 conidia for each spike using the two both inoculation techniques. The spikes on the main culms (one spike per plant) were inoculated during anthesis (Zadok stage 69: Zadoks *et al*., 1974), at greenhouse temperatures ranging from 20–24°C. Subsequently, the spikes were sprayed with sterile distilled water and covered with clear plastic bags for 48 h to maintain (> 80%) high humidity. Unin-

oculated control plants were treated with sterile distilled water supplemented with 0.05 % (v/v) of Tween-20. Disease incidence (%) was determined for the spray inoculated plants by counting the numbers of bleached spikes at 3, 9, 15 and 21 d post infection (dpi). Disease severity (%) was determined for both spray and point inoculated plants by counting the numbers of bleached spikelets for each inoculated spike from 3 to 21 dpi. All inoculation trials were performed in three replicates, and each replicate contained 20 spikes for each variable (genotype × conidium concentration × inoculation technique).

Fungal biomass quantification

At 21 dpi, the 2,500 c/s (the strongest disease pressure condition) point and spray inoculated spikes were collected and immediately stored in liquid nitrogen, for quantification of *F. graminearum* DNA in the chaff and rachis tissues. Fungal DNA quantification was performed following the protocol of Horevaj *et al.* (2011) and Siou *et al.* (2014). Material to obtain the *F. graminearum* calibration curve (60 mg of fresh mycelium) and the wheat calibration curve (60 mg of uninoculated wheat material) and total inoculated wheat chaff and rachis (60 mg of inoculated wheat material), were finely ground using mortars and pestles plus liquid nitrogen, and were stored at -80°C until DNA extractions. Total wheat and fungal DNA extraction were performed following the protocol for the Invisorb® Spin Plant Mini Kit (Stratec Molecular), and DNA was quantified with a Qubit™ fluorometer 1.01 (Invitrogen) using the Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific). DNA from inoculated samples was diluted to 10 ng μL^{-1}, while fungal and wheat calibration curves were obtained preparing three serial 1:10 dilutions from fresh fungal mycelium and uninoculated wheat material DNAs. Real-Time *q*PCR was performed following the instructions from Rotor Gene Q (Qiagen) and Xpert Fast SYBR (uni) Master Mix (Grisp). Real-Time *q*PCR amplification conditions included: an initial denaturation step of 3 min at 95°C; 35 cycles of 5 sec denaturation at 95°C, 30 sec of annealing at 61°C and 20 sec of elongation at 72°C. A final melt cycle was performed to confirm the amplicons unicity. Real-Time *q*PCR was performed using the primer pair Tri6_10F/Tri6_4R for *F. graminearum* DNA quantification (Horevaj *et al.,* 2011), and Act_77F/Act_312R for wheat DNA quantification (Mandalà *et al.*, 2019). Three biological replicates were analysed for each quantification and from each of these, three technical replicates were obtained and tested. Results are reported as ng of fungal DNA per ng of plant DNA.

Thousand kernel weight (TKW)

At maturity, the 20 spikes from 2,500 c/s (the greatest disease pressure condition) for point and spray inoculated plants, and the uninoculated control plants, were collected and stored at 4°C. the spikes were hand threshed to separate kernels from the chaff. Kernels were then weighed to determine and calculate the TKW. The TKW of inoculated plant kernels was then compared with the TKW of control plant kernels, to estimate the percent yield loss due to inoculations.

Statistical analyses

Data were subjected to analyses of variance (ANOVA). The following data were compared: disease incidence (%) among different conidium inoculation concentrations and genotypes for spray inoculated plants; disease severity (%) among different conidium inoculation concentrations, inoculation methods and genotypes; fungal DNA concentrations (ng of fungal DNA per ng of plant DNA); and yield loss (%) between 2,500 c/s spray- and point-inoculated plants. Two levels of significance ($P < 0.05$ and $P < 0.01$) were computed to assess the significance of the F values. When significant F values were observed, a pairwise analysis was carried out using the Tukey Honestly Significant Difference test (Tukey test) at the 0.95 or 0.99 confidence levels.

RESULTS

Several conidium concentrations were tested to assess disease pressure responses in symptom development. The ANOVA test showed that there were no statistically significant differences in trends of FHB incidence (Type I) or severity (Type II) from the different conidium concentrations in the three cultivars tested. Incidence reached 100% between 15 and 21 dpi in all the three wheat cultivars. For each conidium concentration tested; disease severity also reached 100% between 15 and 21 dpi, when the wheat spikes were point inoculated. From the spray inoculations, FHB severity for 'Marco Aurelio' reached 44% at 21 dpi, while severity on 'Palesio' and 'Claudio' was also 100% at 21 dpi.

Figure 1 presents results obtained from the phenotypic evaluations of symptoms. Figures 1a, 1b and 1c show the genotype comparisons, for assessment of FHB differential responses connected to resistance genotype diversity, while Figures 1d, 1e and 1f show the inoculation method comparisons, for assessment of differences in symptom severity (Type II). Figure 1a shows the

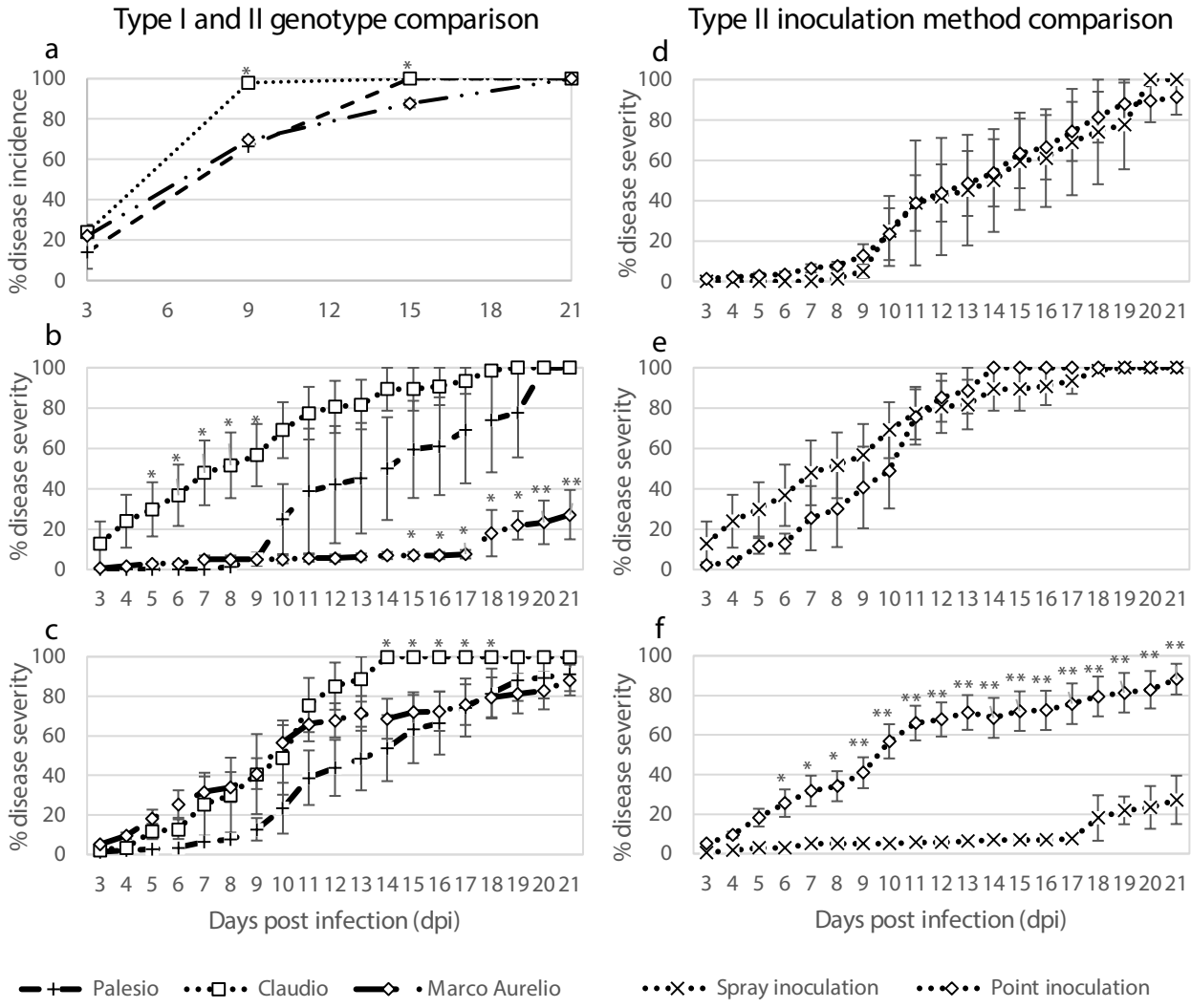

Figure 1. *Fusarium graminearum* symptom development during 21 dpi, following inoculation of three Italian wheat genotypes with 2,500 conidia per spike. a) % disease incidence (Type I); b) % disease severity after spray inoculation (Type II); c) % disease severity after point inoculation (Type II); d), e) and f) % disease severity (Type II) between spray and point inoculation methods for, respectively, 'Palesio, 'Claudio' and 'Marco Aurelio'. Data represent averages and standard errors for three independent replicates with at least 20 plants for each genotype × inoculation combination. Statistical analyses were performed according to a one way analysis of variance (ANOVA) with the Tukey test at a 0.95 confidence level and (*) $P < 0.05$, and at a 0.99 confidence level and (**) $P < 0.01$.

variation in disease incidence at 3, 9, 15 and 21 dpi, for 2,500 c/s, inoculated onto the three Italian wheat cultivars tested. 'Claudio' was the most susceptible reaching 98% of symptomatic spikes at 9 dpi ($P < 0.05$). At 15 dpi, 'Marco Aurelio' showed reduced symptom progression ($P < 0.05$), suggesting Type I tolerance for most of the trial duration.

Figures 1b and 1c indicate the FHB severity trend comparisons between the genotypes at 2,500 c/s. The spray inoculation technique (Figure 1b) gave disease severity at 9 dpi of 5% in 'Palesio' and 'Marco Aurelio', and 57% in 'Claudio'. This indicated the presence of initial Type II resistance in 'Palesio' and 'Marco Aurelio' ($P < 0.05$). From 10 dpi until the end of the trial, the statistically significant differences in symptoms between 'Claudio' and 'Palesio' disappeared, due to the high variability of results obtained with the spray inoculation method. In contrast, symptoms in 'Marco Aurelio' reached a maximum of 27% at 21 dpi ($P < 0.05$ for 15 and 19 dpi and $P < 0.01$ for 20 and 21 dpi), compared to 'Claudio' and 'Palesio'.

Figure 1c shows the same genotype comparisons as previously described, but after point inoculations, to assess putative FHB Type II resistance under more

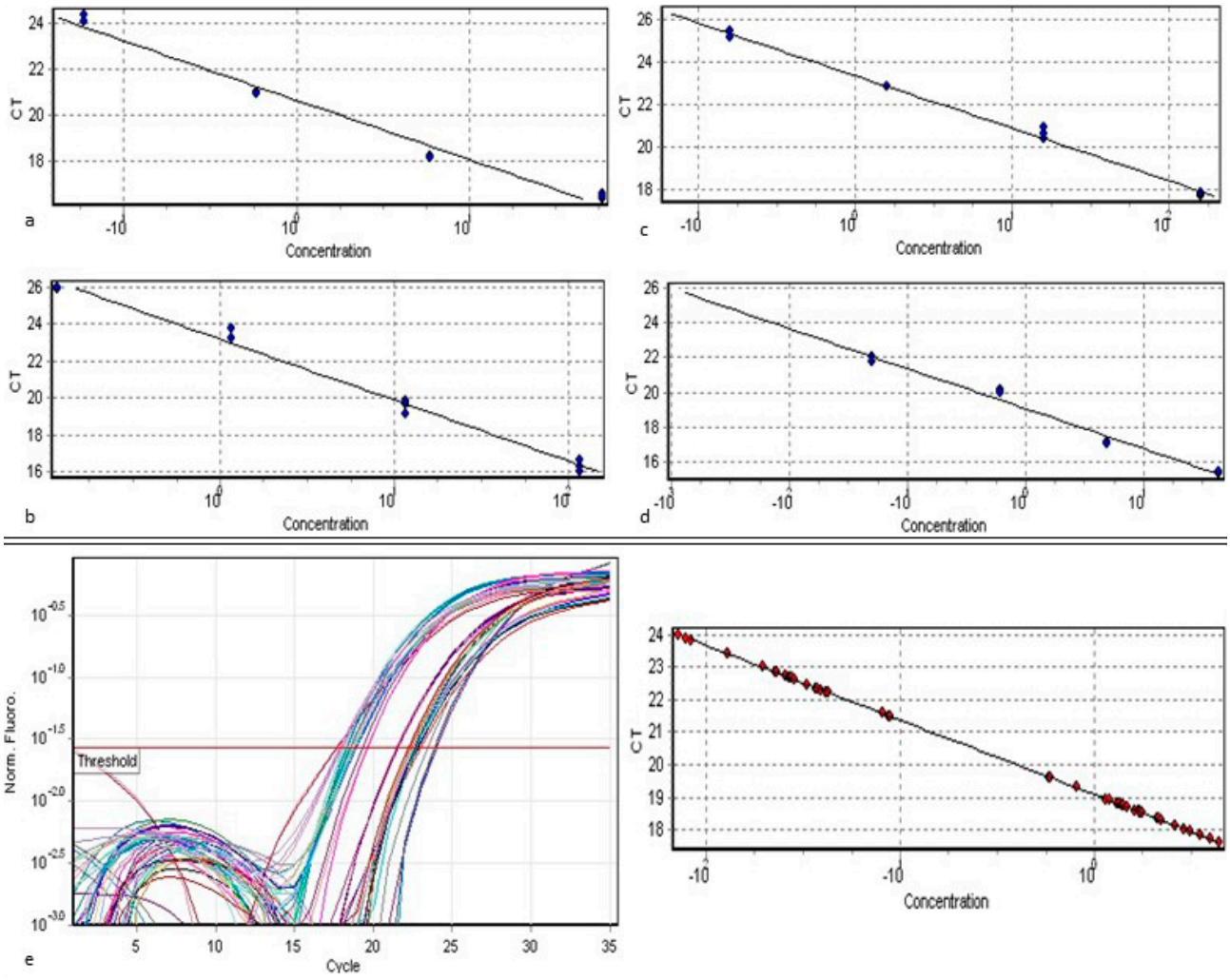

Figure 2. Standard curves resulting from Real-Time *q*PCR quantifications: a), b) and c) show, respectively, *Act* standard curves for wheat genotypes 'Palesio', 'Claudio' and 'Marco Aurelio' pure DNAs. d) shows the *Tri6* standard curve for *F. graminearum* pure DNA. e) shows amplification curves of the *Tri6* gene (left) and interpolations with the standard curve (right).

aggressive disease conditions. Between 3 and 13 dpi, no statistically significant differences were observed among the three wheat cultivars. Subsequently, 'Claudio' again demonstrated high susceptibility, reaching 100% severity at 14 dpi, while significantly less symptom development was observed on 'Palesio' and 'Marco Aurelio' at 14-18 dpi ($P < 0.05$). These results confirm that Type II resistance was present in 'Palesio' and 'Marco Aurelio' under harsh disease conditions. However, at 19–21 dpi, no symptom differences were detected among the three wheat genotypes.

Disease severity differences observed between the spray and point inoculation methods at 2,500 c/s for the three wheat genotypes are shown in Figures 1d, 1e and 1f. Figures 1d and 1e show the severity progression, respectively, in 'Palesio' and 'Claudio'. Under both spray and point inoculation, these two wheat cultivars did not show any FHB resistance or tolerance. In contrast, Figure 1f shows the severity trend in 'Marco Aurelio', where, starting from 6 to 8 dpi, symptom progression was less after spray inoculation than point inoculation ($P < 0.05$). These differences in symptom development were enhanced from 9 to 21 dpi ($P < 0.01$), and at the end of the trial, disease severity reached 27% after spray inoculation, and 88 % after point inoculation.

Additional estimations of FHB Type II tolerance or resistance were made using fungal DNA quantification after spray and point inoculations with 2,500 c/s. Figure 2 shows the Real-Time *q*PCR curves: Figures 2a, 2b, 2c and 2d show standard curves for DNA quantification

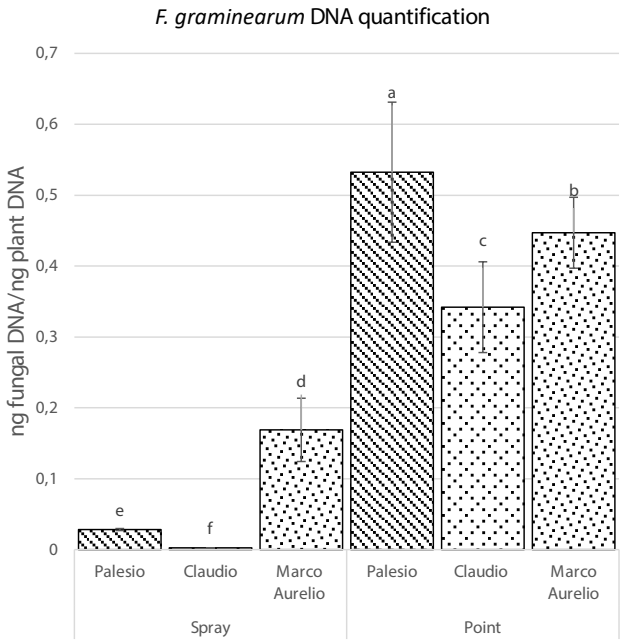

Figure 3. *Fusarium graminearum* biomass quantification from wheat chaff and rachides sampled from spikes that were either spray or point inoculated with 2,500 conidia per spike. Data represents averages and standard errors for three independent technical replicates derived from three independent biological replicates for each genotype × inoculation method. Statistical analyses were performed according to two way analysis of variance (ANOVA), with Tukey test at 0.95 confidence level ($P < 0.05$).

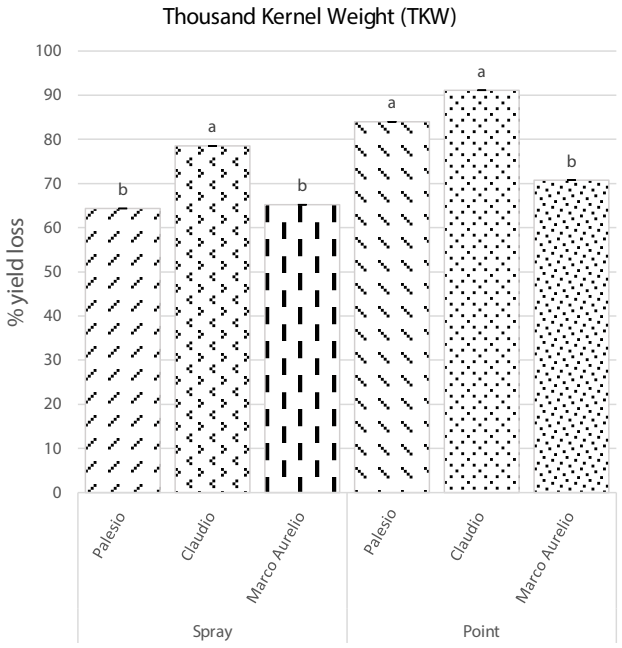

Figure 4. Yield losses (%) based on TKWs for inoculated and control plants after spray or point inoculations with *F. graminearum* at 2,500 conidia per spike. Data represent averages and standard errors for three independent replicates performed with at least 20 plants for each genotype × inoculation combination. Statistical analyses were performed according to two way analysis of variance (ANOVA) with Tukey test at 0.95 confidence level ($P < 0.05$).

of the *Actin* gene ('Palesio', 'Claudio', 'Marco Aurelio'), and the *Tri6* gene (*F. graminearum*). Figure 2e shows the amplifications results. Final results (Figure 3) showed that spray inoculation resulted in a less accumulation of fungal DNA in the wheat chaff and rachis than from point inoculation. 'Palesio' (0.028 ± 0.00146 ng of fungal DNA ng^{-1} per plant DNA and 'Claudio' (0.0025 ± 0.00012 ng) had less pathogen DNA than 'Marco Aurelio', (0.168 ± 0.0446 ng). After point inoculations, greater fungal DNA concentrations were detected, reflecting the aggressiveness of this inoculation method. 'Claudio' again had reduced pathogen accumulation (0.342 ± 0.064 ng of fungal DNA/ng per plant DNA) compared to 'Marco Aurelio' (0.447 ± 0.05 ng) and 'Palesio' (0.532 ± 0.099 ng).

TKW was measured for kernels derived from 2,500 c/s inoculated and uninoculated plants, to determine the pathogen impacts on potential wheat yields for the wheat genotypes. Figure 4 shows results reported as % yield losses. Point inoculations resulted in greater losses compared to spray inoculations ($P < 0.05$), since losses from point inoculations reached 84% in 'Palesio', 91% in 'Claudio' and 71% in 'Marco Aurelio'. These differences were not significant with 'Marco Aurelio', where spray and point inoculation had similar effects. 'Claudio' was more susceptible after spray (79% yield loss) or point (91%) inoculation, while 'Marco Aurelio' was the least affected cultivar, under both spray (65% yield loss) and point inoculation (71%). However, all the yield losses were very high for all of the inoculation and cultivar treatments.

DISCUSSION

FHB is normally a sporadic disease of wheat and other cereals, because infection and colonisation by *Fusarium* spp. are largely dependent on the prevailing weather conditions, which also determine disease severity (Xu, 2003; Burlakoti *et al.*, 2010). The risks of infection are associated with warm and humid conditions (Xu and Nicholson, 2009). As a result, FHB incidence and severity usually vary from year to year (Sutton, 1982), and from region to region (Jelinek *et al.,* 1989).

To develop resistant cereal germplasm, artificial inoculation is essential to ensure disease development, to optimise host genotypic differentiation, and to reduce

the influence of host morphological characters that can contribute to disease avoidance (Mesterházy, 1995; Vaughan *et al.,* 2016).

Evaluating FHB resistance using natural infections is often not possible as disease incidence and severity vary over time and space due to changes in environmental conditions such as temperature and precipitation that are difficult to control (Mesterházy *et al.*, 2003; Kriss *et al.*, 2012).

Obtaining consistent differentiation of FHB resistance levels relies on the use of comparative inoculation methods and different screening tests because of FHB's multigenic nature and complexity (Parry *et al.*, 1995; Browne, 2009). In the present study, Type I and Type II FHB responses were evaluated in three Italian wheat cultivars, to identify the presence of genotypic resistance/tolerance, and to assess two screening protocols, by testing different aspects and components of FHB resistance.

Different conidium concentrations in inocula were assessed to evaluate disease incidence and severity responses connected to different disease pressure. FHB incidence increased in response to conidium concentration, but the differences observed were not statistically significant. Differences in disease severity were more pronounced after spray inoculations, but again, these differences were not statistically significant. These observations could be due to increases in disease development with increasing conidium concentration until a maximum was reached where additional inoculum does not increase the level of disease (Stein *et al.,* 2009). We also observed that results obtained from spray inoculations were characterized by high variability, compared to point inoculations. Kiecana and Mielniczuk (2013) explained that, despite spray inoculation resembling natural routes to infection by FHB pathogens, disease assessment could be arduous due to heterogeneity of conidium spatial location on wheat heads. When a pathogen is spray-inoculated, inoculum can also partially germinate, resulting in reduced symptom development (Parry *et al.*, 1995; Al Masri *et al.*, 2017). This could be due to close relationships between pathogen assessment and plant phenological stage. First establishment of FHB is related to host floret anthesis, which is not uniform within each spike: anthesis begins in the central floret, and then occurs in the upper and lower flowers (Dweba *et al.*, 2017; Kheiri *et al.*, 2019). Point inoculations, on the other hand, is reported to be more environmentally stable and results from this method are more reproducible, since the inoculum is applied directly into the central florets at anthesis. This ensures that equal amounts of inoculum are delivered to individual plants and reduces the chance of disease escape, which has been observed after spray inoculations (Engle *et al.*, 2007; Geddes *et al.*, 2008; Mesterházy *et al.*, 2015). Despite this advantage, point inoculation does not represent the most natural source of *F. graminearum* inoculum and is more labour intensive and time-consuming to carry out. However, point inoculation likely mimics the fungal inoculum transferred onto cereal florets by tiny insects such as aphids and thrips that are often found in wheat crops (Usele *et al.*, 2013; Imathiu *et al.*, 2014; Sørensen *et al.*, 2016).

The genotype comparisons showed that 'Claudio' durum wheat was the most Type I and Type II susceptible, 'Palesio' bread wheat had initial Type I and II resistance after spray inoculation, and Type II tolerance after point inoculation. 'Marco Aurelio' durum wheat showed Type I tolerance and Type II resistance after spray inoculation, and Type II tolerance after point inoculation. These results are similar to those of Miedaner *et al.* (2003). They tested the covariation between spray and point inoculations, and compared host heritability of reactions to pathogens for the two methods. Point and spray inoculations resulted in similar mean disease severities among host genotypes, while the most important source of variance was observed between inoculation methods, reflecting the different disease severities achieved with the two methods.

We observed differential FHB responses within the diverse wheat genotypes analysed. To our knowledge, there are no published studies on the FHB reactions of 'Palesio' and 'Marco Aurelio' wheat cultivars, so our results cannot be compared to others. Amoriello *et al.* (2018) screened a number of durum wheat cultivars, including 'Claudio', for resistance to DON contamination, and 'Claudio' was one of the most contaminated. The ability to degrade DON has been described as Type III FHB resistance, and tolerance of high DON concentrations as Type IV resistance'.(Mesterházy, 1995; 2002; Gunupuru *et al.*, 2017). We cannot associate the susceptibility we observed as resulting from high DON accumulation, since we did not analyse mycotoxin content as an FHB resistance factor.

Regarding the Type II tolerance observed in 'Palesio', it is known that bread wheat is naturally more resistant than durum wheat, because of its hexaploid nature (Buerstmayr *et al.*, 2014; Haile *et al.*, 2019). Nevertheless, the most interesting FHB Type II responses were observed in 'Marco Aurelio' durum wheat. Sources of resistance are limited in durum wheat (Stack *et al.*, 2002) and they reside mainly in other cultivated tetraploid wheat subspecies, such as ancient cereal crops (Oliver *et al.,* 2008). Some of these old cereal crops are *Triticum*

turgidum subspecies, such as *T. turgidum* subsp. *turgidum*, which is native to Mediterranean countries, as is 'Marco Aurelio'. It can therefore be assumed that the Italian wheat genotype 'Marco Aurelio' possess some FHB resistance characteristics that also occur in the ancient cereals.

Results from fungal biomass quantification of the wheat chaff and rachides between the two inoculation techniques reflect the results obtained from the phenotypic evaluations of disease severity. When spray inoculated, wheat cultivars gave better contrast of pathogen spread, than when they were point inoculated. Kumar *et al.* (2015) assessed a Real-Time *q*PCR technique to detect and quantify *F. graminearum* biomass in rachides of barley and wheat resistant and susceptible cultivars, using the *Tri6* gene for fungal quantification and the *Actin* gene for the normalization, as done here. They observed that disease severity could not discriminate resistance before 9 dpi, while spikelet resistance was discriminated in all the wheat and barley genotypes tested, based on *q*PCR quantification of the fungal biomass. We therefore conclude that the wheat cultivars used in our study possess levels of tolerance to pathogen colonisation when spray inoculated, but not when point inoculated. These results agree with those of Brennan *et al.* (2005), who showed that visual disease assessment clearly reflected yield losses, but that no significant relationship was present between symptom severity and fungal DNA content in grain.

The phenotypic FHB scoring after spray inoculation at 2,500 c/s (disease incidence and severity) revealed that 'Claudio' durum wheat reached 100% of diseased spikes and spikelets sooner than the other cultivars, and was also likely best limit pathogen spread. The rapid bleaching of spikes but low amounts of fungal DNA could be explained in several ways. During pathogen infection and FHB development, plant vessels become blocked, preventing water and nutrient supplies as a defence mechanism, and causing sudden spike wilting (Kang and Buchenauer, 2000; Kheiri *et al.*, 2019). head bleaching due to natural absence of water and nutrients could be confused with FHB symptoms (Zwart *et al.*, 2008). Regarding the low amount of fungal biomass present in the chaff and rachides, it is known that DON is an important virulence factor that facilitates infection spread (Bai *et al.*, 2002; Jansen *et al.*, 2005). Ilgen *et al.* (2009) observed that the *Tri5* gene, which controls the trichodeine synthase involved in DON synthesis, is highly induced in the transit zone of host rachis nodes, where the rachilla and rachides divide. It could be possible that wheat genotypes possessing Type III or Type IV resistances (ability to degrade DON and to tolerate high DON levels) will give contrasting pathogen spread into the host vessels. TKW evaluation revealed that 'Marco Aurelio' was the least damaged wheat genotype, after spray and point inoculations. Resistance evaluation concentrates on visual head disease symptoms, since most QTL analyses have evaluated this trait. The TKWs were generally neglected in early wheat breeding programmes for resistance to FHB. Resistance to kernel infection arises from the premise that those genotypes should have a resistance type that does not affect levels, but their TKW values differ significantly. Therefore, it is not by chance that yield stability was developed as a major trait in plant breeding. However, FHB resistance, like yield, is governed by many QTLs, and infection severity has a strong impact on yield. It is therefore necessary to consider TKW in FHB resistance screening protocols (Canci *et al.*, 2004; Mesterházy *et al,.* 2015).

Current wheat breeding programmes for FHB resistance focus more on Type II than Type I resistance (Buerstmayr *et al.*, 2003; Burlakoti *et al.*, 2010; Xiao *et al.*, 2016). Type II resistance has also been reported to be more genetically and environmentally stable than Type I resistance, and provide a more reliable indication of cultivar resistance (Bai and Shaner, 2004). It is desirable, when possible, to replicate FHB resistance testing within and across environments (years and/or locations), in order to obtain meaningful results and to assess reproducibility of the data obtained (Buerstmayr *et al.*, 2014). Different FHB screening methods do not provide answer to the same question. FHB traits differ, and visual symptoms, fungal biomass and TKWs do not closely follow the similar patterns (Mesterházy *et al.,* 2015). No genotype in the present study was completely resistant to FHB. Thus, most genotypes probably quantitatively combine different levels of FHB resistances. This supports the assumption that FHB resistance types are probably governed by different loci and measure different resistance reactions, as has been suggested by Schroeder and Christensen (1963). For routine screening of FHB resistance in large breeding populations, a fast, cheap, and reliable inoculation method is desirable. Spray inoculation is advantageous over point inoculation. It is based on whole-plot inoculation (Martin *et al.*, 2017), which has similarity to natural disease situations and requires less time and labour for inoculation and disease assessments. Spray inoculation can also be useful to establish rapid and low cost assays to evaluate FHB resistance using wheat seedlings (Soresi *et al.*, 2015). However, spray inoculation is environmentally influenced, resulting in high variability and less replicable results. Point inoculation mimics infection through insects, but it is genetically and environmentally stable (Imathiu *et*

al., 2014). From our experience, we suggest, when possible, that both inoculation methods are used, and that as many FHB characteristics as possible are evaluated, since we observed differences in responses within inoculation techniques and the parameters assessed. In addition, screening different FHB parameters allowed us to recognize the FHB responses in 'Marco Aurelio', one of the most cultivated durum wheat cultivars in Italy.

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New or Unusual Disease Reports

First report of *Erysiphe elevata* causing powdery mildew on *Catalpa bignonioides* in Montenegro

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Summary. The first record of powdery mildew caused by *Erysiphe elevata* in Montenegro is presented in this paper. The fungus was detected on leaves, fruits and flowers of *Catalpa bignonioides,* causing severe disease of some trees, and was identified on the basis of morphological and molecular characteristics. In pathogenicity tests, inoculation of leaves of healthy young plants of southern catalpa resulted in typical powdery mildew symptoms.

Keywords. Catalpa trees, invasive fungal pathogen, morphological and molecular characterisation.

INTRODUCTION

Catalpa bignonioides is a tree native to the south-eastern United States. It is known as southern catalpa (Olsen *et al*., 2006). These trees are planted as ornamentals in all temperate areas of the world, especially eastern North America and Europe (Olsen and Kirkbride Jr, 2017). The species is an important decorative plant providing urban greenery in central and southern parts of Montenegro. The trees are usually located along footpaths, in parks or allées.

Powdery mildews are common plant diseases, but some of these pathogens have become invasive due to their introduction to, and spread throughout, new territories. This is the case with *Erysiphe elevata* (syn. *Microsphaera elevata* Burrill), a well-known species in North America (Braun, 1987) that appeared recently in Europe and caused severe infections on *Catalpa bignonioides* trees in some European countries (Ale-Agha *et al*., 2004; Kiss, 2005) and Asia (Cho *et al*., 2014). The first report of the pathogen on *Catalpa* trees in Europe was from Hungary in 2002 (Vajna *et al*., 2004). Since then there have been several reports from different European countries: including the

United Kingdom, Slovakia, Slovenia, Romania, the Czech Republic, Germany, Switzerland, Ukraine and Turkey (Ale-Agha *et al.*, 2004; Cook *et al.*, 2004; Milevoj, 2004; Pastirčakova *et al.*, 2006; Heluta *et al.*, 2009; Fodor and Vlad, 2013; Erper *et al.*, 2018).

During 2016 to 2018 in central and southern parts of Montenegro, powdery mildew symptoms were observed on numerous *Catalpa bignonioides* plants in parks, along footpaths and also in a nursery. The aim of the present study was to identify the causal agent of the disease.

MATERIALS AND METHODS

Morphology and pathogenicity tests

In the period of 2016 to 2018, flowers, leaves and fruits of *Catalpa bignonioides* plants were collected from two localities in Podgorica (city in central Montenegro), where powdery mildew symptoms were observed. Samples were examined using a microscope (Axioskop 2 Plus, Zeiss) equipped with a Zeiss Axiocam ERc 5s camera, which was operated using the AxioVision release 4.8.2 software. Morphological features of the anamorph and teleomorph stages of the fungus were studied.

A voucher specimen was deposited in the Plant Pathology Herbarium at the Biotechnical Faculty in Podgorica, Montenegro and in the phyto-pathological herbarium of the Slovenian Institute of Hop Research and Brewing. Pathogenicity tests were performed according to the method described by Cho *et al.* (2014), inoculating the leaves of four healthy young southern catalpa plants with conidia scraped off diseased leaves. Four control plants were not inoculated. All plants were maintained in a laboratory at 24 to 28°C, not bagged, and under natural photoperiod (15h light / 9h dark).

DNA extraction, PCR, sequencing and data analysis

Genomic DNA was extracted using the CTAB method (Weising *et al.*, 1991) from mycelium and conidia obtained from infected leaves of two representative samples (IHPS-F46 and IHPS-F47). PCR was carried out using the internal transcribed spacer (ITS) region primers ITS1/ITS4 (White *et al.*, 1990) and ITS5/P3 (Takamatsu *et al.*, 2009). The PCR products were subjected to both strand direct Sanger sequencing by the commercial sequencing service Eurofins Genomics, Germany. Consensus sequences were assembled using CodonCode Aligner 8.0.1 (United States of America), and submitted to GenBank under the following accession numbers: MK253282 (isolate IHPS-F46, primers ITS1/ITS4), MK253283 (isolate IHPS-F46, primers ITS5/P3), MK253284 (isolate IHPS-F47, primers ITS1/ITS4) and MK253285 (isolate IHPS-F47, primers ITS5/P3). To confirm the identity of the isolates, the BLAST search algorithm was used for sequence comparison in the GenBank nucleotide database. Sequence alignments were made using the MUSCLE algorithm, and phylogenetic analysis was conducted in MEGA6 (Tamura *et al.*, 2013) using the Maximum Likelihood method based on the Jukes-Cantor model performing 10,000 bootstraps.

RESULTS AND DISCUSSION

In the surveyed localities, symptoms on trees (Figure 1) were mostly expressed on leaves, but also on flowers and fruit. Symptoms on leaves included greyish white powdery zones on the upper leaf surfaces, and were especially visible on younger leaves, which were totally covered by ashy coatings. Leaves with powdery mildew became deformed because of growth inhibition in the colonized areas. On older foliage, many chasmo-

Figure 1. Symptoms of powdery mildew on severely-affected catalpa tree.

Figure 2. Brown necrosis of infected catalpa flowers.

Figure 4. Chasmothecium of *Erysiphe elevata* with long appendages.

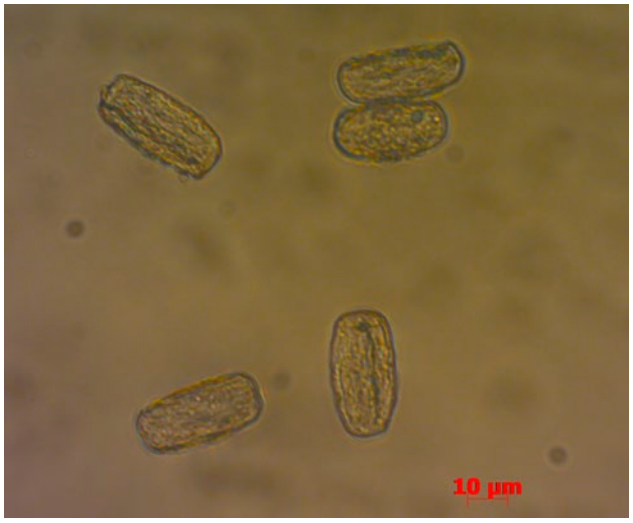

Figure 3. Conidia of *Erysiphe elevata*.

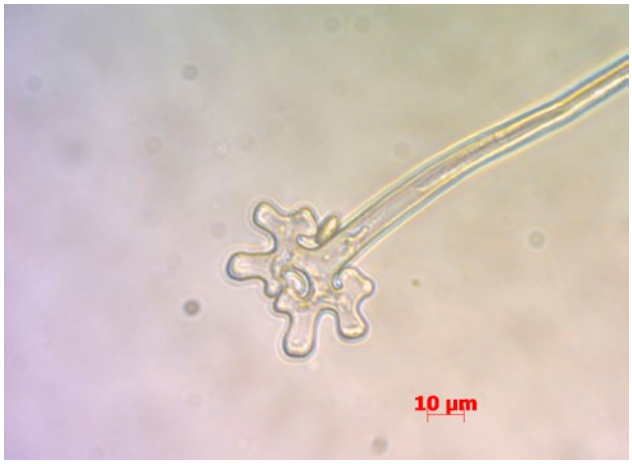

Figure 5. Dichotomously branched tip of appendage in *Erysiphe elevata*.

thecia were seen on the adaxial surfaces of the leaves. The infections led to premature defoliation, leaving bare branches with fruit. Infected flowers were mostly found in June, and they were brownish in colour (Figure 2). Diseased fruit, observed mostly in September, were discoloured, with white, bleached areas, and were cracked if severely affected. The infected plants had reduced growth and reduced decorative value.

Microscopic examinations of the samples revealed the presence of anamorph and teleomorph stages of a powdery mildew fungal pathogen. Mycelium was epiphytic with colourless, branched and septate hyphae. Cylindrical to elliptical conidia (Figure 3) were hyaline and 22 to 39 µm (mean = 29.9 µm) × 12 to 20 µm (mean = 15.0 µm). Conidia were mostly found on the upper leaf surfaces, but were also detected on flowers and fruit of catalpa trees. Chasmothecia were numerous, dark brown to black, globose, 91 to 130 µm (mean = 103.7 µm) in diameter, with several appendages whose lengths were 5-6 times greater than the diameters of the chasmothecia (Figure 4). Chasmothecia were abundant, scattered or in groups on the adaxial leaf surfaces, but rarely on fruit. Appendages were hyaline, slightly thicker towards the bases and ending in dichotomously branched tips (Figure 5). The chasmothecia contained four to seven asci (Figure 6), which were sessile or on short stalks (Figure 7a and b), and measured 29 to 64 µm (mean = 55.0 µm) × 27 to 44 µm (mean = 34.2 µm), and each ascus contained 4 to 6 ascospores. Ascospores were ellipsoid to ovoid, measuring 20 to 29 µm (mean = 24.1 µm) × 11 to 15 µm (mean = 12.8 µm) (Figure 8).

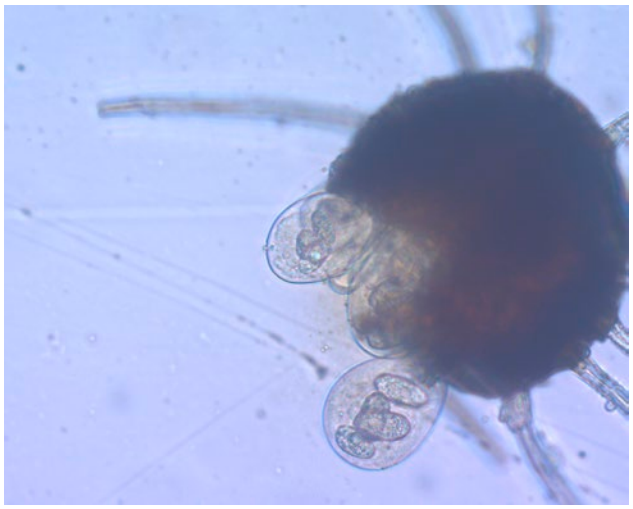

Figure 6. Chasmothecium of *Erysiphe elevata* containing asci with ascospores.

Pathogenicity tests gave positive results, as powdery mildew colonies appeared on the leaves 6 days after inoculation (Figure 9). The leaves of the non-inoculated control plants remained symptomless.

Although seven different species of powdery mildews have been recorded on *Catalpa* spp., only two are host-specific to catalpa: *Erysiphe catalpae* Simonian and *E. elevata* (Burrill) U. Braun & S. Takam. (Olsen *et al.*, 2006). A detailed study of differentiation between these two species was carried out by Ale-Agha *et al.* (2004), who showed that the anamorph and the teleomorph of *E. catalpae* can be distinguished from *E. elevata*. The anamorph of *E. elevata* is sparsely developed, while in *E. catalpae* the anamorph develops abundant conidia. However, for morphological identification, the length and branching of the chasmothecium appendages are key taxonomic characteristics. Ascomata in *E. elevata* are abundant, with long flexuous appendages, which are (1) 2–4 (6) times as long as the ascoma diameter. The appendages have dichotomously branched apices, while ascomata in *E. catalpae* are rarely formed, and their appendages are short, 0.5–1.5 times as long as the ascoma diameter and have unbranched apices.

Based on the morphological features of the fungus studied in our research, the pathogen was tentatively identified as *Erysiphe elevata*. Dimensions of conidia, chasmothecia, asci and ascospores also coincided with the observations of other authors (Braun, 1987; Ale-Agha *et al.*, 2004; Cook *et al.*, 2004; Vajna *et al.*, 2004; Pastirčakova *et al.*, 2006).

Species identity was confirmed by molecular analysis of two representative isolates (IHPS-F46 and IHPS-F47), carried out using the internal transcribed spacer (ITS) region primers ITS1/ITS4 and ITS5/P3. Both primer sets gave PCR products which were subjected to direct sequencing. BLAST analysis of the 646-bp ITS1/ITS4 (MK253282, MK253284) and 715-bp ITS5/P3 (MK253283, MK253285) sequences revealed a 99% similarity with several *E. elevata* sequences (Figure 10) available in GenBank. The four closest Genbank sequences were included in the phylogenetic analysis, together with the sequences derived in this study and sequences of *E. catalpae* and *Neoerysiphe galeopsidis*, which are two additional powdery mildew species pathogenic to *C. bignonioides*. The consensus tree (Figure 10) grouped the Montenegro isolates together with other *E. elevata* isolates (100% bootstrap value). This confirmed the previous findings of differentiation of *E. elevata* from *E. catalpae* and *Neoerysiphe galeopsidis* (Cook *et al.*, 2006).

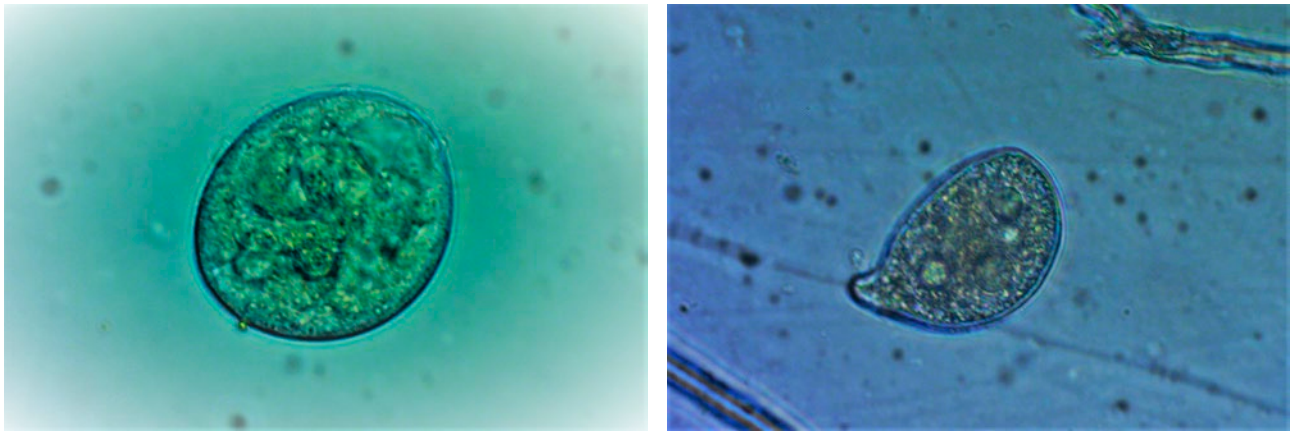

Figure 7. Asci of *Erysiphe elevata*, sessile (a) or on a short stalk (b).

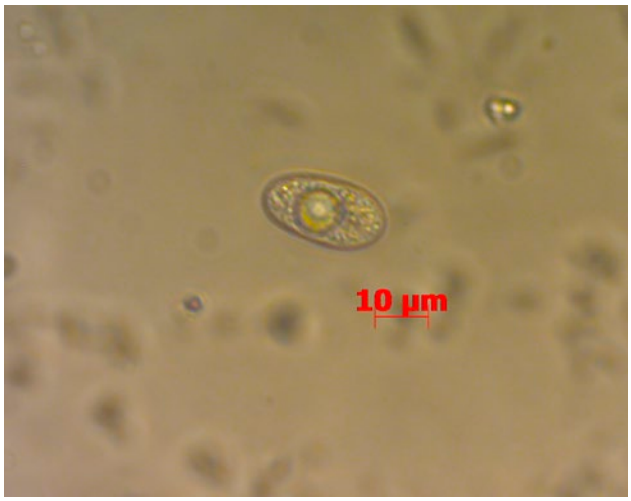

Figure 8. Ascospore of *Erysiphe elevata*.

Figure 9. Powdery mildew colonies developed on inoculated catalpa leaves.

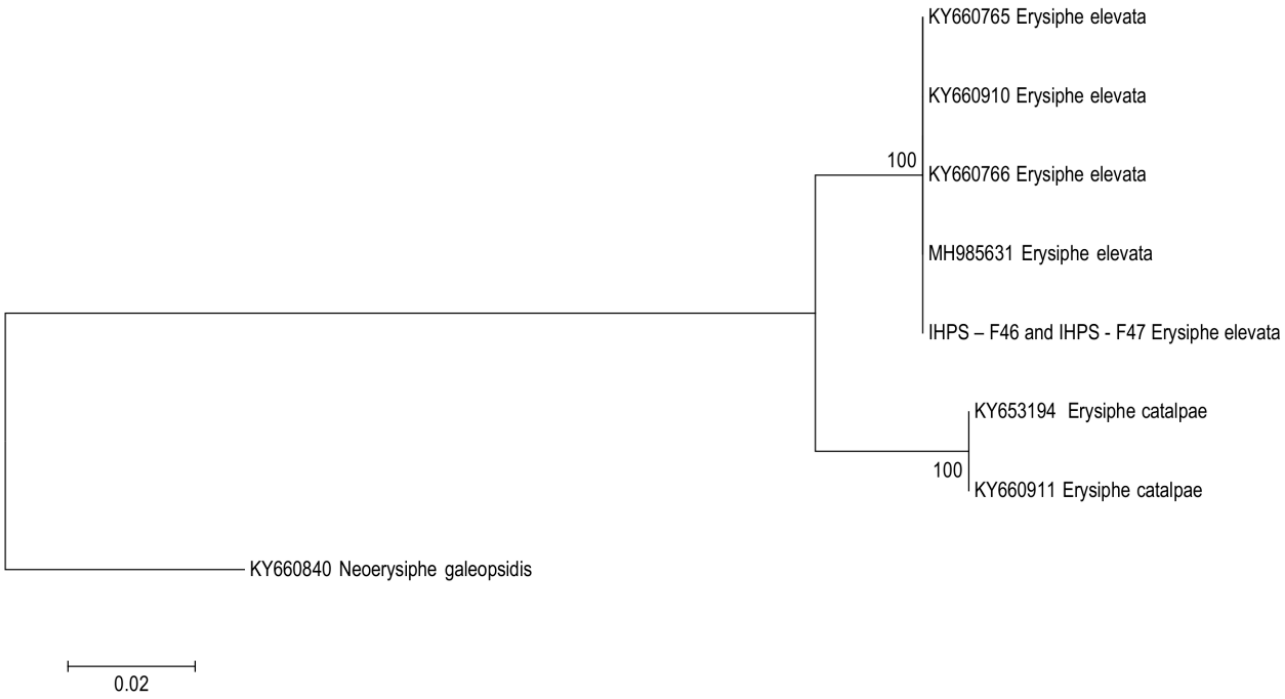

Figure 10. Neighbour-joining phylogenetic tree of *Erysiphe elevata* isolates IHPS-F46 and IHPS-F47, based on rDNA – ITS sequences. Numbers at the nodes indicate bootstrap values generated from 10,000 replicates. The scale bar indicates the number of nucleotide substitutions.

This is the first report of *Erysiphe elevata* causing powdery mildew of *Catalpa bignonioides* in Montenegro. This confirms the further spread of *E. elevata* in Europe. Appropriate control measures need to be taken to prevent or minimize the damage caused by this pathogen to catalpa trees, which are important ornamental trees.

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New or Unusual Disease Reports

Characterization of *Eutypa lata* and *Cytospora pistaciae* causing dieback and canker of pistachio in Italy

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Summary. During the winter of 2017, dieback and canker symptoms were observed on pistachio (*Pistacia vera*) in two orchards in the Bronte area, Catania Province, Sicily, Southern Italy. Two different fungi were consistently isolated from infected tissues. Morphological observations and multi-locus phylogenies using five genomic loci (ITS, *act*, *rpb2*, *tef1* and *tub2*) identified these fungi as *Cytospora pistaciae* and *Eutypa lata*. Pathogenicity tests on 5-y-old potted plants of *P. vera* grafted on terebinth (*P. terebinthus*) reproduced similar symptoms as those observed in nature, and Koch's postulates were fulfilled for these two pathogens. This study is the first to report dieback and canker diseases of pistachio caused by *C. pistaciae* and *E. lata* in Italy.

Keywords. Pathogenicity, molecular analysis, disease symptoms, *Pistacia vera*.

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INTRODUCTION

Pistachio is cultivated in the southern regions of Italy, of which Sicily is the main production area. The province of Catania (with 430 ha of pistachio), followed by the provinces of Caltanissetta (with 220 ha) and Agrigento (with 145 ha) are the largest pistachio-producing areas, with a total production of 3,878 tons (AGRISTAT, 2017). Currently, the commune of Bronte in Catania Province represents the most important area of Sicily for pistachio production, and pistachio is an important economic resource for this territory (Barone and Marra, 2004). In this area, different pistachio cultivars are grafted on terebinth plants which are grown on volcanic soils (Barone *et al*., 1985). Few studies have been conducted to investigate pistachio diseases occurring in Italy, and only a few diseases have been reported to date. These include branch dieback (caused by *Botryodiplodia* sp.), leaf spot (*Alternaria alternata*), anthracnose, branch and twig cankers (*Botryosphaeria dothidea*)

and phylloptosis and leaf spots (mainly caused by *Septoria pistaciae*) (Casalicchio, 1963; Schilirò and Privitera, 1988; Frisullo *et al*., 1996; Vitale *et al*., 2007). In eastern Sicily, cankers and decline caused by *Liberomyces pistaciae* Voglmayr, Vitale, Aiello, Guarnaccia, Luongo & Belisario are the most important pistachio diseases (Vitale *et al*., 2018). Blight caused by *Arthrinium xenocordella* Crous was also recently reported on pistachio fruit in the Agrigento Province (Aiello *et al*., 2018).

During the winter of 2017, pistachio trees with dieback, canker and gummosis symptoms were observed in the area of Bronte. Following culturing from necrotic tissues, two fungal species were consistently isolated. Cankers from one orchard generated colonies of *Cytospora* while cankers from a second orchard generated *Eutypa* colonies.

The aim of the present study was to investigate the etiology of pistachio canker diseases, which could represent new threats for the pistachio production of Sicily.

MATERIALS AND METHODS

Isolation and morphology of fungi

Surveys were conducted in ten pistachio orchards with histories of branch canker and dieback in eastern Sicily (Catania Province). Approximately 20 symptomatic pistachio branches with canker were collected from each orchard for analyses. Sub-cortical and wood fragments (about 5 × 5 mm) were cut from the margins between affected and healthy branch tissues. Tissue pieces were disinfected in 1.2% sodium hypochlorite for 60 s, rinsed in sterile water and dried on sterile filter paper. The fragments were then placed into Petri plates containing potato dextrose agar (PDA, Oxoid) amended with 100 mg L^{-1} of streptomycin sulfate (Sigma-Aldrich), and incubated at room temperature (25 ± 5°C). Fungal colonies consistently growing from symptomatic tissues were cultured into new PDA plates. To obtain pure cultures, single-conidium or hyphal-tip isolations were performed after 1 month incubation at room temperature under natural light conditions. Isolates for each putative fungal pathogen (four isolates of *Eutypa* and three of *Cytospora*) were characterized by morphological, molecular and phylogenetic analyses (Table 1). These cultures were deposited in the working collection of Dr Pedro Crous (CPC), at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands (Table 1). Size and shape of conidia were recorded for each fungal isolate grown on PDA for 2 weeks at 25 ± 1°C.

DNA extraction, PCR amplification and sequencing

Extractions of genomic DNA were performed from pure cultures, as reported elsewhere (Guarnaccia and Crous, 2017), using the Wizard Genomic DNA Purification Kit (Promega Corporation). Partial regions of five loci were amplified. The primers ITS5 and ITS4 (White *et al*., 1990) were used to amplify the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA operon, including the 3' end of the 18S rRNA, the first internal transcribed spacer region, the 5.8S rRNA gene; the second internal transcribed spacer region and the 5' end of the 28S rRNA gene. The primers ACT-512F and ACT-783R (Carbone and Kohn, 1999) were used to amplify part of the actin gene (*act*). The partial beta-tubulin (*tub2*) gene was amplified with primers Bt-2a and Bt-2b (Glass and Donaldson, 1995). The primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) were used to amplify part of the translation elongation factor 1-α gene (*tef1*). The primers 5f2/7cr were used to amplify part of *rpb2* (O'Donnell *et al*., 2010). The regions ITS, *act*, *tef1* and *rpb2* were amplified for the species of *Cytospora* using the PCR programmes adopted by Lawrence *et al*. (2018) and Jami *et al*. (2018). The regions ITS and *tub2* were amplified for the species of *Eutypa* following the PCR programmes used by Moyo *et al*. (2018a). The PCR products were sequenced in both directions using the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems Life Technologies), after which amplicons were purified through Sephadex G-50 Fine columns (GE Healthcare) in MultiScreen HV plates (Millipore). Purified sequence reactions were analyzed on an Applied Biosystems 3730xl DNA Analyzer (Life Technologies). The DNA sequences generated were analyzed and consensus sequences were computed using the program SeqMan Pro (DNASTAR).

Phylogenetic analyses

Novel sequences generated in this study were blasted against the NCBIs GenBank nucleotide database, to determine the closest relatives to be included in the phylogenetic analyses. Blast analyses indicated that three isolates belonged to *Cytospora* and the remaining four to *Eutypa*. Sequence alignments of the different gene regions, including sequences obtained from this study and sequences from GenBank, were initially performed using the MAFFT v. 7 online server (http://mafft.cbrc.jp/alignment/server/index. html) (Katoh and Standley, 2013), and then manually adjusted in MEGA v. 7 (Kumar *et al*., 2016). To establish the identity of the fungal isolates, phylogenetic analyses were conducted using

one locus (data not shown) as well as concatenated analyses of four loci (ITS, *act*, *tef1* and *rpb2*) for *Cytospora* spp. and two loci (ITS and *tub2*) for *Eutypa* spp., as indicated by blast analysis. Additional reference sequences were selected based on recent studies on *Cytospora* and *Eutypa* species (Lawrence *et al*., 2018, Moyo *et al*., 2018a, b). Phylogenetic analyses were based on Maximum Parsimony (MP) for all the individual loci and for the multi-locus analyses. The MP analyses were carried out using PAUP (Swofford, 2003). Phylogenetic relationships were estimated by heuristic searches with 100 random addition sequences. Tree bisection-reconnection was used, with the branch swapping option set on 'best trees' only, with all characters weighted equally and alignment gaps treated as fifth state. Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC) were calculated for parsimony and the bootstrap analyses (Hillis and Bull 1993) were based on 1,000 replicates. Sequences generated in this study were deposited in GenBank (Table 1).

Pathogenicity of representative isolates

Pathogenicity tests with one representative isolate of *C. pistaciae* (CPC34208) and one of *E. lata* (CPC34213; Table 1) were carried out to satisfy Koch's postulates. These tests were carried out in a growth chamber maintained at 25 ± 1°C. Potted 5-y-old plants of *P. vera* grafted onto *P. terebinthus* were used for artificial inoculations. Three plants were inoculated with each isolate. Six wounds were made on individual plant stems approx. 8-10 cm apart from each other.

Inoculations were made on stems after removing of bark discs with a cork borer, placing a 5 mm plug from a 14-d-old PDA culture of test isolate into the wound and covering with Parafilm® (Pechney Plastic Packaging Inc.) to prevent desiccation. An equivalent number of plants and inoculation sites were inoculated with sterile PDA plugs to serve as controls. The inoculated plants were observed once each month for symptoms development, and a final assessment was conducted 5 months after inoculation. To fulfil Koch's postulates, re-isolations were carried out following the procedure described above, where tissue fragments were plated onto PDA. Each re-isolated fungus was identified through morphological characteristics.

RESULTS AND DISCUSSION

Symptomatic plants showed cankers with cracking and gum exudation, and often branches or shoots showed dieback. Under the bark of affected branches, cankers were characterized by discolouration and necrosis, and in some cases discolouration extended to the vascular tissue (xylem) and pith. Two different fungal colony types were consistently obtained from isolations from symptomatic tissues (Figure 1) taken from the two orchards. Cankers from one orchard generated *Cytospora* colonies while cankers from the other orchard generated *Eutypa* colonies. The same symptoms in the remaining orchards investigated in the Bronte area produced colonies of *L. pistaciae* (Vitale *et al*., 2018).

Conidia of three representative isolates of *Cytospora* were in accordance with the description by Lawrence *et al*. (2018) of *C. pistaciae* Lawr., Holland & Trouillas. The four MP trees derived from the single gene sequence alignments (ITS, *act*, *tef1* and *rpb2*) were topologically similar, confirming that the three isolates used for the molecular analyses were *Cytospora*. The combined phylogeny of *Cytospora* species consisted of 35 sequences, including the outgroup sequences of *Diaporthe limonicola* (culture CBS 142549; Guarnaccia and Crous, 2017). A total of 2,056 characters (ITS: 1–574, *act*: 581–890, *tef1*: 897–1289, *rpb2*: 1296–2056) were included in the phylogenetic analysis of *Cytospora* spp. For the phylogeny of *Cytospora* species, 489 characters were parsimony-informative, 336 were variable and parsimony-uninformative and 1,213 characters were constant. A maximum of 1,000 equally most parsimonious trees were saved (Tree length = 1 552, CI = 0.743, RI = 0.782 and RC = 0.581). Bootstrap support values from the parsimony analysis were plotted on the phylogenetic trees presented in Figure 2. In the combined analyses, the three representative isolates clustered with four reference strains of *C. pistaciae*. The individual alignments and trees of the single loci used in the analyses were compared with respect to their performance in species recognition. *Cytospora pistaciae* was differentiated and identified in all single-gene analyses.

Cytospora terebinthi Bres. has been reported in Italy as the causal agent of cankers and gummosis of pistachio (Corazza *et al*., 1990; Furnitto, 1984), while other *Cytospora* species have been reported in other crops, including peach (Hampson and Sinclair., 1973; Banko and Helton, 1974). The taxonomy of *Cytospora* species associated with fruit and nut crops was recently revised, and *C. pistaciae* was described as a new species on pistachio in California, but the pathogenicity of this species was not investigated (Lawrence *et al*. 2018).

Conidia of four isolates of *Eutypa* were in accordance with the description of *E. lata* by Moyo *et al*. (2018b). The two MP trees derived from the single gene sequence alignments (ITS and *tub2*) were topologi-

cally similar, and this confirmed that the four isolates used in this study were *Eutypa*. All the species belonging to *Eutypa* and other Xylariales used in the multilocus phylogeny consisted of 29 sequences with the outgroup sequences of *L. pistaciae* (CBS 144255; Vitale *et al*., 2018). A total of 1,076 characters (ITS: 1–582, *tub2*: 589–1,076) were used for the Xylariales analysis, and 453 characters were parsimony-informative, 166 were variable and parsimony-uninformative and 451 characters were constant. A maximum of 1,000 equally most parsimonious trees were saved (Tree length = 1 669, CI = 0.648, RI = 0.786 and RC = 0.509). Bootstrap support values from the parsimony analysis were plotted on the phylogenetic trees presented in Figure 3. In the combined analyses, the four isolates were related to reference isolates of *E. lata*. The individual alignments and trees of the single loci used in the analyses were compared with respect to their performance in species recognition. *Eutypa lata* was differentiated and identified in all single-gene analyses.

Eutypa lata is a pathogen with a wide host range, occurring in more than 160 hosts (Farr and Rossman, 2017). In Italy, *E. lata* has been reported on *Acer* sp. in Sicily (Greuter *et al*., 1991), *Ribes rubrum* (Prodorutti *et al*., 2008), olive trees (Tosi and Natalini, 2009) and *Vitis vinifera* (Acero *et al*., 2004). Eutypa dieback and gummosis of pistachio caused by *E. lata* has been reported only in Greece (Rumbos, 1986).

Five months after artificial inoculation, symptoms produced from each fungus in trees were similar to those present on trees in the field. These consisted of external cankers and gumosis produced around the inoculation sites, with small cracks present in each sunken lesion. After removing the bark, a dark discolouration and necrotic tissues were visible (Figure 1). The respective inoculated pathogens were re-isolated from symptomatic tissues, thus fulfilling Koch's postulates. No symptoms were observed on control (uninoculated) plants.

This is the first report of *E. lata* and *C. pistaciae* associated with cankers on pistachio in Europe. Further

Figure 1. Symptoms reproduced from mycelial plug inoculation with *Cytospora pistaciae* (a) and *Eutypa lata* (b) on 5-y-old potted plants of *Pistacia vera* 5 months after inoculation with respective fungi. Cultural characteristics of *Cytospora pistaciae* (c) and *Eutypa lata* (d) colonies grown on PDA are also illustrated.

Table 1. Collection details and GenBank accession numbers for isolates included in this study.

Species	Culture No.	Host	Locality	GenBank Number[a]				
				ITS	*act*	*tef1*	*rpb2*	*tub2*
Cytospora austromontana	**CMW 6735**	*Eucalyptus pauciflora*	Australia	NR137542	-	-	-	-
C. berkeleyi	**StanfordT3T**	*Eucalyptus globulus*	California, USA	AY347350	-	-	-	-
C. californica	**9c–24 = CBS 144234**	*Juglans regia*	California, USA	MG971935	MG972083	MG971645	-	-
	KARE264	*Pistacia vera*	California, USA	MG971920	MG972069	MG971630	-	-
C. cincta	CFCC 89956	*Prunus cerasifera*	China	KR045624	-	-	KU710953	-
C. cinereostroma	**CMW 5700**	*Eucalyptus globulus*	Chile	AY347377	-	-	-	-
C. diatrypelloidea	**CMW 8549**	*Eucalyptus globulus*	Australia	AY347368	-	-	-	-
C. disciformis	**CMW 6509**	*Eucalyptus grandis*	Uruguay	AY347374	-	-	-	-
C. eriobotryae	**IMI136523**	*Eriobotrya japonica*	India	AY347327	-	-	-	-
C. eucalypticola	**ATCC 96150**	*Eucalyptus nitens*	Tasmania, Australia	AY347358	-	-	-	-
	CMW 5309	*Eucalyptus grandis*	Entebbe, Uganda	AF260266	-	-	-	-
	CMW 40051	*Eucalyptus camaldulensis*	Zimbabwe	KF923249	-	-	-	-
	CMW 40048	*Eucalyptus camaldulensis*	Zimbabwe	KF923248	-	-	-	-
C. gigaspora	**CFCC 89634**	*Salix psammophila*	China	KF765671	KU711000	-	KU710960	-
C. granati	**CBS 144237**	*Punica granatum*	USA	MG971799	MG971949	MG971514	-	-
C. joaquinensis	**CBS 144235**	*Populus deltoides*	USA	MG971895	MG972044	MG971605	-	-
C. leucostoma	CFCC 50015	*Sorbus pohuashanensis*	China	KR045634	-	-	-	-
C. nivea	MFLUCC 15–0860	*Salix acutifolia*	Russia	KY417737	KU711006	-	KY417805	-
C. parapistaciae	KARE232	*Pistacia vera*	California, USA	MG971807	MG971957	MG971522	-	-
	KARE268	*Pistacia vera*	California, USA	MG971806	MG971956	MG971521	-	-
	KARE269	*Pistacia vera*	California, USA	MG971805	MG971955	MG971520	-	-
	KARE270 = CBS 144506	*Pistacia vera*	California, USA	MG971804	MG971954	MG971519	-	-
C. parasitica	**MFLUCC 15–0507**	*Malus domestica*	Russia	KY417740	-	-	KY417808	-
C. pistaciae	KARE441	*Pistacia vera*	California, USA	MG971800	MG971950	MG971515	-	-
	KARE442	*Pistacia vera*	California, USA	MG971803	MG971953	MG971518	-	-
	KARE443 = CBS 144238	*Pistacia vera*	California, USA	MG971802	MG971952	MG971517	-	-
	KARE444	*Pistacia vera*	California, USA	MG971801	MG971951	MG971516	-	-
	CPC 34208 = CBS 144226	*Pistacia vera*	Italy	*MN078066*	*MN078063*	*MN078077*	*MN078080*	-
	CPC 34209	*Pistacia vera*	Italy	*MN078067*	*MN078064*	*MN078078*	*MN078081*	-
	CPC 34211	*Pistacia vera*	Italy	*MN078068*	*MN078065*	*MN078079*	*MN078082*	-
C. punicae	5A-80 = CBS 144244	*Punica granatum*	USA	MG971943	MG972091	MG971654	-	-
C. sacculus	CFCC 89624	*Juglans regia*	China	KR045645	-	-	KU710976	-

(Continued)

Table 1. (Continued).

Species	Culture No.	Host	Locality	GenBank Number[a]				
				ITS	*act*	*tef1*	*rpb2*	*tub2*
C. salicacearum	**MFLUCC 16–0509**	*Salix alba*	Russia	KY417746	-	-	KY417814	-
C. salicicola	**MFLUCC 14–1052**	*Salix alba*	Russia	KU982636	-	-	-	-
Cryptosphaeria subcutanea	CBS 240.87	*Salix borealis*	Norway	KT425232	-	-	-	KT425167
Diaporthe limonicola	**CBS 142549**	*Citrus limon*	Malta	MF418422	-	MF418501	MH797629	-
Diatrypella atlantica	**HUEFS 194228**	unknown	Brazil	KM396615	-	-	-	KR363998
Eutypa cremea	CBS 120837	*Prunus salicina*	South Africa	KY752762	-	-	-	KY752791
Eutypa lata	CBS 121430	*Prunus armeniaca*	South Africa	KY752766	-	-	-	KY752794
	ADSC300	*Schinus molle*	Australia	HQ692610	-	-	-	HQ692493
	SACEA01	*Ceanothus* sp.	Australia	HQ692615	-	-	-	HQ692499
	EP18	*Vitis vinifera*	Australia	HQ692611	-	-	-	HQ692501
	CPC 34213	*Pistacia vera*	Italy	*MN078069*	-	-	-	*MN078073*
	CPC 34214	*Pistacia vera*	Italy	*MN078070*	-	-	-	*MN078074*
	CPC 34215	*Pistacia vera*	Italy	*MN078071*	-	-	-	*MN078075*
	CPC 34216	*Pistacia vera*	Italy	*MN078072*	-	-	-	*MN078076*
Eutypa leptoplaca	ADFIC100	*Ficus macrophylla*	Australia	HQ692608	-	-	-	HQ692485
Eutypa maura	CBS 219.87	*Acer pseudoplatanus*	Switzerland	AY684224	-	-	-	DQ006967
Eutypa tetragona	CBS 284.87	*Sarothamnus scoparius*	France	DQ006923	-	-	-	DQ006960
Eutypella citricola	STEU 8098	*Vitis vinifera*	South Africa	KY111634	-	-	-	KY111588
Eutypella microtheca	STEU 8107	*Vitis vinifera*	South Africa	KY111629	-	-	-	KY111608
Eutypella vitis	MSUELM13	*Vitis vinifera*	USA	DQ006943	-	-	-	DQ006999
Liberomyces pistaciae	**CBS 144225**	*Pistacia vera*	Italy	MH797562	-	-	-	MH797697
Peroneutypa alsophila	CBS 250.87	*Arthrocnemum fruticosum*	France	AJ302467	-	-	-	-
Peroneutypa curvispora	HUEFS 136877	unknown	Brazil	KM396646	-	-	-	-
Peroneutypa diminutiasca	**MFLUCC 17–2144**	unknown	Thailand	MG873479	-	-	-	MH316765
Peroneutypa diminutispora	**HUEFS 192196**	unknown	Brazil	KM396647	-	-	-	-
Peroneutypa kochiana	F-092,373	*Atriplex halimus*	Spain	AJ302462	-	-	-	-
Peroneutypa longiasca	**MFLUCC 17–0371**	*Hevea brasiliensis*	Thailand	MF959502	-	-	-	-
Peroneutypa rubiformis	**MFLUCC 17–2142**	unknown	Thailand	MG873477	-	-	-	MH316763
Peroneutypa scoparia	DFMAL100	*Robinia pseudoacacia*	France	GQ293962	-	-	-	GQ294029
	CBS 242.87	*Robinia pseudoacacia*	France	AJ302465	-	-	-	-
	MFLUCC 17–2143	unknown	Thailand	MG873478	-	-	-	MH316764

[a] ITS: internal transcribed spacers 1 and 2 together with 5.8S nrDNA; act: actin; tef1: translation elongation factor 1-α gene; rpb2: RNA polymerase second largest subunit; tub2: beta-tubulin. Sequences generated in this study indicated in italics. Ex-type and ex-epitype cultures are indicated in bold.

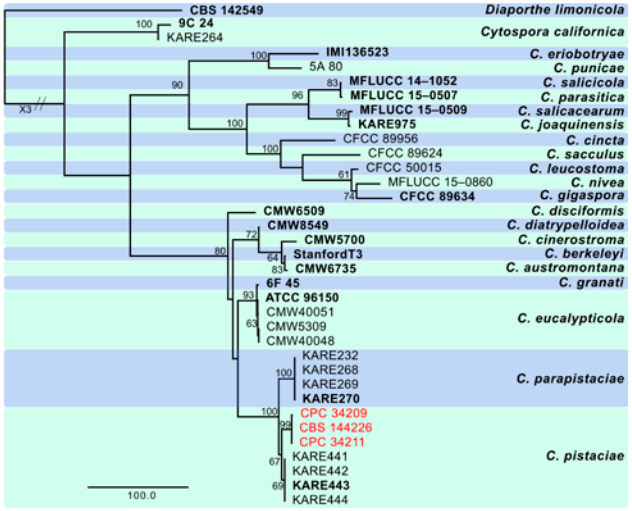

Figure 2. The first of two equally most parsimonious trees obtained from a heuristic search of the combined ITS, *act*, *tef1* and *rpb2* sequence alignments of *Cytospora* spp. Bootstrap support values are shown at the nodes. The strains isolated in this study are shown in red and the scale bar represents the number of changes. The tree was rooted to *Diaporthe limonicola* (CBS 142549).

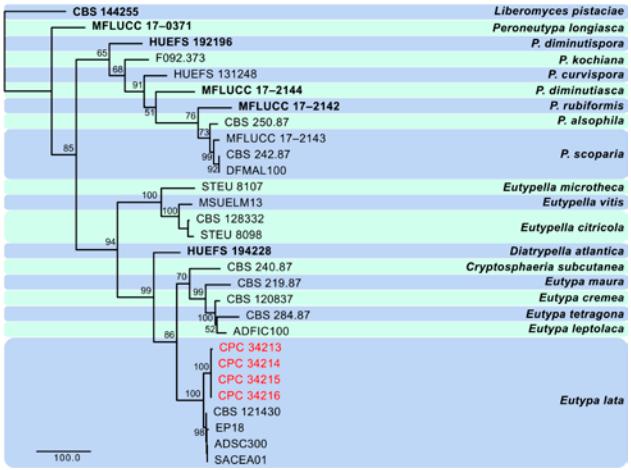

Figure 3. The first of four equally most parsimonious trees obtained from a heuristic search of the combined ITS, and *tub2* sequence alignments of species belonging to *Eutypa* and other genera of Diatrypaceae. Bootstrap support values are shown at the nodes. The strains isolated in this study are shown in red and the scale bar represents the number of changes. The tree was rooted to *Liberomyces pistaciae* (CBS 144255).

studies should investigate the role of propagation material, mechanical injuries and pruning wounds in disease transmission and spread.

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Punica granatum (pomegranate) as new host of *Erysiphe platani* and *Podosphaera xanthii*

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Summary. Pomegranate is important as an ornamental tree with spectacular flowers and delicious fruits, consumption of which has potential health benefits. In 2018 and 2019, pomegranate leaves infected by powdery mildew were collected at two locations in Hungary. One collection of the pathogenic fungi from each location was identified based on morphology and internal transcribed spacer (ITS) region analysis. One sample had pseudoidium-type conidiophores and lobed appressoria, and the other sample had catenate conidiophores and conidia with fibrosin bodies. Chasmothecia were absent in both cases. Based on morphology and ITS sequence analysis one powdery mildew fungus was identified as *Erysiphe platani*, and the other latter as *Podosphaera xanthii*. Pathogenicity tests were conducted with both species. This is the first record of powdery mildew on *Punica granatum* caused by *E. platani* and *P. xanthii*. *Erysiphe platani* has been reported only from *Platanus* species and *Ailanthus altissima*, while *P. xanthii* has a broad host range including more than 12 plant families.

Keywords. Powdery mildew, Erysiphales, *Erysiphe punicae*, host range expansion.

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INTRODUCTION

Pomegranate (*Punica granatum* L.) is a widely cultivated shrub throughout the Middle East and Caucasus region, and in North and tropical Africa, South Asia, Central Asia, the dry regions of Southeast Asia, and parts of the Mediterranean Basin (Holland *et al.*, 2009). Powdery mildew infection on pomegranate was reported first from Azerbaijan in 1964, and the causal agent was described as *Erysiphe punicae* (Braun and Cook, 2012). Later this pathogen was also found in Ethiopia, Greece, India, Iraq and the Ukraine, Crimea (Amano, 1986), and then in Iran (Khodaparast *et al.*, 2000). Recently, a new powdery mildew fungus, with cylindrical conidiophore foot cells and lobed appressoria, was reported on pomegranate in Italy (Pollastro *et al.*, 2016). Conidia were ellipsoid to cylindrical and without fibrosin bodies. Based on the analysis of the ITS sequence the fungus was identified as *Erysiphe* sp., belonging to the unresolved *E. aquilegiae* clade (Pollastro *et al.*, 2016).

The aim of the present study was to identify the causal agent of the powdery mildew on pomegranate plants in Hungary.

MATERIALS AND METHODS

Fungus samples and morphology

In September 2018, severe powdery mildew symptoms were observed on a bonsai pomegranate tree in Budapest and in a nursery garden in Győr (Hungary). Samples from fresh collections were examined by being mounted in 3% KOH solution to determine the presence of fibrosin bodies in the conidia. Dried specimens were rehydrated as described by Shin and La (1993). The morphological characteristics of the fungal structures were examined with bright-field and phase contrast microscopy, using a ZEISS AxioScope2 microscope (Germany) equipped with an AxioCam ICc5 camera (Zeiss). At least 30 measurements were made for each fungus structure.

PCR and sequence analyses

Genomic DNA was extracted from infected pomegranate leaves with the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. The internal transcribed spacer (ITS) region was amplified using the powdery mildew specific primers PMITS1 and PMITS2 (Kiss *et al*., 2001). One microliter of the first amplification mixture was used for a second amplification using the nested primer set ITS1F (Gardes and Bruns, 1993) and ITS4 (White *et al*., 1990). All PCR amplifications were performed in a final volume of 20 µL. Reaction components included 1 µL of 10 µM forward and reverse primers (Sigma-Aldrich), 1 µL DNA template and 10 µL Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher Scientific). The cycling times and temperatures for both primer pairs were as follows: 98°C for 2 min, followed by 36 cycles of 5 s at 98°C, 5 s at 60°C and 15 s at 72°C, and a final extension step at 72°C for 5 min. The nucleotide sequences of the amplicons were determined with primers ITS1F and ITS4, and were deposited in GenBank under accession numbers MK211158 and MK211159. ITS sequences were compared with accessions in the National Center for Biotechnology Information database (NCBI, http://www.ncbi.nlm.nih.gov/Blast.cgi) by applying the Basic Local Alignment Search Tool (BLAST; Altschul *et al*., 1990) using the nucleotide search option (BLASTn).

Pathogenicity tests

Pathogenicity of the specimens were confirmed through gently pressing infected leaves onto the leaves of four asymptomatic pomegranate seedlings, each with ten fully expanded true leaves. In all pathogenicity tests four non-inoculated plants served as controls.

Plants were maintained in growth chambers and visually evaluated for disease up to 10 d after inoculation.

RESULTS AND DISCUSSION

Light microscopy revealed that the infections of pomegranate in Budapest (Figure 1A) and Győr (Figure 2A) were caused by two morphologically different powdery mildew species. The specimen from Budapest had epiphytic hyphae with lobed hyphal appressoria (Figure 1B), and conidiophores producing single conidia. The foot-cells of the conidiophores measured 76 to 211 × 4 to 8 µm and were slightly sinuous or straight (Figure 1D). Conidia were hyaline, ellipsoid or doliiform, measuring 46 to 53 × 17 to 22 µm. Fibrosin bodies were absent from the conidia, and the conidium germ tubes were terminal (Figure 1C). Examination of the specimen from Győr revealed that the powdery mildew infection of pomegranate was caused by a fungus with catenate conidium development, that produced conidia in chains. Hyphal appressoria are indistinct to slightly nipple-shaped and solitary. The foot-cells of the conidiophores were straight (Figure 2D) and the dimensions were 70 to 140 × 10 to 18 µm. Conidia were ellipsoid, 38 to 45 × 21 to 27 µm, and contained fibrosin bodies (Figure 2B). Conidia each germinated at lateral positions (Figure 2C). No chasmothecia were found in the two specimens.

The BLASTn search of the ITS sequences revealed 100% identity of the pseudoidium (Budapest) sample to *Erysiphe platani*, previously described on *Platanus occidentalis* in China (MG680940), *Platanus* x *hispanica* in the United Kingdom (KY660927) and *Ailanthus altissima* in Switzerland (KX086214). Ninety-nine percent similarity was found with *E. platani* infecting *Platanus occidentalis* in Greece (KM068123) and *Platanus* × *acerifoli* in China (KX611158). The BLASTn analysis of the sample with catenate conidia (from Győr) showed 100% identity to *Podosphaera xanthii*, previously described on *Abelmoschus esculentus* in China (MK439611), *Gynostemma pentaphyllum* in Korea (KP120971), *Senna occidentalis* in Mexico (JQ728480), and on other host plants.

The results of the pathogenicity tests confirmed *E. platani* and *P. xanthii* to be pathogenic to pomegranate. Inoculated plants developed powdery mildew signs and symptoms after 5 d, whereas the control plants remained healthy. The fungi present on the inoculated plants were, respectively, morphologically identical to those originally observed on the diseased pomegranate plants from the two locations.

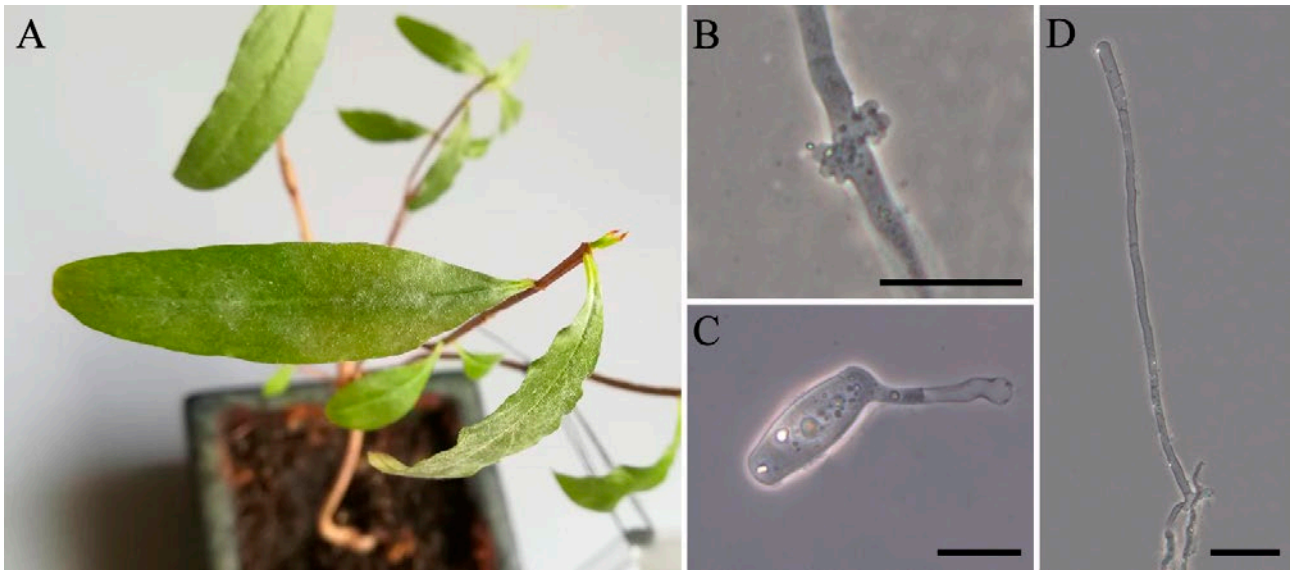

Figure 1. *Erysiphe platani* on *Punica granatum*. A: Symptoms of powdery mildew on *Pu. granatum* leaves. B: hyphal appressoria. C: germinating conidium. D: conidiophore. Bars = 50 μm.

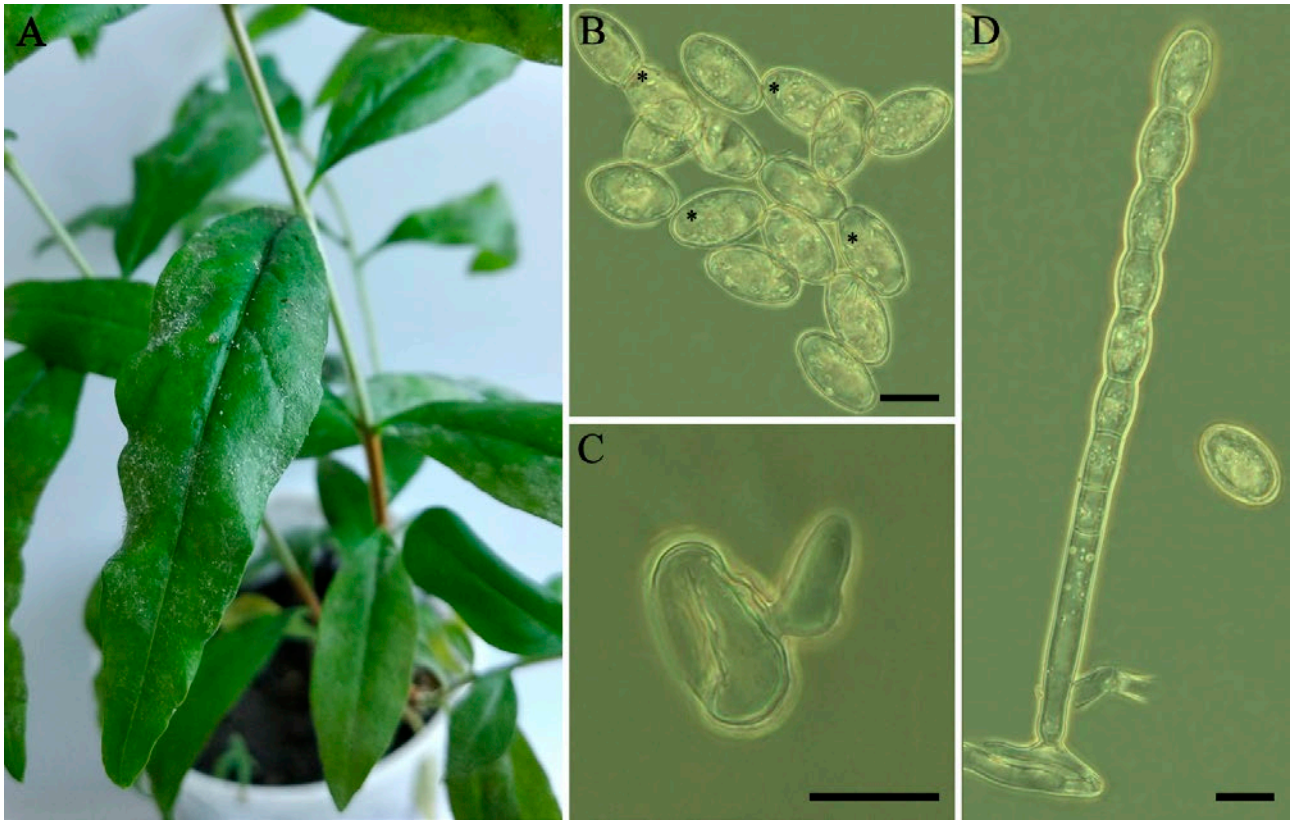

Figure 2. *Podosphaera xanthii* on *Punica granatum*. A: White colonies of *P. xanthii* on *Pu. granatum*. B: conidia, asterisks show fibrosin bodies. C: germinated conidium. D: conidiophore. Bars = 25 μm.

To our knowledge, this is the first report of *E. platani* and *P. xanthii* causing powdery mildew on pomegranate. Both specimens were deposited at the Herbarium of the Hungarian Natural History Museum, Budapest, Hungary, under accession numbers BP110664 (*E. platani*) and BP110665 (*P. xanthii*).

Podosphaera xanthii has a broad host range with worldwide distribution, and is considered a species complex rather than a single species. *Podosphaera xanthii sensu lato* consists of morphologically undistinguishable cryptic species infecting several plant species from at least 12 families (Braun and Cook, 2012). Recently this pathogen was reported from the inflorescence of the carnivorous plant, bladderwort (*Utricularia gibba*; Wu *et al*., 2019) and from *Peperomia tetragona* (Cho *et al*., 2017), which confirms the broad and expanding recorded host range of this fungus. *Erysiphe platani* was first recorded in the United States of America by Howe (1874). This pathogen has a much narrower host range than *P. xanthii*, infecting mainly plants in the genus *Platanus* in the *Platanaceae* (Braun and Cook, 2012). To date, a report of *E. platani* on the invasive tree-of-heaven (*Ailanthus altissima*) has been the only record of *E. platani* on a host that was not in *Platanus*. (Beenken, 2017). The ITS sequence of our sample was identical to that of *E. platani* infecting *A. altissima*. However, the foot cells of conidiophores of our collection were slightly longer than those reported by Beenken (2017).

Our results indicate possible host range expansions or host jumps, of the two powdery mildew species. Expansion of host ranges has been found in many other powdery mildew species (e.g. Ito and Takamatsu, 2010; Takamatsu *et al*., 2013; Vagi *et al*., 2007). To our knowledge, this is the first report of *E. platani* and *P. xanthii* causing powdery mildew on *Punica granatum*.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country where they were performed.

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Corrigendum

New records of *Penicillium* and *Aspergillus* from withered grapes in Italy, and description of *Penicillium fructuariae-cellae* sp. nov. Marilinda Lorenzini, Maria Stella Cappello, Giancarlo Perrone, Antonio Logrieco, Giacomo Zapparoli, Phytopathologia Mediterranea Vol 58, No 2 (2019) 325-341

In the published version of the article "New records of *Penicillium* and *Aspergillus* from withered grapes in Italy, and description of *Penicillium fructuariae-cellae* sp. nov." by Lorenzini *et. al*., Phytopathologia Mediterranea 58 (2): 325–341, 2019, the culture designated as holotype of *Penicillium fructuariae-cellae* was not indicated as being preserved in a metabolically inactive state. As a consequence, the proposed new species is invalid due to Art. 40.8 of the Shenzhen Code (Turland *et al.* 2018).

The species is validated herein.

Penicillium fructuariae-cellae M. Lorenzini, G. Zapparoli & G. Perrone, sp. nov. MB 833218
Description: see Lorenzini *et al*., Phytopathologia Mediterranea 58 (2): 337 (2019).
Holotype: CBS 145110 (preserved in a metabolically inactive state) collected by Lorenzini M. and Zapparoli G., isolated from withered grapes of Corvina variety (*Vits vinifera*) (Marano di Vaplolicella, Italy), December 2013.

The author apologizes for any inconvenience caused.

REFERENCE

Turland NJ, Wiersema JH, Barrie FR, Greuter W, Hawksworth DL, *et al.* (2018) *International Code of Nomenclature for algae, fungi, and plants (Shenzhen Code) adopted by the Nineteenth International Botanical Congress Shenzhen, China, July 2017*. [Regnum Vegetabile no. 159.] Glashütten: Koeltz Botanical Books.

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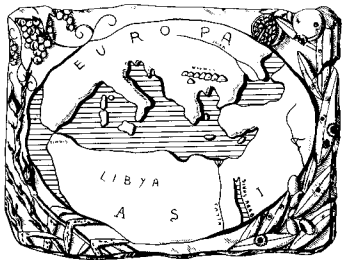

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