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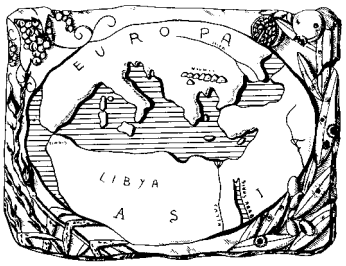

PHYTOPATHOLOGIA MEDITERRANEA

Plant health and food safety

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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

Wheat rust evolution in Spain: an historical review

Fernando MARTÍNEZ–MORENO*, Ignacio SOLÍS

ETSIA, Dep. Ciencias Agroforestales, University of Seville, Ctra de Utrera km1, 41013 Seville (Spain)
*Corresponding author: fernan@us.es.

Abstract. Rusts are important wheat diseases worldwide. The three rust diseases of wheat are yellow rust, leaf rust and stem rust, and each has characteristic features. The Guadalquivir valley in the south–west and Girona in the north–east are the areas Spain most affected by these diseases. Key factors for understanding the history of wheat rust epidemics in Spain are historical weather records in the rust–prone areas and characterization of rust resistance in historical varieties currently preserved in seed banks. These diseases in Spain have been of moderate importance, with stem rust being the most significant disease. During the second half of the 20th century several major epidemics occurred. In 1958 and 1978 severe outbreaks of yellow rust occurred in the Guadalquivir valley. These were probably associated with crop intensification, especially a large number of host landraces replaced by just a few cultivars, as well as immigration of external pathogen inoculum. From the early 1970s, CIMMYT elite cultivars arrived in Spain. These possessed good resistance to leaf and stem rust (*Sr2*), and had early heading dates. Subsequently, stem rust severity rapidly decreased in Spanish fields, but leaf rust epidemics became frequent during 1998–2008 on durum wheat in south–west Spain. In 2013, races virulent on *Lr14a* gene were first reported in Spain, but they did not result in disease epidemics. In 2012–16 yellow rust epidemics were recorded at many locations due to incursion of the 'Warrior' race. Despite the availability of effective fungicides and resistant cultivars to manage the three rust diseases, these diseases continue to threaten wheat production in Spain. In 2016, stem rust caused epidemics on durum wheat in Sicily (Italy), which has similar climatic conditions to those found in the south and east of Spain. Alert systems and international co-operation are needed to characterize the resistance of cultivars, and to monitor the movement and virulence of the wheat rust fungi.

Keywords. *Triticum aestivum*, *Triticum turgidum*, *Puccinia triticina*, *Puccinia striiformis*, *Puccinia graminis*, rust diseases.

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INTRODUCTION

Wheat rusts are diseases caused by obligate biotrophic pathogenic fungi that belong to the division *Basidiomycota*, order *Pucciniales*, and genus *Puccinia*. The diseases are caused by three distinct species producing three different diseases. Yellow rust is caused by *Puccinia striiformis* West. f. sp.

tritici, leaf rust is caused by *P. triticina* Eriks., and stem rust is caused by *P. graminis* Pers. f. sp. *tritici* Eriks & E. Henn. Wheat rusts have complex life cycles (macrocyclic) that involve five types of spores, i.e., urediniospores, teliospores, basidiospores, pycniospores, and aeciospores. These pathogens are heteroecious, requiring alternate hosts to complete their life cycles (Kolmer, 2013). On wheat, uredioniospores are produced asexually from uredinia ("pustules") that develop on the leaves, and each pustule may produce up to 3,000 spores per day over a period of 20 days (Roelfs *et al.*, 1992). Polycyclic infections occur in wheat field within growing seasons, when the newly formed urediniospores re–infect the same populations of wheat plants, and these infections have potential to develop into epidemics. Urediniospores are airborne and may travel as far as several thousand kilometres from their initial sources (Ordoñez *et al.*, 2010).

CHARACTERISTICS OF WHEAT RUSTS

Yellow (or stripe) rust

Typical symptoms of *Puccinia striiformis* f. sp. *tritici* infections are yellow–orange pustules forming stripes on the leaves, and awns can also be affected. Since the discovery that barberry (*Berberis* spp.) are the alternate hosts, *P. striiformis* is now considered as a macrocyclic and heteroecious rust (Jin *et al.*, 2010). Temperatures for infection and disease development are low (2–15°C), with an optimum temperature of approx. 10°C (Roelfs *et al.*, 1992). Yellow rust is the prevailing rust at high altitudes or in places with moderate or cold winter, although new races with tolerance to warmer temperatures have been recently reported (Hovmøller *et al.*, 2011). Yield losses can reach 24 to 39% in winter wheat cultivars in Central Asia (Sharma *et al.,* 2016). According to McIntosh *et al.* (2013), 67 yellow rust resistance genes (*Yr1* to *Yr67*), and 42 with temporary designations, have been discovered.

Leaf (or brown) rust

Leaf rust is the most common wheat rust worldwide. Pustules of *P. triticina* are reddish–brown in colour. Leaf rust is heteroecious, with *Thalictrum speciosissimum, Isopyrum fumaroides* and *Anchusa azurea* reported as alternate hosts (Anikster *et al.*, 1997; Bolton *et al.*, 2008). The disease progresses at temperatures from 10 to 30°C, with an optimum temperature of 20°C (Roelfs *et al.*, 1992), and causes yield losses attributable mainly to reduced kernel weights (Saari and Prescott, 1985; Huerta–Espino *et al.*, 2011). However, in environments conducive to leaf rust, losses can surpass 30% of potential yield (Cátedra and Solís, 2003). Cultivars resistant to leaf rust carry *Lr* genes. More than 100 of these genes and their alleles have been identified in wheat, and 71 of these are officially named (Singh *et al.,* 2013).

Stem (or black) rust

Stem rust is characterized by dark reddish–brown pustules. *Puccinia graminis* f. sp. *tritici* can cause disease at temperatures ranging from 15 to 35°C, with an optimum of approx. 25°C (Roelfs *et al.*, 1992). In the past this was considered to be the most harmful wheat rust, causing yield losses of up to 50% (Leonard and Szabo, 2005). Stem rust is heteroecious with several species of barberry and *Mahonia* spp. as alternate hosts (Leonard and Szabo, 2005). Cultivars resistant to stem rust carry *Sr* genes, and at least 60 *Sr* genes are known (Chen *et al.*, 2018).

CLIMATIC CONDITIONS AND HISTORIC WHEAT AREAS OF SPAIN

Spain has a prevailing Mediterranean climate, that is characterized by dry, hot summers and mild, wet winters, in the *Csa* 'Köppen–Geiger' classification (Kottek *et al.*, 2006). In the north of Spain, where temperatures are generally less than in the south, most areas have *Csb* (Mediterranean climate with a warm summer) or *Cfb* classifications (temperate oceanic climate). It is also important to discriminate between the 'standard Mediterranean' climate of lowland regions with moderately–cool winters, and 'continentalized Mediterranean' climate of the interior with cold winters. The standard Mediterranean climate covers coastal areas (excluding the northern Atlantic coast), the Guadalquivir river basin, and the lower reaches of the Tagus and Guadiana basins to the west of the country (Figure 1). The continentalized Mediterranean climate predominates in Spain's plateau (*Meseta*) of the interior, at more than 600 m above sea level (AEMET, 2011).

Water deficits are the main constraints to high yields in Spanish field crops. Two thirds of the country receives less than 400 mm of annual rainfall, and the amount of solar radiation is high, especially in the south (Rivero, 2013). Winds coming from Morocco blowing from SW to NE are important for transport of rust inoculum from early to late maturing wheat varieties (Bout–Roumazeilles *et al.*, 2007).

Figure 1. Main wheat growing regions (green) and provinces (red font) in Spain, that are prone to rust outbreaks.

Wheat has been, and continues to be, cultivated in the best dryland soils of Spain. These are located near the basins of the main Spanish rivers that are, from north to south, the Ebro, Duero, Tagus, Guadiana, and Guadalquivir (Figure 1). The deep clay soils of these areas are suitable for wheat production (Rivero, 2013). Sowing of wheat takes place during October through to December each year, while harvest dates range from late May (Guadalquivir basin) to mid–July (Ebro and Duero basin). Extreme heat in summer (especially in the south) prevents extended growing cycles. Winter and facultative wheat cultivars are planted in the Ebro, Duero, Tagus, and Guadiana basins. Spring wheat cultivars are winter–sown in southern Spain (Guadalquivir basin). Bread wheat is the main wheat class, with a current area of approx. 1.8 million ha in north and central Spain, while durum wheat is grown on approx. 0.3 million ha and is the prevailing class in southern Spain (AETC, 2018).

ORIGIN OF WHEAT RUSTS AND THEIR PRESENCE IN SPAIN

Wheat was domesticated as cereal grain approx. 8,000 years BCE, in the Fertile Crescent (Zohary *et al.*, 2012). The most important species of cultivated wheat are *Triticum aestivum* L. subsp. *aestivum* (bread wheat) and *Triticum turgidum* L. subsp. *durum* (Desf.) Husnot (durum wheat). Leaf rust first originated from rust infected *Aegilops speltoides* in Israel while rust isolates from durum wheat derive from virulent genotypes on bread wheat (Liu *et al.*, 2014). The putative centre of origin of yellow rust is reported to be near the Himalaya region (Ali *et al.*, 2014), while that of stem rust is Central Asia, which is also the origin of barberry (Leonard and Szabo, 2005).

Three rust diseases probably adapted quickly to domesticated wheat in a 'host tracking' manner (Stukenbrock and McDonald, 2008). Cultivated wheat spread eastward and westward at approx. 1 km per year (Zohary *et al.*, 2012). In the west, wheat reached Turkey and later spread to the entire Mediterranean Basin, first arriving in Spain approx. 5,600–5,700 years BCE when the 'Neolithic culture' entered Spain. The first two introductions were likely via maritime travel on the Mediterranean Sea from Italy to the Valencia region (east of Spain), and by land through the Pyrenees to northern Spain. Another route is recorded via Morocco to southern Spain approx. 200 years later (García–Martínez de Lagrán, 2015). The expansion of the Neolithic culture was rapid in the Iberian Peninsula (during less than 300 years), and was likely facilitated by the network of Mesolithic peoples. The rust diseases presumably spread along with the cultivation of wheat.

Wheat rusts were important diseases in ancient times. Aristotle (384–322 BCE) mentioned that humidity produced the 'rust disease', and Theophrastus (371–287 BCE) recorded that rust was caused by sunshine and dew (Arthur, 1929). Stem rust uredospores have been retrieved from excavations in Israel from sites established in the late Bronze Age (1,300 BCE) (Kislev, 1982).

In Roman times (8th BCE to 5th ACE centuries) a divinity dedicated to wheat rusts was created, i.e. the god *Robigus*. The celebration *Robigalia* was established on April 25, and was one of the many agricultural festivals celebrated in the month of April. A prayer was recited and a red dog puppy was sacrificed on an altar in a forest outside Rome, Italy (current Via Cassia–Via Sesto Miglio cross, coordinates; 41.967156 N, 12.439740 W). There is also evidence of the Robigalia celebration taking place in *Hispania* (Roman Spain). At the archaeological sites of Mas Castellar de Pontós (Girona) and La Huelga (Palencia), remains of sacrificed dogs have been found that could be connected to Robigalia ceremonies (Adroher *et al.*, 1993; Lettow–Vorbeck *et al.*, 2014). The Spanish word for rust, *roya* derives from the Latin *Robigus*.

Pope Gregory I declared in 590 ACE that April 25 was a day to pray for a good harvest, i.e. the (major) Rogation Day. In western Christianity, a rogation is a public supplication consisting of blessing the fields, and asking for God's mercy through prayers like the Litany of the Saints (Gozalo de Andrés, 2003; Wikipedia, Rogation days, 2018). The coincidence of Rogation Day with Saint Mark's day is chance. Saint Mark died on that day in 68 ACE as a martyr in Alexandria (Egypt). Saint Mark's day (*día de San Marcos*) is still celebrated as

a feast day in many villages and small towns throughout Spain, and it is common that people gather and eat together in the countryside on April 25 (Table 1).

DESCRIPTIONS OF WHEAT RUSTS IN THE MIDDLE AND MODERN AGES (5TH TO 19TH CENTURIES ACE)

It is not known whether the occurrence of wheat rust increased in Spain in the Middle Ages compared to Roman times. The 'honeymoon' hypothesis suggests that the severity of grain, especially wheat, diseases was low in Roman times and increased in the Middle Ages in North Europe, as bread wheat became the prevalent cereal (Dark and Gent, 2001). It is possible that Spain also underwent this 'honeymoon' effect, but the scanty records of rust attacks does not allow corroboration of this hypothesis. In the Book of Agriculture of G. Alonso de Herrera (16th century) there is a mention of 'rust', but on barley, and it is not clear if this referred to rust or to any foliar disease favoured by fog (Alonso de Herrera, 1818).

Barberry (*Berberis vulgaris*) is present in Spain, and three subspecies are reported to occur, subsp. *vulgaris*, *seroi* and *australis*. Moors may have planted barberry bushes as thorny hedges to border fields and gardens of *Al–Andalus* (Muslim Spain), and they also produced good quality jam from the berries (Roelfs *et al.*, 1992). Andalusian botanist Abu'l–Khayr cited the presence of barberry bushes in his 'Book of Agriculture' in the 11th century (Harvey, 1993). The expansion of barberry plantings may have contributed to increases in rust outbreaks in Spain, since this these plants are alternate hosts for stem rust and, as recently reported by Jin *et al.* (2010), also for yellow rust. As barberry only grows at altitudes above 1,000 m in southern Spain, but in the north can grow at lower elevations (300 m and above; López, 2007), it is likely that rust outbreaks due to barberry proximity first occurred in wheat cultivated in northern Spain.

Drought, locusts, birds, and ants were frequently cited as the main constraints to Spanish wheat production in the Late Middle and Modern Ages (12th to 19th century) (Alberola–Romá, 2012; Zadoks, 2013; Páscoa *et al.*, 2017), but rusts were rarely mentioned. Throughout the Middle Ages there were periods of climatic instability. From 1645 to 1715, the 'minimum of Maunder' (a reduction of solar activity) led to global cooling resulting in the so–called 'Little Ice Age' (Barriendos, 1997; Alberola–Romá, 2014). In the 40 year period of 1760 to 1800 the 'Maldà Oscillation' produced a 'moody' climate throughout the Spanish Mediterranean area, with years of severe drought and others of heavy rains (Barriendos and Llasat, 2009). Rust epidemics were probably not frequent, but they may have occurred with some regularity, especially in years with heavy rains in winter and spring.

A possible consequence of increased rust severity during this period was the retrieval of ancient wheat hulled species, einkorn, emmer and spelt, which are considered to be more resistant to wheat rusts than non-hulled varieties (Campbell, 1997; Hussien *et al.*, 1998; Zapata *et al.*, 2004). These cereal types, dominant in the Neolithic Period but in declining production at the time of the Roman Empire, may have been re-introduced to some extent to many places in Spain and Europe in the Middle Ages, especially in humid and mountainous land as found in many places in northern Spain (e.g. Asturias). Their cultivation continued until recently in that region (Dugan, 2008).

In S. Clemente's supplement (*adición*) to the book *Libro de Agricultura General* from Alonso de Herrera, it is stated that "*chamorro* [awnless] wheat is more resistant to rust because dew droplets do not build up in their spikes", but *candeal velloso* is more infected by rust due to its awned heads. To quote Alonso de Herrera (1818), "*Chapado velloso* and *moruno velloso* wheat [two kinds of durum wheat] resist well the rust".

DESCRIPTIONS OF WHEAT RUSTS IN THE 19TH AND EARLY 20TH CENTURIES

Throughout the 19th century European scientists reported the presence of rusts in wheat fields, but severe epidemics were rarely noted. Epidemics that occurred were of only local or regional importance, probably because of low crop intensification, long rotation periods between wheat crops, and diversity of wheat landraces. Yellow rust was the first to appear in February–March each growing season, followed by leaf rust in March–April and stem rust, the most damaging, in May. In Castille, straw and other materials were commonly burned to avoid dew formation on the leaves, and dew was removed by ropes each held by two people walking along the crop furrows. In 1877 stem rust epidemics were reported in Navarra (Ruiz de Casaviella, 1878), and leaf rust was also recorded in Teruel, Castellón and Guadalajara (González–Fragoso, 1918).

In 1923, during a trip to northern Spain, American plant pathologist E.C. Stakman observed greater presence of stem rust in fields close to barberry bushes than in other areas (Stakman, 1923). A brochure by the Spanish Ministry of Agricuture (*Ministerio de Agricultura de España*) in 1932 described the state of wheat rusts in

Table 1. Selected locations in Spain where Saint Mark's day is a popular feast.

Location	Province	Population[1] (2017)	Characteristics
Adra	*Almería*	24,697	Celebration
El Ejido	*Almería*	88,096	Local pilgrimage
Noreña	*Asturias*	5,210	Gastronomic fair
El Barraco	*Ávila*	1,886	Local pilgrimage
Almendralejo	*Badajoz*	33,540	Local pilgrimage
Talayuela	*Cáceres*	7,338	Celebration
Puente Genil	*Córdoba*	30,173	Local pilgrimage
Arroyo del Ojanco	*Jaén*	2,353	Local pilgrimage
Beas de Segura	*Jaén*	5,275	Local pilgrimage, bullfighting festival
Noia	*La Coruña*	14,295	Horse, livestock and machinery fair
Oleiros	*La Coruña*	35,198	Celebration
San Martín de la Vega	*Madrid*	18,824	Celebration, procession, bullfighting festival
Valdemoro	*Madrid*	73,976	Meal on park, typical sweet (*hornazo*)
Benaoján	*Málaga*	1,497	Festival, local pilgrimage
Cuevas de San Marcos	*Málaga*	3,722	Local pilgrimage, own name village, typical sweet (*hornazos de San Marcos*)
Alfarnate	*Málaga*	1,113	Local pilgrimage
Bullas	*Murcia*	11,546	Local pilgrimage
Yecla	*Murcia*	34,092	Local pilgrimage
Palencia	*Palencia*	78,892	Local pilgrimage
Agulo	*S.C. Tenerife*	1,066	Bonfire jumping
Tegueste	*S.C. Tenerife*	11,108	Local pilgrimage
Numancia de la Sagra	*Toledo*	4,755	Celebration
Gatika	*Vizcaya*	1,672	Celebration

[1] Locations shown are those with populations of more than 1,000 inhabitants.

Spain. The most common rust was listed to be stem rust, and the main control measures suggested were removal of barberry bushes near wheat fields, hot water treatment of wheat seed to kill the spores, and use of resistant wheat varieties. Praying to God was still considered as a disease control measure at the time (*Ministerio de Agricultura*, 1932).

Unamuno Irigoyen (1943), in his reception speech to the *Real Academia de Ciencias Exactas, Físicas y Naturales*, stated that, "from all known rust fungi there is no doubt that wheat rusts are the most important from an agricultural economy point of view, because of the great losses they cause on harvest". He also confirmed the absence of published data about the importance of rust fungi in Spain.

DESCRIPTIONS OF WHEAT RUST EPIDEMICS, RACES, AND RESISTANCE BREEDING FROM THE SECOND HALF OF THE 20TH CENTURY

The most severe rust attacks from 1950 onwards were in areas where wheat crop areas were largest and most intensified, with the Guadalquivir basin region (western Andalusia, south–west Spain) having the greatest rust severity. There are also reports of rust attacks in Girona (Catalonia, north–east). Until the 1950s, there was no information about rust races in Spain, and on many occasions wheat rust diseases and their causal species were confounded, especially leaf and stem rust. The first scientific studies were carried out by M.J. Urríes after his stay in 1947 with W.Q. Loegering at the Minnesota Agricultural Experiment Station (Co-operative Rust Laboratory at St. Paul, Minnesota, USA) to study stem rust. He later collaborated with agronomists R. Cañamas and J. Salazar from INIA in Madrid (Fernández, 1964). J. Salazar continued his research at the *Centro de Cerealicultura* (Center for Cereal Research) in Madrid in the 1950s, 1960s, and 1970s. He surveyed the virulence of the main stem and leaf rust races in Spain. The main highlights relating to epidemics, race surveys, and breeding for resistance to the three wheat rust diseases are presented here.

YELLOW RUST

Yellow rust epidemics in Andalusia

Yellow rust rarely produced epidemics in Spain, but in 1957 and 1960 severe yellow rust outbreaks were reported on bread wheat in the Guadalquivir basin (Zadoks, 1960). Only a few years earlier, French (Florence Aurore) and Italian (Mara, Impeto) cultivars had been imported in an attempt to increase the low yields of typical older Spanish landrace varieties. Concurrently, changes in cultural practices such as increased seed rates and enhanced N fertilization were implemented for wheat crops. In 1978 another more severe yellow rust epidemic occurred in the Guadalquivir basin. According to Nagarajan *et al.* (1984), these epidemics were caused by a 'perfect storm' of conditions favourable to yellow rust, including:

1. Replacement of varieties. The cultivars Mahissa 1 and Siete Cerros (of CIMMYT origin) had replaced a multitude of landraces and varieties, but the replacements were highly susceptible to yellow rust. However, other new cultivars such as Cajeme, Yecora, Cocorit and Mexicale were resistant (Alvarado and Morillo, 1978).

2. Presence of yellow rust inoculum. In 1976 and 1977, severe yellow rust epidemics were recorded in many parts of the south–west Mediterranean Basin, such as Tunisia and Algeria. Those countries were also replacing old landraces with high yielding, semi–dwarf CIMMYT cultivars, and airborne spores could easily have reached Andalusia from these countries.

3. Favourable weather for the development of the disease. Heavy rains in the Guadalquivir basin were reported in the spring of 1978, with many cloudy days and mild temperatures.

However, Nagarajan *et al.* (1984) made *P. striiformis* isolate surveys, and ruled out the presence of a new virulent race. The main races were 40E008, 40E136 and 41E136 (Johnson *et al.*, 1972), which belonged to the 'French group' and the 'Levantine group'. At this time wheat cultural practices also intensified (increased plant density and fertilizer applications). The epidemics prompted the former *Instituto Nacional de Semillas y Plantas de Vivero* (National Institute of Seed and Nursery Plants) to demand resistance to yellow rust as a prerequisite to register a variety (Palmero *et al.*, 2008). Yellow rust severity rapidly decreased thereafter.

The 'Warrior' yellow rust race

From 1980 yellow rust did not cause significant damage in Spain, but in 2012 yellow rust epidemics were again reported on bread wheat at several Spanish locations. The newly created Global Rust Reference Center (GRRC) in Denmark (GRRC, 2018) surveyed Spanish yellow rust races, and all matched the 'Warrior' race. This race was first detected in 2011 in Denmark, France, Germany, Sweden, United Kingdom, and Spain, often at high frequencies. The race replaced previous populations, from which it was clearly different, since it originated from a population close to the Himalayas (Hovmøller *et al.*, 2016). The rapid spread of the 'Warrior' race from northern to southern Europe was due to its virulence on most bread wheat cultivars, including the British cultivar 'Warrior', from which the race takes its name. The race displays a high number of virulence alleles, including those for *Yr1, Yr2, Yr3, Yr4, Yr6, Yr7, Yr9, Yr17, Yr25*, *Yr32*, and *YrSp* (Table 2).

Previously, in 2008 and 2009, yellow rust was found on some bread wheat cultivars in Navarra (northern Spain). In 2011 yellow rust severity increased in Navarra, and in 2012 spread to other locations in northern Spain (Aragon, Castilla y León). In 2013 yellow rust spread south (Castilla La Mancha, Andalusia) and east-

Table 2. Races of *Puccinia striiformis* f. sp. *tritici* (yellow or stripe rust) in Spain during 2010–2016, according to the Global Rust Reference Center.

Pu. striiformis f. sp. *tritici*	Year						
	2010	2011	2012	2013	2014	2015	2016
Race name	PstS2v17v27	PstS2v27, PstS2v27v17, PstS3v2v25v27, Warrior	Warrior	Warrior	Warrior	Warrior, Warrior(-), Triticale 2006	Warrior(-)
Frequency (%)	100	29/14/43/14	100	100	100	44/44/12	100
No. isolates	2	8	6	8	6	9	5

[1] Virulence of race PstS2v27 (*Yr2,6,7,8,9,25,27*), PstS2v17v27 (*Yr2,6,7,8,9,17,25,27*), PstS3v2v25v27 (*Yr2,6,7,8,25,27*), Warrior (*Yr1,2,3,4,6,7,9,17,25,32,Sp,Amb*), Warrior (-) (*Yr1,2,3,4,6,7,9,17,25,32,Sp*), and Triticale2006 (*Yr2,6,7,8,10*).

wards (Catalonia). Farmers had to treat bread wheat crops with fungicides in many locations. The GENVCE (Spanish Group for Screening Field Crops New Varieties) field tests in 2013/14 found that the cultivars Artur Nick, Gazul, and Valbona were resistant (Gómez-Caño, 2016).

In 2014 yellow rust epidemics intensified. As an example, in 2013 variety Altamira was susceptible only in Navarra, but in 2014 it was also susceptible in Castilla y León, Aragón, and Madrid. As well, important cultivars in Spain such as CCB Ingenio, Artur Nick and Nogal became susceptible to yellow rust. In 2015, race Triticale 2015, first detected in Scandinavia and virulent on many triticale cultivars, was found in Spain by GRRC researchers (GRRC, 2018).

For durum wheat, in 2013 the cultivars Gigadur, Lecitur, Clovis and Massimo Meridio showed yellow rust symptoms in Andalusia and Extremadura. In 2014 yellow rust was also recorded in Castilla y León in durum wheat crops. Yellow rust severity on durum wheat increased and was similar to that on bread wheat in many regions in 2016. In research on yield losses in durum wheat due to yellow rust, performed at a field trial in Aranjuez (Madrid), Vergara–Diaz *et al.* (2015) reported a mean yield loss of 18%, while two cultivars suffered 57% losses. The numbers and weights of kernels decreased, but head numbers were not affected by the disease.

A new yellow rust race was detected in 2016 in Morocco and Sicily (Italy) by the GRRC. This race, named PstS14 and virulent on *Yr2, Yr3, Yr6, Yr7, Yr8, Yr9, Yr17, Yr25, Yr32,* and *YrSp* was detected in Spain in 2018 (GRRC, 2018). Yellow rust severity on the Avocet differential host set at three locations of Andalusia (2016–2017) is presented in Table 3. Resistance genes *Yr5, Yr10, Yr15* were effective in all locations, and Avocet lines with *Yr1* and *Yr8* genes showed resistance to most tested isolates. Yellow rust infections decreased slightly by 2017 in durum and bread wheat crops throughout Spain.

Table 3. Severity of yellow rust (*Puccinia striiformis*) on the Avocet bread wheat near–isogenic differential, set at three locations in the Guadalquivir basin of Andalusia during 2016 and 2017.

Differential genotype	Yellow rust severity		
	Escacena del campo (Huelva)	Écija (Sevilla)	Jerez de la Frontera (Cádiz)
Year	2016	2017	2017
Avocet S	70	-	15
Avocet (*YrA*)	30	20	30
Yr1/6*Avocet S	30	0	0
Yr5/6*Avocet S	0	0	0
Yr6/6*Avocet S	60	40	15
Yr7/6*Avocet S	60	40	20
Yr8/6*Avocet S	5	8	10
Yr9/6*Avocet S	50	80	10
Yr10/6*Avocet S	0	0	0
Yr15/6*Avocet S	0	0	0
Yr17/6*Avocet S	40	40	5
Yr18/6*Avocet S	40	80	20
Yr24/6*Avocet S	15	30	15
Yr26/6*Avocet S	15	20	5
Yr27/6*Avocet S	20	7	2
Yr32/6*Avocet S	50	40	15
YrSp/6*Avocet S	30	3	0

LEAF RUST

First race surveys (1968–1977)

J. Salazar initiated the first leaf rust surveys in Spain. In 1973 he published research on 159 rust samples collected from 1968 to 1971 (151 from bread wheat, seven from durum, and one from triticale) that were characterized for their virulence. He identified 31 races, using a differential set based on five varieties. The most common race was found in 23 isolates, and the five main races comprised 52% of total isolates (Salazar and Brañas, 1973b). In another study performed during 1972 to 1975, 178 samples were collected and 33 races identified. Rust samples were taken from Tortosa (Tarragona), Borjas Blancas (Lleida), La Alberca (Murcia), and La Coruña. None of the races were virulent on variety Malakoff (*Lr1*), and the prevailing races were similar to those found previously (Salazar and Brañas, 1977). These results were published as reports without details regarding methods, which makes it difficult to fully understand the study.

Leaf rust epidemics on durum wheat (1997–2004)

Durum wheat is a traditional crop in southern Spain with average crop areas of approx. 200,000 ha since the 19th century. In 1992, however, area of durum wheat increased when the European Union implemented a supplement subsidy (in the Common Agricultural Policy) for cultivation of this crop (Royo, 2005). Durum acrea reached a peak of 940,000 ha in 2003/04. Although durum wheat was generally considered more resistant to leaf rust than bread wheat, from 2001/02 leaf rust epidemics on durum wheat were recorded at many locations in the Guadalquivir basin. Most durum cultivars

were susceptible. Only the Italian variety Colosseo (with *Lr14a* gene) was resistant. Other resistant Italian cultivars such as Italo, Vinci, and Virgilio were brought to Spain in the 2004 growing season (RAEA, 2004). However, most bread wheat cultivars were resistant to leaf rust during this period.

In 2001 a severe leaf rust epidemic was reported in Mexico where durum wheat variety Altar C84, resistant to leaf rust for almost 20 years, became susceptible. The new virulent race, designated as BBG/BN, caused losses valued at $US 32 million in Mexico over the course of 3 years (Singh *et al.*, 2004). Many durum cultivars from CIMMYT, including Altar, carried the *Lr72* gene that protected from many races, including leaf rust races from bread wheat (Herrera *et al.*, 2014). A mutation at the virulence locus corresponding to *Lr72* resulted in a race virulent to most durum wheat cultivars. The variety Gallareta, an Altar sibling, was one of the most cultivated cultivars at that time in Spain, and this displayed susceptibility. As most durum wheat cultivars sown in Spain had CIMMYT origins, many probably carried the *Lr72* gene.

In field trials on yield losses due to foliar diseases in durum wheat in the Guadalquivir basin during the 2000–2001 and 2001–2002 growing seasons, yield losses reached 28 to 30% in susceptible cultivars. Both seasons were characterized by high levels of leaf rust infections (Cátedra and Solís, 2003).

Martínez *et al.* (2005) studied the virulence of 56 single pustule leaf rust isolates from durum and bread wheat, collected from several Andalusian locations during 1998–2000 (Table 4). Thirty–five races were identified using the Thatcher near isogenic lines as differentials. No race was virulent to genes *Lr9* and *Lr24*. Bread wheat races were different from durum wheat races, and the bread wheat races were more variable than those from durum wheat (Kolmer *et al.*, 2013). None of the durum races were virulent to *Lr1, Lr3, Lr15, Lr16* and *Lr17* genes, whereas several bread wheat isolates were virulent to these genes.

A worldwide study of durum wheat leaf rust virulence performed by the Cereal Disease Laboratory (CDL) (Minnesota, USA) determined that Spanish and Italian races grouped together (Kolmer and Liu, 2000). The races were classified as DBBDML, having virulence/avirulence spectra of *Lr2c, 10, 14b, 20, 23 / Lr1, 2a, 2c, 3, 9, 16, 24, 26, 3ka, 11, 17, 30, B, 3bg, 14a, 15, 18, 28*. In a later study, seven isolates taken from durum wheat in Andalusia during the period 2000 to 2003 were found to be similar to isolates from Spain, France, and those from America, suggesting a common origin (Ordoñez and Kolmer, 2007).

Table 4. Frequency of *Puccinia triticina* (leaf or brown rust) isolates virulent on Thatcher near–isogenic lines, collected in Spain and Andalusia in different years.

Genes / locations[1]	Salazar 1972–75	Andalusia 1997	Andalusia 1998–2000	Andalusia 1998–2000 (durum)	Andalusia 2000–03 (durum)	Andalusia 2009–13 (durum)
Lr1	3	0	30	0	0	1.2
Lr2a	17	0	2	0	0	0
Lr2b	52	0	34	47		
Lr2c	88	100	73	100	0	100
Lr3	53	40	41	0	0	3.6
Lr3bg		40	41	0	0	3.6
Lr3ka		10	25	0	0	0
Lr9		0	0	0	0	0
Lr10		100	89	87	100	97.6
Lr11	73	0	96	100	0	0
Lr12			100	100		
Lr13			56	20		
Lr14a		20			0	13.2
Lr14b		100	98	93	14.3	98.8
Lr15		0	30	0	0	
Lr16		30	30	0	0	1.2
Lr17		20	32	0	0	0
Lr18		50	96	100	0	57.8
Lr19		0	0	0	0	
Lr20		60	71	93	100	100
Lr21		0	98	100		
Lr22			100	100	14.3	
Lr23		100	79	87	100	
Lr24		0	0	0	0	0
Lr25		0	0	0	0	
Lr26		0	7	7	0	0
Lr28		0	9	0	0	0
Lr30		10	13	0	0	0
Lr32		0	9	0	0	
Lr34			100	100		
Lr35			82	73		
Lr37			100	100		
LrB		60	64	47		
Thatcher		100	100	100	100	100
No. isolates	178	10	56	15	8	83

[1] Modified and here reproduced with the permission of the copyright holder from Martínez *et al.* (2005).

From 2004 onwards, rust attacks in Spain began to decrease. Farmers regularly applied fungicides to their crops, new durum cultivars from breeders were released in the Guadalquivir basin with resistance to leaf rust, and the environmental conditions of subsequent seasons (mainly low rainfall) were unfavourable to rust epidemic development.

Many of the recently released durum wheat resistant cultivars carried *Lr14a* located on chromosome 7BL. This gene, from the emmer cultivar Yaroslav, was transferred to bread wheat and then to durum wheat. The Italian cultivar Creso (released in 1974) carried this gene, and for more than 30 years was resistant to all leaf rust races. Many durum wheat cultivars worldwide inherited the *Lr14a* gene, including Italian variety Colosseo and Spanish variety Don Jaime (Martínez *et al.*, 2005).

New races virulent to Lr14a in Spain

Pustules of high infection type were observed on the cultivars Colosseo and Don Jaime in the Spring of 2013, at widely separated field trials at Conil de la Frontera (Cádiz, southern Spain) and La Tallada d'Empordà (Girona, north–east Spain). This confirmed that virulence to *Lr14a* gene was present in Spain (Table 5). Additionally, this race was different from the reported French races virulent to *Lr14a* that were avirulent to *Lr72*, while the Spanish races were also virulent to *Lr72* (Soleiman *et al.*, 2016).

Durum wheat breeding programmes are also using other genes for resistance to leaf rust. Herrera–Foessel *et al.* (2005) reported the presence of new genes in durum cultivars, such as the complementary genes *Lr27* + *Lr31* (present in CIMMYT variety Jupare or in Spanish Don Ricardo), *Lr61* (present in CIMMYT variety Guayacán), *LrCam* (present in Camayo) and *Lr3* (present in Storlom). At the same time a high level of partial resistance was found in some durum wheat cultivars (Herrera–Foessel *et al.*, 2008). Further research has identified novel resistance genes to be deployed in the near future in durum wheat from wild relatives but already in the bread wheat background, i.e. *Lr19* (from *Lophopyrum ponticum*), *Lr47* (from *Triticum speltoides*), and *Lr37* (from *Triticum ventricosum*). Other research has focused on selecting durum landraces, lines or old varieties with leaf rust resistance. Loladze *et al.* (2016) found usable resistance in the durum varieties Gaza, Amria, Geromtel_3, Geruftel_1, Tunsyr_2, and Biblos, of varied origins.

In a study of gene postulation in bread wheat, the presence of resistance genes *Lr1, Lr10, Lr13, Lr20, Lr26* and *Lr28* was confirmed (Martínez *et al.*, 2007). In durum wheat, the resistance responses of most cultivars when inoculated with several leaf rust isolates did not match with any of the *Lr* genes from the Thatcher differential series. Some cultivars also had some levels of partial resistance (Martínez *et al.*, 2007). Another study was performed by the same group to characterize leaf rust resistance in a collection of 917 accessions from the Spanish Center for Genetic Resources (CRF–INIA). Susceptible reactions were normal, and only 6% of total entries (4.8% in durum, 11.9% bread wheat) had severities less than 20% compared to the susceptible check (Figure 2). Seven susceptible accessions (six bread, one rivet wheat) were also identified with fair levels of partial resistance (Martínez *et al.*, 2001).

Table 5. Resistance responses of 20 near–isogenic lines and eight durum genotypes, each at the 5th leaf growth stage, to two races of *Puccinia triticina* (leaf or brown rust), from 2009–11 and the race of 2013.

Genotype	Race and infection type		
	Isolates 2009–11		Isolate 2013
	DBB/BN	DBB/CN	DBB/BS (Conil, Girona)
Thatcher	S	S	S
Lr1	R	R	R
Lr2a	R	R	R
Lr2c	S	S	S
Lr3	R	R	R
Lr3bg	R	R	R
Lr3ka	R	R	R
Lr9	R	R	R
Lr10	S	S	S
Lr11	R	R	R
Lr13	R	R	R
Lr14a	R	R	S
Lr15	R	R	R
Lr16	R	R	R
Lr17	R	R	R
Lr18	R	S	R
Lr23	S	S	S
Lr24	R	R	R
Lr26	R	R	R
Lr30	R	R	R
Jupare (*Lr27+31,Lr72*)	R	R	R
Gallareta (*Lr72*)	S	S	S
Somateria (*Lr14a+*)	R	R	S
Colosseo (*Lr14a+*)	R	R	S
Don Jaime (*Lr14a+*)	R	R	S
Storlom (*Lr3,Lr72*)	R	R	R
Guayacán (*Lr61*)	R	R	R
Camayo (*LrCam*)	R	R	R

[1] R indicates a resistance response and S a susceptible response. Modified and here reproduced with the permission of the copyright holder from Soleiman *et al.* (2016).

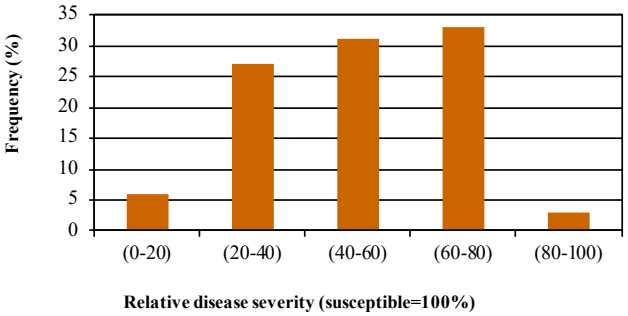

Figure 2. Relative leaf rust (*Puccinia triticina*) severity in a collection of 917 Spanish wheat landraces, planted at Cordoba, Spain in 1996/97. Here reproduced with the permission of the copyright holder from Martínez *et al.* (2001).

STEM RUST

Initial studies (1948–1971)

M.J. Urríes collected stem rust samples during 1948 to 1961 when this rust predominated, for identification of races using the Stakman differential set (Urríes and Arzoz, 1961). Subsequently, J. Salazar and M. Brañas monitored stem rust in the period 1968 to 1971, and reported only mild attacks in the field. From the 120 rust samples they collected, the majority were taken from bread wheat, although some were from barley and *Aegilops* spp. A total of 15 races were found, and their virulence profiles in samples from 1948, 1961 and 1968 to 1971 are displayed in Table 6. Samples collected near barberry bushes belonged to a group of races different from the isolate group collected at distance from barberry. The majority of races tested were avirulent to varieties Reliance (*Sr5*), Vernal (*Sr9e*) and Khapli (*Sr13*) (Salazar and Brañas, 1973a).

Stem rust vanishes by the mid–1970s

The first CIMMYT wheat cultivars were introduced to Spain in the early 1970s and these had earlier heading dates than most cultivars cultivated in Spain at that time. In Andalusia, harvest date was advanced by about a month, to May 25 to June 15. Stem rust did not appear on wheat leaves until the end of April each year, by which time it was too late to cause epidemics and the disease gradually disappeared from wheat fields. Furthermore, these new CIMMYT cultivars carried the *Sr2* gene that provided partial resistance and was effective worldwide (Singh and Rajaram, 2002). However, at present in off–season summer field trials under irrigation performed by some wheat breeding companies, pustules of stem rust can be seen at some locations, e.g. Conil de la Frontera (Cadiz) in southern Spain.

Table 6. Virulence of isolates of *Puccinia graminis* f. sp. *tritici* (stem rust) collected in Spain over several time periods and tested on a set of differentials wheat genotypes.

Differential	*Sr* gene	Year[1]		
		1948	1961	1968–71
Little Club	Susc.	100	100	100
Marquis	*Sr7b*	46.6	37.2	55.8
Reliance	*Sr5*	6.6	0.5	9.2
Kota	*Sr28*	22.6	33.3	32.5
Arnautka	*Sr9d*	86.6	47.6	51.7
Mindum	*Sr9d*	86.6	47.6	52.5
Spelmar	*Sr9d*	86.6	47.6	53.3
Kubanka	*Sr9g*	100	100	81.7
Acme	*Sr9g*	100	100	79.2
Einkorn CI2433	*Sr21*	65.3	21.9	40.0
Vernal	*Sr9e*	2.6	0.2	3.3
Khapli	*Sr13*	0	0	0
No. isolates		–	510	120

[1] Analyses performed in 1948 and 1961 by Urríes and Arzoz (1961). The 1968–71 analyses were performed by Salazar and Brañas (1973a).

The 'Sicily threat' of 2016

In 1999 a new virulent stem rust race, named Ug99, appeared in Uganda, and subsequently spread throughout East Africa (Wanyera *et al.*, 2006). In 2003 this race was found in Ethiopia and then crossed the Red Sea. In 2007 it was detected in Iran, but was controlled by releasing resistant cultivars led by international co-operation. No sign of stem rust was seen in Spain during this period.

In 2016 a severe stem rust epidemic was recorded in Sicily affecting 20,000 ha of durum wheat. The race involved was different from Ug99 and was virulent to genes *Sr9e* and *Sr13*. Both genes are common in durum cultivars (Bhattacharya, 2017). A risk analysis by the GRRC predicted the possibility that spores of the Sicilian stem rust race (TTRTF) were already in the eastern regions of the Iberian Peninsula (GRRC, 2018). However, to our knowledge stem rust race TTRFT has not yet been detected in Spain.

CONCLUSIONS

Wheat rust outbreaks occur from time to time but are difficult to predict. The main factors promoting

epidemics are: 1) the presence of airborne inoculum that can be dispersed over very long distances, 2) novel pathogen races with virulence to the main *R*–genes, 3) deployment of susceptible host cultivars, 4) particular cultural practices, such as a high crop seed rates, monoculture, and irrigation, and 5) changing weather conditions. These factors are difficult to control, as continents and many countries are involved. International co-ordination is needed to monitor the movement of inoculum, identify prevailing races and their virulence, and gain knowledge of the rust resistance genes present in the cultivated wheat cultivars, in Spain and worldwide. As such, and emphatically, the monitoring and research carried out by the Borlaug Global Rust Initiative (BGRI, 2018), and the Global Rust Reference Center (GRRC, 2018) are crucial. Additionally, at the Spanish national level, it is important that disease alert systems such as RAIF in Andalusia (RAIF, 2018) are adequately maintained into the future.

The use of rust resistant cultivars is important, preferably deploying effective *R*–genes against the prevailing pathogen races prevailing in a region. Many plant breeding companies and institutions are attempting to combine the common type of resistance (monogenic, vertical and hypersensitive) with more difficult to manage partial resistance (polygenic, and horizontal), to enhance the durability of the resistance.

Diversification of crop genetics is also relevant. Rust epidemics in the second half of the 20th century were partly the result of crop intensification and reductions in the number of wheat cultivars grown, which were often genetically related and shared the same *R*–genes. Diversity solutions such as extended crop rotations, cultivar mixtures, segregating host populations, regional control to deploy cultivars with different *R*–genes, and genes for partial resistance, are all advisable rust management strategies (McDonald and Linde, 2002). The consequences of new cultural practices for wheat (including irrigation, new fertilizers, increased seeding rates, hybrid cultivars, and high–yielding susceptible cultivars) with respect to rust epidemics are yet to be seen. This may be controversial, as cultivars with *R*–genes normally have small yield penalties, and many farmers prefer to use high–yielding but susceptible cultivars, and apply fungicides to maximize their incomes. It is not always possible, however, to apply fungicides or do so in a timely manner. In addition, susceptible cultivars may act as inoculum reservoirs for any of the three wheat rust pathogens.

An array of fungicide classes (triazoles, strobilurins, carboxamides) are available for farmers. Fungicides are relatively affordable, effective, and they can control other foliar diseases as well as rusts. However, their application should be discouraged when resistant cultivars are available, as fungicide use will reduce farmer profitability and contribute to increased environmental footprints. Fungicides are certainly warranted when resistance is not available, or is not fully effective, particularly if rust outbreaks are forecasted to cause significant yield losses.

What may be expected of the three rusts in future

Yellow rust is the most unpredictable rust. The epidemics of 1958, 1978, and 2012 in Spain appeared suddenly and caused significant yield losses. Potential damage from epidemics decreased in importance when breeders released new resistant wheat cultivars and farmers applied fungicides. New races adapted to warm temperatures may become more prevalent and infect wheat for longer periods than expected.

Leaf rust is the most common rust in Spain, although it rarely causes major problems. Most of the durum and bread wheats grown contain effective *Lr* genes. However, virulence analyses of the prevailing races, and severity assessments in rust–prone regions, must continue to be carried out.

Stem rust has not been detected in Spain since the early 1970s although it may be present at low frequencies. It is important to carefully monitor this rust in view of the durum wheat stem rust outbreak of 2016 in Sicily.

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Review

Grapevine, esca complex, and environment: the disease triangle

Michael FISCHER[1,*], Samira PEIGHAMI ASHNAEI[2]

[1] *JKI-Julius Kühn-Institute, Federal Research Centre for Cultivated Plants, Plant Protection in Fruit Crops and Viticulture, D-76833 Siebeldingen, Germany*
[2] *Plant Disease Research Department, Iranian Research Institute of Plant Protection (IRIPP), Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran*
*Corresponding author: michael.fischer@julius-kuehn.de

Abstract. This review compiles the available knowledge on the triple impact of host-pathogens-environment in the progress of the esca disease complex of grapevine. The perennial crop grapevine encounters different biotic and abiotic factors responsible for numerous changes at the various growth stages. This review provides increased understanding of the esca disease complex, with emphasis on (1) the nature of esca-associated fungi as endophytes or pathogens in grapevine, (2) the importance of grapevine genotype and age in relation to resistance or susceptibility to the pathogens, (3) the significant effects of climatic changes, especially drought, on pathogen development and symptomatology, and (4) the physio-biochemical changes in the grapevines arising from the biotic and abiotic interactions. Drought often provides conditions favouring disease development in plants. Physiological and biochemical changes in plants play critical roles in this topic. The constantly increasing economic impacts of esca disease in many grape-producing countries, and the broad lack of knowledge so far, require precise studies on the transcriptional responses to biotic and abiotic factors in grapevines, as effects of "climate change" develop. On the viticultural side, improved management of water and adjusted nutrition balance in vineyards may become useful strategies to mitigate the widespread damage caused by grapevine wood pathogens.

Keywords. Climate change, esca disease complex, grapevine, water stress.

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INTRODUCTION

Viticulture, like other fields of agriculture, is deeply affected by a wide variety of biotic and abiotic factors. For the past three decades, the economic burden of trunk diseases such as esca has become a limiting factor for grapevine production in many countries (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Dubos *et al*., 2002; Bertsch *et al*., 2009; Úrbez-Torres, 2011; Bruez *et al*., 2013; Mohammadi *et al*., 2013; De la Fuente *et al*., 2016). Advances in control of grapevine leaf strip disease (GLSD), the main disease in esca complex, have only recently been made with the introduction of *Trichoderma* as a biological treatment for wound protection, and with the possibility to reduce

symptom expression by applications of mixtures of foliar fertilizers (Di Marco *et al*., 2004; Calzarano *et al*., 2014; Calzarano and Di Marco, 2018).

Esca is a disease complex, generally characterized by the development of typical inner necrosis in grapevine wood tissues and external symptoms known as "tiger-striped" leaves or black measles on the berries, assigned to infection by pathogenic fungi that invade the perennial plants and their vascular systems (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Graniti *et al*., 2000). Petri disease, as part of the esca complex (Surico, 2001; Gramaje and Armengol, 2011) is caused by several fungi in the genera *Phaeomoniella* and *Phaeoacremonium* (Kubátová *et al*., 2004; Mostert *et al*., 2006; Essakhi *et al*., 2008; Gramaje *et al*., 2009; Gramaje and Armengol, 2011). Mature vineyards (10 years or more) are also affected by white rot linked with Basidiomycete taxa such as *Fomitiporia mediterranea* and related species (Fischer, 2002; Fischer and Kassemeyer, 2003; Fischer and Binder, 2004; Fischer *et al*., 2005; Fischer, 2006; Péros *et al*., 2008; Luque *et al*., 2009; Cloete, 2015; Fischer and González García, 2015).

In recent years there has been progressive evolution in the concept of esca and the diseases that are included within this complex (for example see Mugnai *et al*., 1999; Calzarano *et al*., 2001, 2014; Edwards *et al*., 2001; Larignon *et al*., 2001, 2009; Surico *et al*., 2008; Surico, 2009; Lecomte *et al*., 2012; Bertsch *et al*. 2013; Fontaine *et al*., 2016). Following the suggestion of Surico (2009), GLSD (previously known as "young esca") is considered the most important and widespread disease within the esca complex. Other diseases that are discussed within esca include brown wood streaking, Petri disease (black goo or slow dieback), and esca proper (including white rot). Co-existence of GLSD and esca proper is a common feature in mature vineyards. However, all of the above diseases may overlap, as they also do with canker agents such as those caused by members of the Botryosphaeriaceae or Diatrypaceae (Rolshausen *et al*., 2010; Gramaje *et al*., 2018; Moyo *et al*., 2018).

The above picture is not adopted throughout the community of pathologists and others with interests in esca, and differing views are taken, for instance, by Lecomte *et al.* (2012) or Larignon and collaborators (Larignon *et al*., 2001; 2009). Other, and less specific, designations have also been commonly used, including "manifest and hidden esca" (Marchi *et al*., 2006), "grapevine dieback disease" (Amponsah *et al*., 2011), "esca decline" (Lecomte *et al*., 2008, 2011) or "esca dieback foliar expression" (Guérin-Dubrana *et al*., 2012).

The diseases included in the esca complex are often cryptic and symptoms usually take several years to develop, in some of the diseases discontinuously (Surico *et al*., 2000, 2006; Christen *et al*., 2007; Calzarano *et al*., 2018). A potentially important group of pathogens are endophytic fungi (Petrini, 1986), which may be pathogenic or mutualistic, depending on the circumstances (Schulz and Boyle, 2005; Alvarez-Loayza *et al*., 2011). In the endophytic phase they asymptomatically colonize their hosts to form latent infections, but may modify their behaviour and become invasive when the plant gets stressed (Verhoeff, 1974; Gubler *et al*., 2005; Aroca *et al*., 2006; Surico *et al*., 2006; Slippers and Wingfield, 2007; Sakalidis *et al*., 2011; Hofstetter *et al*., 2012). There has been considerable debate during the past two decades whether, and to what extent, environmental factors influence fungal development in vineyards and the expression of symptoms (Calzarano *et al*., 2018). Several biotic and abiotic factors (Cramer *et al*., 2007; McDowell *et al*., 2008; Deluc *et al*., 2009; Andreini *et al*., 2014; Kovacs *et al*., 2017) naturally affect the physio-biochemical processes of grapevines, and subsequently their responses to pathogens, as in the case of GLSD phytoalexin patterns during leaf symptom development (Calzarano *et al*., 2016; 2017a; 2017b).

It is well known that exposure of plants to different stresses generally induces the expression of various genes. As a result, various enzymes and plant hormones (Vanholme *et al*., 2008; Vogt, 2010) with multiple biological functions are produced in response to different environmental stimuli. In this way, the variable virulence of potential pathogens, the host defense responses, and environmental conditions constitute the disease triangle (Agrios, 2005).

This eventually leads to an even more diffused picture of esca and the related diseases, making it challenging to clearly separate between diseases and fungal endophytes/pathogens and stress related symptomatology. In the case of grapevine and esca, whenever possible we refer to the specific designations of the diseases.

In this review, we refer to a large amount of literature, most of which is original research papers. In addition, we cite several review articles, which provide useful summaries of certain aspects of the "plant and stress" topic. A considerable proportion of the references refers to plants other than grapevine. While we particularly emphasize grapevine and the esca complex, numerous entries in Tables 1 and 2 also refer to other groups of plants. First, this underlines the overall significance of the subject, i.e. interrelations between stress factors and host plants. Secondly, data generated for non-grapevine hosts may assist understanding of esca and related disease phenomena.

ESCA-ASSOCIATED FUNGI: ENDOPHYTES OR PATHOGENS?

Some vascular-inhabiting fungi isolated from declining vines (Ferreira *et al*., 1999) or other hosts have been considered as latent opportunistic pathogens, and cause diseases when their hosts are subjected to abiotic stresses (Pearce, 1996; Slippers and Wingfield, 2007; Jactel *et al*., 2012).

One of the main features of GLSD is the variability in external symptom expression, whereby symptoms on leaves and berries may be obvious one year but are not apparent in another. The significant increase in foliar symptoms in 20-year-old potted vines induced by excessive watering (Surico *et al*., 2010), and the relationship between growing season rainfall (particularly in July) and leaf symptoms expression (Calzarano *et al*., 2018) have confirmed the role of rainy seasons and water in the soil in GLSD symptom development (Surico *et al*., 2000; Marchi *et al*., 2006). Factors extraneous to the plant-pathogen interactions (e.g. light, soil type, nutrients, and water availability) have been hypothesized to play roles in the erratic nature of the foliar symptoms of esca (Mugnai *et al*., 1999; Calzarano *et al*., 2007; 2009; 2014). The observed rates of mortality of vines are not necessarily, or not always, in accordance with the incidence of external symptoms (Andreini *et al*., 2014). However, Calzarano and collaborators (2018) observed greater mortality of GLSD affected vines that showed severe leaf symptoms at the first appearance, compared to vines with low symptom severity.

Although several studies have dealt with various aspects of esca related pathogens, the causes of foliar symptom development still remain elusive (Surico *et al*., 2006; Larignon *et al*., 2009; Camps *et al*., 2010). It was hypothesized that toxic metabolites produced by fungi in colonized wood reach leaves through the xylem vessels, triggering defense responses that result in the development of leaf symptoms (Evidente *et al*., 2000; Tabacchi *et al*., 2000; Andolfi *et al*., 2011; Bertsch *et al*., 2013). These plant responses include the formation of necrotic lesions on leaves as a hypersensitive reaction. In GLSD symptomatic vines, phytoalexins increased with increasing severity of leaf symptoms, confirming that these substances are synthesized as a reaction to lesions occurring on leaves, usually followed by formation of antimicrobial compounds such as stilbene derivatives (Heath, 2000; Calzarano *et al*., 2016; 2017a; 2017b).

No strict correlation has been found between the occurrence of symptoms and the extent of pathogen colonization or wood necrosis (Calzarano and Di Marco, 2007; Liminana *et al*., 2009; Fischer and Kassemeyer, 2012). In contrast, Lecomte *et al*. (2008) showed that vines exhibiting leaf symptoms had, on average, greater proportions of necrotic wood than asymptomatic plants. The degree of wood necrosis may also be positively correlated with the mortality of vines (Liminana *et al*., 2009).

No significant difference was observed between the fungal communities that inhabit apparently healthy and visibly diseased individual plants (Hofstetter *et al*., 2012). Both *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. have been isolated from asymptomatic tissue (Mugnai *et al*., 1999; Bruez *et al*., 2014; Elena *et al*., 2018), supporting the concept of latent phases for these pathogens. Furthermore, the time delay between infection and symptom expression (Di Marco and Osti, 2008) may reinforce the role of other factors in esca disease development. A possible explanation for this situation may be the different environmental factors prevailing. The varying interactions between such factors and symptom expression caused by vascular pathogens has been extensively reviewed by Mundy and Manning (2011). Characterizing the impacts of abiotic factors in grapevine responses may be a key step towards obtaining complete knowledge of physicochemical changes in plants that lead to disease development and the appearance of symptoms.

GRAPEVINE GENOTYPE AND AGE

Genotype

Genotype relationships with possible tolerance of *Vitis* spp. to biotic and abiotic stresses has been the focus of many studies. Scion cultivars and the rootstocks may differ in their responses to stress. Rootstocks provide benefits, such as drought stress tolerance (Alsina *et al*., 2007; Koundouras *et al*., 2008; Gambetta *et al*., 2012). However, information is scarce on the genetic backgrounds of these benefits, and specific responses to environmental factors.

Grapevine rootstocks have been shown to play important roles in adaptation to water deficit (Soar *et al*., 2006; Marguerit *et al*., 2012; Parker *et al*., 2013), while scion transpiration rates and their acclimation to water deficit are, through different genetic architecture, controlled genetically by rootstocks (Marguerit *et al*., 2012). Alsina *et al*. (2011) showed that scion stomatal conductance was more strongly down-regulated with drought-sensitive rootstocks (*V. riparia* × *V. rupestris* cv. 101-14Mgt) than with drought-tolerant ones (*V. berlandieri* × *V. rupestris* cv. Paulsen 1103).

Rootstock impacts may be mediated by chemical (Soar *et al*., 2006; Alsina *et al*., 2011), hormonal (particularly abscisic acid: ABA) and hydraulic signaling in responses to water deficit (Soar *et al*., 2006; Vandeleur *et al*., 2009; Marguerit *et al*., 2012). At the hydraulic level in roots, radial water movement mediated by aquaporins was shown to have a significant potential contribution to drought stress adaption (Vandeleur *et al*., 2009; Lovisolo *et al*., 2010). Aquaporins also affect root hydraulic conductance (Perrone *et al*., 2012), water uptake by fine roots (Koundouras *et al*., 2008, 2009; Gambetta *et al*., 2012) and, consequentially, vine growth (Pouzoulet *et al*., 2014). Selection of appropriate rootstocks may increase tolerance to extended drought stress periods, and even resistance to pathogens.

Rootstock genotype may affect the frequency of external symptoms (Marchi, 2001; Murolo and Romanazzi, 2014). However, leaf symptoms are not necessarily related to alterations observed in wood (Eskalen *et al*., 2001; Feliciano *et al*., 2004; Calzarano and di Marco, 2007; Romanazzi *et al*., 2009; Andreini *et al*., 2013; Murolo and Romanazzi, 2014).

The comparative transcriptome analysis in Pinot Noir grapevine highlighted rootstock-dependent differences in the response of genes involved in the jasmonic acid (JA) metabolism and pathogenesis-related (PR) proteins (Berdeja *et al*., 2014). JA is a well known regulator of wound responses against pathogens, and plays a role in water stress perception and regulation and, subsequently, in expression of several related genes (Bell and Mullet, 1991; Zhang and Memelink, 2009; see Table 1 for more information on gene expression induced by environmental factors in different plants). In grapevine, production of PR proteins is the most frequent defense reaction to fungal infection (Derckel *et al*., 1999); they accumulate in leaves and berries after pathogen infection and contribute to grapevine resistance (Giannakis *et al*., 1998). Berdeja *et al*. (2014) indicated that, under drought stress, the transcript abundance of some specific PR genes, e.g. PR1 and PR4, differed between rootstock cultivars. This indicates a link between JAs and ABA in water-stressed grapevines (Suhita *et al*., 2004).

As for scion cultivars, *V. vinifera* Chardonnay and Merlot have been classified as less susceptible to esca than Cabernet Sauvignon, which is considered as susceptible due to the high proportions of symptomatic vines observed in different climatic conditions (Christen *et al*., 2007; Andreini *et al*., 2009; Bruez *et al*., 2013; Murolo and Romanazzi, 2014). Varieties with small xylem vessels are considered less likely to express foliar symptoms because of a limited drought-induced xylem cavitation (Pouzoulet *et al*., 2014). Furthermore, own rooted vines sometimes show lower incidence of esca disease than grafted vines (Andreini *et al*., 2014), possibly because they avoid contamination by fungal spores on wounds at graft unions during propagation processes (Halleen *et al*., 2003; Hofstetter *et al*., 2012). In contrast, vines grafted on SO4, with low drought resistance, and those grafted on 1103P, conferring high drought resistance, had very similar amounts of apoplexy, while the rate in own-rooted vines, also with high drought resistance, was much higher (Marchi, 2001).

With other combinations of cultivar and rootstock, the rootstocks may be less important than other factors, both internal and external. This suggests that the susceptibility of vine genotypes to esca depends on environment and genotype factors (Marchi, 2001). The complexity of esca, and the effects of several environmental factors in appearance of symptoms, make evaluation of cultivar susceptibility very difficult. In-depth and broad genetic studies of varieties in response to biotic and abiotic stresses are necessary.

Age

Age of plants is a key factor governing the interaction between responses to the biotic and abiotic stresses, and whether plants are tolerant or susceptible to stress factors; the overall effect of stress combination on plants largely depends on their age (Pandey *et al*., 2015). Age of vines also affects disease incidence, resulting in greater incidence of diseased plants in old vineyards. Older plants experience more infection cycles than young plants, through wounds they have received (McCutcheon *et al*., 1993; Mugnai *et al*., 1999; Zabalgogeazcoa, 2008; Amponsah *et al*., 2011; Kovacs *et al*., 2017). As a result, there are positive correlations between vine age and trunk disease incidence, and, on a global scale, the disease remains the greatest limitation to maintaining optimum yields (Brown *et al*., 2016).

CLIMATIC AND ENVIRONMENTAL CHANGES AS ABIOTIC FACTORS

Climatic and edaphic factors have been reported to influence the incidence of esca and other grapevine trunk diseases (Graniti *et al*., 2000; van Niekerk *et al*., 2011; Sosnowski *et al*., 2011; Calzarano *et al*., 2018). This indicates that the pathogens are not the only agents responsible for the disease symptoms. Changes in environmental conditions are known to exacerbate disease symptoms in plants (Boyer, 1995). In latent vine infections, the presence of pathogens does not necessar-

Table 1. Gene expression in response to genotype and various environmental factors and the putative action in different plants.

Gene Expression	Plant species (as cited in respective research papers)	Environmental factors	Putative action	Reference
Pathogenesis-related proteins	*Vitis* ssp. *V. vinifera* *V. vinifera*	Pathogen infection; Genotype	Defense genes against pathogens	Giannakis *et al*., 1998 Derckel *et al*., 1999 Letousey *et al*., 2010
Phenylalanine ammonia lyase (PAL)	*Vitis* sp. *V. vinifera* *V. vinifera* *V. vinifera*	Pathogen infection; Water stress	Related to synthesis of the phytoalexin in grapevine and general responses against biotic and abiotic stresses	Melchior and Kindl, 1991 Grimplet *et al*., 2007 Deluc *et al*., 2009 Letousey *et al*., 2010
Abscisic acid (ABA)	*Zea mays* *Glycine max* *Z. mays* *V. vinifera* *Arabidopsis thaliana* *V. vinifera* *A. thaliana* *Solanum lycopersicum* *V. vinifera* *Vitis* spp. *Pinus taeda*	Water stress; Rootstock genotype	Stomatal closure; Regulation of transpiration and root hydraulic traits; Affecting SA-, JA and ET-related defense genes; Suppressing PAL activity and ROS production	Zhang and Davies, 1990 McDonald and Cahill, 1999 Kizis and Peges, 2002 Soar *et al*., 2004 Kariola *et al*., 2006 Soar *et al*., 2006 Adie *et al*., 2007 Asselbergh *et al*., 2008 Vandeleur *et al*., 2009 Lovisolo *et al*., 2010 Lorenz *et al*., 2011
Cytokinins	*S. lycopersicum* *Z. mays*	Water stress	Influencing shoot responses	Kudoyarova *et al*., 2007 Alvarez *et al*., 2008
Sugars	*V. vinifera* *Prunus persica* *V. vinifera* *V. vinifera* *V. vinifera* *P. persica* *P. persica*	Water stress; Genotype	Sugar accumulation in fruit	Freeman and Kliewer, 1983 Golding *et al*., 2006 Castellarin *et al*., 2007 Deluc *et al*., 2009 Koundouras *et al*., 2009 Lopresti *et al*., 2014 Cirilli *et al*., 2016
Amino acids	*Ocimum* sp. *V. vinifera* *V. vinifera*	Water stress; Rootstock genotype	Adjusting the osmotic potential in cytoplasm; Affecting berry composition	Khalid, 2006 Deluc *et al*., 2009 Berdeja *et al*., 2014
Polyamines (PAs)	*Oryza sativa* *Z. mays*	Saline stress; Pathogen infection	Involved in many physiological processes, such as cell growth and response to stress tolerance	Krishnamurthy and Bhagwat, 1989 Rodriguez-Kessler *et al*., 2008
Anthocyanins	*Pisum sativum* *V. vinifera*	UV-B radiation; Water stress	Developing a light red color in leaves and playing a primary role in photosynthesis; Playing a major role in wine quality, including colour, flavor and bitterness	Noguees *et al*., 1998 Berdeja *et al*., 2014
Lignin	*Z. mays*	Water stress	Important in plant defense	Alvarez *et al*., 2008
Stilbenes	*Sorghum bicolor* *V. vinifera*	Pathogen infection; Water stress	Protection against environmental challenges	Yu *et al*., 2005 Grimplet *et al*., 2007
Flavonoids	*P. sativum* *Hordeum vulgare*	UV-B radiation; Water stress; Saline stress	Decreasing the auxin level in plant tissues and causing growth retardation	Noguees *et al*., 1998 Ali and Abbas, 2003

ily result in the immediate appearance of symptoms (Di Marco and Osti, 2008), as abiotic factors affect the appearance and the severity of the disease (Lecomte *et al*., 2011). As a result, incidence and symptom profiles of diseases associated with the different esca pathogens may vary between different climatic areas (van Niekerk *et al*., 2011). Climate changes will not occur uniformly in different regions, particularly for host and pathogen exposure to drought (Schultz, 2016).

Environmental changes may affect the nature of host responses to the pathogens. Firstly, plants adjust to environmental challenges by tightly and differentially regulating their transcriptomes (Baker *et al*., 1997; Chen *et al*., 2002; Yamaguchi and Shinozaki, 2006). These alterations, often found associated with duration and severity of stresses, determine a plant's ability to respond to internal and external signals, and to adjust to changing conditions (Eastburn *et al*., 2011; Pandey *et al*., 2015). Plant responses to environmental changes, e.g. development of thicker wax layers on leaves or changes in stomatal densities, could impact the processes of infection and expression of symptoms (Campbell and Madden, 1990). Abiotic stresses have impacts on the defense-growth trade-off that plants face whenever there is a pathogen attack (Huang *et al*. 2008; Leakey *et al*. 2009; Kontunen-Soppela *et al*., 2010), causing resistance or susceptibility to the pathogens (Kuldau and Yates, 2000; Amtmann *et al*., 2008; Mittler and Blumwald, 2010). Environmental changes can also have direct effects on the pathogens. With esca, as indicated above, climatic conditions influence not only the distribution of pathogens but also the disease symptoms they cause (Surico *et al*., 2000; Marchi *et al*., 2006; van Niekerk *et al*., 2011; Calzarano *et al*., 2018).

Climatic changes have the potential to alter the incidence and severity of plant disease epidemics, as well as to reshape the co-evolutionary relationships between pathogens and host plants (Burdon *et al*., 2006; Ziska and Runion, 2007; Crowl *et al*., 2008). Drought and temperature, as the most influential factors, affect pathogens by altering growth rates, propagule germination, and the rates of inoculum production (Huber and Gillespie, 1992). Eventually, increased colonization of plants by pathogens may occur in the presence of abiotic stresses (Koga *et al*., 2004).

Drought

With the background of "climate change", the frequencies and intensities of drought periods are increasing worldwide. Increased drought leads to enhanced plant respiration, resulting in carbon (C) losses at the plant level (Schultz, 2000; Moriondo *et al*., 2011), and plant death due to C starvation (Martinez-Vilalta *et al*., 2002; Breda *et al*., 2006; McDowell *et al*., 2008; Adams *et al*., 2013; see Table 2 for additional information on the effect of water stress on different plants).

Low soil water content and resulting water deficit have been considered as causes of stress on grapevines (Lovisolo and Schubert, 1998; Lovisolo *et al*., 2010; Lanari and Silvestroni, 2015). Among different stress combinations that occur in vineyards, the influential interaction between esca and drought has often been considered (Surico *et al*., 2000, 2006, 2010; Edwards *et al*., 2007b, c; Luque *et al*., 2010; Fischer and Kassemeyer, 2012; Bostock *et al*., 2014; Ramegowda and Senthil-Kumar, 2015).

Analysis of *V. vinifera* plants subjected to individual drought stress and/or pathogen infection revealed down-regulation of transcripts involved in photosynthesis, nutrient assimilation, and cellular homeostasis (Choi *et al*., 2013). Two mechanisms have been suggested to illustrate how water deficit increases the susceptibility of grapevine to pathogen attack: first, limitation of photosynthesis (Flexas *et al*., 1999; Escalona *et al*., 1999) eliminates the plant's ability to produce defensive compounds, and, second, plant growth is reduced in the presence of the pathogen, allowing further progression of the pathogen and eventually expression of symptoms.

Drought-induced plant death under pathogen attack is influenced by the type of interaction between pathogen and host (Oliva *et al*., 2014). Studies reviewed by Boyer (1995) showed that predisposition to disease is often observed in host plants experiencing soil water deficits, and, among other cases, this has been illustrated in increases of bacterial leaf scorch symptoms (in *Parthenocissus*: McElrone *et al*., 2001,), and esca-related wood symptoms (caused by *P. chlamydospora* in *Vitis*: Fischer and Kassemeyer, 2012). Conversely, it was shown that resistance is typically restored when water stress is remediated (in *Pinus*: Johnson *et al*., 1997).

The pathogen may act: i) simultaneously with drought, as an opportunistic agent taking advantage of the effects of water stress on the host; or ii) prior to drought events (Oliva *et al*., 2014). Drought occurring during infection may have greater impact than previous drought episodes (Croise *et al*., 2001). For example, vascular wilt pathogens can accelerate drought-induced mortality by damaging the xylem vascular system, causing phloem impairment and foliage wilting (Oliva *et al*., 2014). These conditions tend to increase evapotranspiration, producing favourable conditions for the development of xylem embolism, as is also observed in esca (Pouzoulet *et al*., 2014).

Table 2. Different plant species and their adaptive changes in response to water stress.

Plant species (as cited in respective research papers)	Physio-biochemical changes	Reference
Acer platanoides, *Populus tremula*, etc.	Stomatal closure	Aasamaa *et al*., 2001
Juglans regiax×nigra		Cochard *et al*., 2002
Betula alleghaniensis, *B. davurica*, etc.		Gu and Rom, 2007
Vitis vinifera		Letousey *et al*., 2010
Zea mays	Changes in photosynthetic rate and Carbon reserves	Westgate and Boyer, 1985
V. vinifera		Christen *et al*., 2007
Arabidopsis thaliana		Hummel *et al*., 2010
Pinus edulis		Sevanto *et al*., 2014
V. vinifera	Enhanced respiration	Schultz, 2000
A. saccharum, *Thuja occidentalis*, etc.	Lower shoot hydraulic conductance and leaf specific conductivity	Tyree and Sperry, 1988
V. vinifera	Lower transectional areas in xylem vessels	Lovisolo and Schubert, 1998
Eucalyptus globulus	Impaired function of phloem	Pate and Arthur, 1998
Vitis sp.	Aquaporin gene expression	Galmes *et al*., 2007
V. vinifera	Growth reduction	Shellie and Brown, 2012
Melissa officinalis	Chlorophyll loss	Munne-Bosch and Alegre, 1999
Z. mays	Alteration in root structure and function	Zhang *et al*., 1995
V. berlandieri×V. rupestris		Dry *et al*., 2000
Lycopersicom esculentum		Mingo *et al*., 2004
Glycine max	ABA-responsive signaling pathway, e.g. the activation of JA-related defense genes, alteration in PAL activity, etc.	Ward *et al*., 1989
Z. mays		Zhang and Davies, 1990
G. max		McDonald and Cahill, 1999
L. esculentum		Audenaert *et al*., 2002
A. thaliana		Kariola *et al*., 2006
A. thaliana		Adie *et al*., 2007
V. vinifera		Grimplet *et al*., 2007
Solanum lycopersicum		Asselbergh *et al*., 2008
V. vinifera		Deluc *et al*., 2009
Vitis sp.		Lovisolo *et al*., 2010
S. lycopersicum	Cytokinin production	Kudoyarova *et al*., 2007
Z. mays		Alvarez *et al*., 2008
V. vinifera	Sugar accumulation	Castellarin *et al*., 2007
V. vinifera		Deluc *et al*., 2009
V. vinifera		Koundouras *et al*., 2009
Ocimum sp.	Accumulation of amino acids, *e.g.* proline	Khalid, 2006
V. vinifera		Deluc *et al*., 2009
V. vinifera		Berdeja *et al*., 2014
G. max	Reduction of isoflavone content	Gutierrez-Gonzalez *et al*., 2010
Z. mays	Changes in lignin content	Alvarez *et al*., 2008

Evidence of esca complex and drought interactions in grapevine

The effects of esca pathogens and water stress on grapevines have been intensively studied. Water stress exacerbated decline symptoms associated with *P. chlamydospora* in young plantations (Ferreira *et al*., 1999). In glasshouse experiments, leaf water potentials in vines subjected to water stress were reduced when the vines were infected by *P. chlamydospora*, indicating that infection altered host plant responses to water stress, making it difficult for vines to get water to their leaves (Edwards *et al*., 2007b; c). Pasquier *et al*. (2013) showed that foliar symptoms of esca proper may impact stress-related pathways in grapevines, e.g. sHSP (small heatshock protein) which is induced during water, salt, and oxidative stresses (Löw *et al*., 2000; Scharf *et al*., 2001). Christen *et al*. (2007), however, showed that foliar symptoms (in the case of GLSD) was not simply a water transport-deficit disease, but that xylem dysfunction due to patho-

gen spread partly explained the appearance of the foliar symptoms.

Intensity and timing of water deficit

Water supply plays an important role in plants under stress. Several studies have shown that water availability induces modifications of vessel diameter in different plants, including *V. vinifera* (Lovisolo and Schubert, 1998; Fichot *et al*., 2009; Bauerle *et al*., 2011). Annual changes in esca symptom expression may be partly due to differences in the size of new vessels formed under different water regimes (Pouzoulet *et al*., 2014).

Root systems that are able to maintain water uptake under low water availability may increase drought resistance (Passioura, 2002; Comas *et al*., 2013; Barrios-Masias and Jackson, 2014). Hydraulic conductivity and suberization of grapevine roots can change dramatically when they are subjected to water deficit, so irrigation management could be used to modify the water uptake capacity of root systems (Barrios-Masias *et al*., 2015). Moderate water deficit increases root growth of grapevine, as this is required for water uptake from deeper layers of soil, and maintains stomatal conductance under declining soil moisture (Alsina *et al*., 2007, 2011). Water use efficiency is consequentially optimized (Shellie and Brown, 2012).

Intensity and timing of water deficit may play critical roles in grapevine health and productivity, particularly in semi-arid regions. Schreiner and Lee (2014) suggested that there will be only small negative impacts of late-season water deficit in Pinot noir grapevines, from evidence with plants grown in pot-in-pot microplots.

Wine grapes are often intentionally grown under water deficit regimes to meet wine quality goals (Castellarin *et al*., 2007). In contrast, increased amounts of rainfall during summer and soils with high water reserves were shown to increase severity of GLSD and other diseases in the esca complex (Calzarano *et al*., 2016; 2017a, 2017b; Guérin-Dubrana *et al*., 2005, 2012; Marchi *et al*., 2006). Apoplectic symptoms are often correlated with excess of soil water combined with hot weather, leading to dramatic imbalance between foliar transpiration and root absorption (Surico *et al*., 2006).

OTHER ABIOTIC FACTORS

Adding plant nutrients (fertilizers) to soils, to promote plant growth, was reported to enhance esca disease expression. For esca proper, high availability of nutrients in a growing season increased the proportion of diseased vines with symptoms, and decreased the proportion of infected but symptomless vines (Calzarano and Di Marco, 2007; Calzarano *et al*., 2009).

Soil salinity also is important. High salinity causes damage to plants from water deficit due to osmotic stress and by ion toxicity from excessive sodium ions (Zhu, 2003; Munns and Tester, 2008; Hasegawa, 2013), and this may provide favourable conditions for esca-related pathogens. However, grapevine is adapted to semi-arid environments, where drought and salinity are prevalent growth restraints, and is considered moderately tolerant to salinity stress (Walker *et al*., 2002; Gil *et al*., 2013). Combined drought and salt stresses led to enhanced sodium accumulation in roots and shoots (Ahmed *et al*., 2013). Ma *et al*. (2015) characterized physiological and molecular responses of grapevine to short-term osmotic and sodium chloride stresses; a close relationship between drought and salinity may exist, leading to eventual impacts on plant physiological responses.

The duration and intensity of light may affect the life cycles of fungal pathogens, including those causing esca. Mittler *et al*. (1997) and Alvarez-Loayza *et al*. (2011) demonstrated that low light intensity favoured endosymbiotic development, while high light intensity triggered pathogenicity of the fungi. These authors also showed that mycelial melanin, correlated with increased production of reactive oxygen species (ROS) in pathogens and often associated with increased virulence to hosts, increased with light exposure, for instance caused by natural radiation. The rates of spore survival are also influenced by light conditions, mainly due to the UV-B portion of the spectrum, and to increased surface temperatures caused by radiation (Rotem *et al*., 1985; Stevenson and Pennypacker, 1988; Braga *et al*., 2015). However, no such studies, for instance with respect to colonization of pruning wounds, have been conducted with esca pathogens.

Pandey *et al*. (2015) reviewed enhanced damage caused by heat and drought stress combinations in plants. Both stress types share a number of physiological traits; their overall effects on plants are additive and aggravate both conditions. Sosnowski *et al*. (2007) reported that temperature and rainfall were related to the symptom development of Eutypiosis. Cool, rainy summers favoured GLSD or esca proper (chronic esca), and hot dry summers favoured apoplexy (acute esca) (Surico *et al*., 2000). Marchi *et al*. (2006) reported on the relationship between annual incidence of manifest esca (diseased plants with foliar symptoms), hidden esca (asymptomatic plants through a growing season) and rainfall. Other data show that rainfall and temperature in (Northern Hemisphere) May-July or only in July were, respec-

tively, directly and inversely related with esca leaf symptoms (Calzarano *et al*., 2018). Incidence of *P. chlamydospora* and *Phaeoacremonium* spp. infections were greater in winter rainfall regions than in marginal and summer rainfall regions, and this was attributed to the climatic preferences of these fungi (van Niekerk *et al*., 2011). Expression of PR-10 protein was found to be dependent on climatic conditions; therefore, it is possible that yearly temperature differences could influence the appearance of foliar symptoms in esca proper (Pasquier *et al*., 2013).

PHYSIO-BIOCHEMICAL CHANGES IN GRAPEVINE IN ADAPTATION TO ENVIRONMENTAL CHANGES

Acclimation of plants to environmental factors leads to adaptive changes in root and shoot growth and physio-biochemical processes, which subsequently can alter molecular host-pathogen interactions. Beckman (1964) noted that physiological changes in plants, such as increased respiration and changes in water balance, result in wilting due to lack of water rather than toxins produced by vascular pathogens, such as *Phaeomoniella* or *Phaeoacremonium*.

Physiological changes

Advanced soil drying decreases the amount of nitrate taken up by plant roots, and this raises the xylem sap pH, increasing the sensitivity of stomata to the hormone ABA (Wilkinson and Davies, 1997; see also Woodall and Ward, 2002; Benjamin and Nielsen, 2006). The effects of stress on xylem sap were investigated by Agüero *et al*. (2008), who reported that sap from water stressed grapevines enhanced the *in vitro* growth of *P. chlamydospora* and *Phaeoacremonium* spp.

Xylem morphology

During developmental stages, xylem is responsive to environmental signals, and biotic and abiotic stresses may threaten xylem function (Pouzoulet *et al*., 2014). Grapevine xylem is very efficient for water transport and by the formation of tyloses and gels is particularly vulnerable to cavitation (Alsina *et al*., 2007). This results in possible dysfunction due to water stresses (Hacke *et al*., 2000; McDowell *et al*., 2008; Choat *et al*., 2012; Vilagrosa *et al*., 2012), and the presence of pathogens (Kuroda, 1991; Edwards *et al*., 2007a; Raimondo *et al*., 2010). Resistance to drought-induced cavitation is correlated with xylem vessel dimensions (Hacke *et al*., 2001; Jacobsen *et al*., 2005; Sperry *et al*., 2006), which are the function of the plant's genetic makeup (Fichot *et al*., 2009). Pouzoulet *et al*. (2014) documented that the grape varieties Cabernet Sauvignon and Thompson Seedless, both considered susceptible to esca, had wider and longer vessels than other varieties. Occlusion processes taking place in vessels of wide diameter, with loose clusters of tyloses, compared to smaller diameter vessels with more compact clusters, would create favourable environments for *P. chlamydospora* (Pouzoulet *et al*., 2017).

Several studies in different plant species have observed increases of vessel grouping (Tyree *et al*., 1994; Lopez *et al*., 2005; Robert *et al*., 2009; Lens *et al*., 2011; Carlquist, 2012), and fewer solitary vessels (Arx *et al*. 2013), with increasing water limitation. Vessel grouping improves hydraulic redundancy and reduces the potential loss of water transport capacity associated with cavitation (Pouzoulet *et al*., 2014). However, it was also hypothesized that the mechanism of tolerance towards esca is similar to that displayed by elms (*Ulmus* spp.) against Dutch elm disease, and therefore may not be correlated with vessel morphology (Venturas *et al*., 2013). Thorough evaluation of xylem structure in different grapevine varieties may offer increased understanding of xylem vulnerability to drought stress, and susceptibility to vascular pathogens.

BIOCHEMICAL CHANGES

Plant biochemical responses to biotic and abiotic factors are complex and based on a number of molecular factors.

Photosynthetic responses

Esca-affected and drought-stressed grapevines provided information on the distinct functional behaviour patterns of photosynthetic response for these two stress types. In a study by Christen *et al*. (2007), esca-infected plants showed greater fluorescence intensity than drought stressed plants. However, the pool size of electron carriers, the electron transport per cross section and the electron transport per active reaction centre increased in the drought stress plants, whereas they decreased in the esca-infected plants.

Defense-related metabolism

In response to biotic and abiotic factors, a "trade-off" exists between growth and defense-related metabo-

lism in plants (Treutter, 2005). When defense compounds are produced, plant resources are used, including amino acids, carbohydrates and other nutrients. Plants with symptoms reduce their carbohydrate reserves during winter dormancy (Petit *et al*., 2006), and the lower pool of reserves may contribute to decreased plant development and vigour during the subsequent year (for grapevine, shown by Fontaine *et al*., 2016).

Vines infected with *P. chlamydospora* showed reduced carbohydrate reserves compared to the control vines during winter dormancy, and were characterized by an overall loss of plant vigour (Petit *et al*., 2006). Rudelle *et al*. (2005) suggested that high metabolic activity associated with secretory defense responses results from reduced starch storage. This associates with the first appearance of GLSD symptoms in a growing season in Trebbiano d'Abruzzo vineyards, at pre-bunch closure, and is when carbohydrate reserves in the wood are at their least. This condition could stimulate release of toxins by the pathogens in the vine wood, causing host leaf responses (Sumarah *et al*., 2005; Eaton *et al*., 2015). The reconstitution of these reserves between pre-bunch closure and veraison (Lebon *et al.,* 2008) was accompanied by reduced leaf symptom expression (Calzarano *et al*., 2016; 2017a; 2017b). In contrast, if a vine has already experienced water stress, and has allocated amino acids to proline production in order to maintain osmotic pressure (Keller, 2005; Deluc *et al*., 2009), less raw material will be available to produce plant defense compounds.

CONCLUSIONS

Considering the fact that grapevine culture and wine production are highly dependent on climatic conditions (van Leeuwen *et al*., 2004; Ollat *et al*., 2016), this review tried to provide new insights regarding biotic and abiotic factors in esca disease incidence.

Grapevines are economically important woody perennial fruit crops, with approx. 7.12 million ha cultivated and 74.5 million t of fruit harvested in 2014 (Gramaje *et al*., 2018). Viticulture is facing important environmental challenges that need to be addressed through coordinated research. To achieve this goal, it will be important to assess the effects of biotic and abiotic stressors on grapevine performance, since plant disease responses may change significantly under the influence of environmental parameters. Evidence for global climate change is generally accepted (Solomon *et al*., 2007; Bradley *et al*., 2012; Matyssek *et al*., 2012), and concomitant changes can lead to the disappearance or emergence of diseases in particular regions.

Water stress and the esca disease complex have been identified as the most important economic threats for viticulture (Luque *et al*., 2010; Sosnowski *et al*., 2011; van Niekerk *et al*., 2011). The worldwide economic cost for the replacement of dead grapevines is estimated to be in excess of 1.5 billion dollars per year (Hofstetter *et al*., 2012; see also De la Fuente *et al*., 2016). Since plant responses to environmental stressors are known to affect responses to pathogens, deciphering how grapevines adapt to drought conditions is an essential step to improving water use efficiency, and to adequately managing the linked pathogens. Understanding the role of xylem plasticity among grapevine varieties, and its consequences for xylem vulnerability to drought stress and susceptibility to vascular pathogens, are essential. Further research is needed to determine the physiological and molecular mechanisms underlying the plant-environment-pathogen triangle. Bio-informatic analyses may be useful for comparing the expression of various sets of biotic and abiotic stress-related genes involved in general plant responses to pathogen infection (Fontaine *et al*., 2016), and these analyses could improve understanding of the regulatory networks that control the plant responses to various stresses. To date, not enough information has been gathered on the effects of environmental stresses on the transcriptional responses of grapevine to the esca disease complex.

Long-term application of controlled conditions should be a priority, to increase understanding of the interactions between grapevine and the esca-related pathogens. The goal of this research should be to manipulate field growth conditions to favour vine hosts. Sustainable esca disease management will consist of a combination of appropriate grapevine cultivars and water regimes to mitigate the economic losses. Due to the extended periods of drought expected in the coming decades, the targeted use of protective endophytes, such as arbuscular mycorrhiza fungi, to improve plant nutrient uptake and osmotic stress tolerance, could provide improved drought tolerance (Schreiner, 2003; Schreiner and Linderman, 2005; Schreiner and Mihara, 2009; Trouvelot *et al*., 2015).

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Current topics

Xylella fastidiosa subsp. *pauca* on olive in Salento (Southern Italy): infected trees have low *in planta* micronutrient content

Marco SCORTICHINI[1,*], Danilo MIGONI[2], Federica ANGILÈ[2], Laura DEL COCO[2], Chiara Roberta GIRELLI[2], Luigi ZAMPELLA[1], Francesco MASTROBUONI[1], Francesco Paolo FANIZZI[2]

[1] *Council for Agricultural Research and the Analysis of Agricultural Economics (CREA), Research Centre for Olive, Fruit Trees and Citrus, Via Torrino 3, I-81110 Caserta, Italy*
[2] *Department of Biological and Environmental Sciences and Technology (Di.S.Te.B.A.), University of Salento, Campus Ecotekne, Via Prov.le Lecce-Monteroni, I-73100 Lecce, Italy*
*Corresponding author: marco.scortichini@crea.gov.it

Abstract. *Xylella fastidiosa* subsp. *pauca* is associated with the olive quick decline syndrome in Salento (Apulia region, Southern Italy). The first outbreak of the disease was noticed in the Gallipoli district, from where it subsequently reached nearby areas. To date, no specific study has verified if abnormal soil or leaf micronutrients is associated with the disease. Soil and leaf samples were taken from 23 olive farms showing symptoms of the syndrome located in Gallipoli and nearby areas. Each sample was analyzed for magnesium and micronutrients content using inductively coupled plasma atomic emission spectroscopy. Real-time PCR indicated that *X. f.* subsp. *pauca* was present in each sampled tree. There is a general lack of molybdenum in soil and low bioavailability of copper and molybdenum in tree leaves. Low content of manganese in soil was also found in some farms located in Gallipoli, Galatone and Trepuzzi. Olive trees grown in Gallipoli area also had low content of boron. Principal component analysis showed that soil and leaf samples from this area had lower micronutrient contents compared with other areas. General copper depletion in leaves was uncommon and has not been previously recorded in Italy in a large area. This could indicate that *X. f.* subsp. *pauca* infection causes a depletion of copper within olive leaves. Reduced copper content has been previously recorded in leaves infected with *X. f.* subsp. *multiplex* and for other bacterial pathogens. The role of copper in relation to *X. fastidiosa* infection is discussed.

Keywords. Olive Quick Decline Syndrome, quarantine bacteria, micronutrients, Inductively Coupled Plasma Atomic Emission Spectroscopy Analysis.

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INTRODUCTION

First evidence of a severe and spreading decline of olive trees (shoot, twig and branch dieback, and tree death) growing in Gallipoli area of the Salento peninsula, Apulia, Southern Italy) was noticed by farmers dur-

ing 2008 (Martelli *et al*., 2016). Some fungi, such as *Phoeoacremonium parasiticum*, *P. rubrigenum*, *P. aleophilum* and *P. alvesi* as well as *Phaemoniella* spp. were reported to be associated with the diseased olive trees (Nigro *et al*., 2013). DNA sequences belonging to *Xylella fastidiosa* were detected at the same time from olive trees showing symptoms of decline collected in the same area (Saponari *et al*., 2013). The area affected by the disease was estimated to be from 8000 to 10000 ha (Nigro *et al.,* 2013; Martelli *et al*., 2016). Subsequently, *X. f.* subsp. *pauca* was isolated from diseased olive specimens, and the disease was named as the "olive quick decline syndrome" (OQDS) (Cariddi *et al*., 2014; Loconsole *et al*., 2014). Once colonizing host tissues, *X. fastidiosa* can be ransmitted and spread by insect vectors (Almeida *et al*., 2005), and many wild plants are reservoirs of the pathogen (EFSA PLH Panel, 2018). After possible introduction from abroad (Marcelletti and Scortichini, 2016; Giampetruzzi *et al*., 2017) and the colonization of olive trees, vectors and wild plant species have played fundamental roles in the subsequent spread of *X. f.* subsp. *pauca* from the Gallipoli district to neighboring areas of Salento (Strona *et al*., 2017; White *et al*., 2017).

In recent years, several studies aimed at elucidate the genomic structure of *X. fastidiosa*, its origin and detection, the host range where the bacterium can survive, the role of the insect vectors and the potential distribution of *X. fastidiosa* in the Mediterranean basin (EFSA PLH Panel, 2018) were performed. However, no specific study has verified if abnormal soil or leaf availability of micronutrients is associated with the outbreak of *X. f.* subsp. *pauca* on olive in the Salento peninsula.

The area where the OQDS was first observed (i.e., Gallipoli and nearby municipalities) is characterized by distinctive lithological types. According to the geological map of Italy (i.e., leaf 214), in that area there are four types, namely QP, Q^1S-Q^1C and C^{11-7}. QP indicates the "Salento calcarenite" (i.e., chalky and sandy-chalky substrates), Q^1S and Q^1C indicates the so-called "Gallipoli formation" (i.e., Q^1S, sandy-clay and marly substrates; Q^1C, sandy-clay soils mixed with lithic calcarenite and arenite), whereas C^{11-7} is the calcareous substrates of the "Galatina dolomite" (Largaiolli *et al*., 1969). The soils of this area are also distinct from those of other areas of Apulia (Costantini *et al*., 2012). However, all the Salento and most of Apulia soils are characterized by their calcareous substrates (Costantini *et al*., 2012). The soils of Gallipoli area have good texture (no of silty or very sandy soils), and are from moderate to good depth, with presence of rock fragments, high drainage capacity and low salinity (Ancona *et al*., 2010).

Recent studies have stressed the importance of the element composition in plant tissues and the host ion changes in relation to *X. fastidiosa* virulence and the development of disease symptoms (Cobine *et al*., 2013; De La Fuente *et al*., 2013; Oliver *et al*., 2014; 2015; Navarrete and De La Fuente, 2014; 2015). Consequently, the element content and availability in the soil and in the host leaves could be linked with olive tree infection by *X. fastidiosa*. In the present study, soil and leaf content was assessed for nutrients that have basic physiological roles in plant metabolism. The putative relationships and role of nutrients with *X. f.* subsp. *pauca* infection in Salento area is also discussed.

MATERIALS AND METHODS

Sampling, and soil and leaf analyses

To verify the micronutrient content of soils and nutrient availability to olive trees, soil and leaf samples (from cv. Ogliarola salentina) were taken during summer of 2018, and analyzed. The samples were from the Gallipoli area and from other districts where *X. f.* subsp. *pauca* was found during last 5 years, and where OQDS symptoms were observed since at least 8-10 years. In these areas, the incidence of *X. f.* subsp. *pauca* is very high (Boscia *et al*., 2017). Samples were taken from 23 farms located in the following municipalities: Alezio, Gagliano del Capo, Galatone, Gallipoli, Leverano, Presicce, Sannicola, Trepuzzi (Salento, Lecce province). All farms had trees with visible symptoms putatively attributable to OQDS (leaf scorching, twig and branch dieback). The following elements were analyzed in soil and leaf tissue: magnesium (Mg), iron (Fe), zinc (Zn), copper (Cu), boron (B), manganese (Mn) and molybdenum (Mo). Leaf calcium content and soil pH were also assessed. Soil pH was measured in bi-distilled water using a suspension of 1:5 solid to liquid phase. For each farm, leaf samples (four subsamples from each tree) were taken from three olive trees, following a distribution of a triangle on the farm (Papadia *et al*., 2011) and according to the methods suggested by Sanzani *et al.* (2012). Samples were taken from part of the crown of each tree not showing any visible symptom of OQDS. In addition, for beneath each tree from which leaves were collected, four soil slice samples were taken from within the external limit of the foliage projection (Papadia *et al*., 2011). Samples were taken at 30–35 cm of depth where there was abundance of tree roots (Fernandez and Moreno, 1999), avoiding collection of rocks. This procedure was adopted to ascertain the soil and leaf composition related to the trees assessed.

All samples were placed in plastic bags and transported to the laboratory for preparation. Leaves were washed with distilled water to remove all the soil particles and then dried. Each soil (1 g) and leaf (0.5 g) subsample was analyzed separately at the University of Salento by using the Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES), by following the standard procedures. Briefly, samples of known dry weight were mixed with 4 mL H_2O_2 and 6 mL HNO_3 at 180 °C for 10 min, using a microwave digestion system (Milestone START D). They were then cooled, diluted with ultrapure water to a final volume of 20 mL, filtered through Whatman No. 42 filter papers, and measured for elemental content using an inductively coupled plasma atomic emission spectrometer (ThermoScientific iCap 6000 Series). Results were expressed as $mg \cdot kg^{-1}$ dry weight (ppm).

Statistical and principal component analysis

The Shapiro-Wilk test was applied for checking the normality of the data obtained from the ICP-AES analyses, using the R statistical environment, version 3.5.1 (R Development Core Team, 2013), and correlation matrix based on Pearson's coefficient was calculated for all the measured elements using MetaboAnalyst 4.0, a web-based tool for visualization of metabolomics (Xia *et al*., 2009; Xia and Wishart, 2016). This approach was used to assess possible linear associations between the variables of the two datasets considered. Multivariate statistical analyses and graphics were obtained using SIMCA-P software SIMCA 14 software, (Sartorius Stedim Biotech, Umeå, Sweden) (Bro and Smilde, 2014). Exploratory data analyses were carried out using Principal Component Analysis (PCA), applying well-established procedures (Papadia *et al*., 2011), and used to obtain a general overview of the natural data grouping. The original dataset was rearranged in a new multivariate coordinate space where the dimensions were ordered by decreasing variance in the data. The principal components were displayed as a set of scores, which highlights clustering or outliers, and a set of loadings (p), which explain the influence of input variables on principal components. Autoscaling, also known as unit or unit variance scaling, was applied to the data (Van den Berg *et al*., 2006). The PCA models were validated using the internal cross-validation default method (7-fold), and were further evaluated with the permutation test (400 permutations) (Trygg and Wold, 2002; Triba *et al*., 2015). The quality of the models was described by R^2 and Q^2. The R^2 value is a cross validation parameter defined as the proportion of variance in the data explained by the models, while the Q^2 parameter is an internal cross validation parameter, which indicates the predictability of the model. Loadings plots were evaluated to investigate the role of the measured variables in the models (Van den Berg *et al*., 2006; Wheelock and Wheelock, 2013).

Occurrence of Xylella fastidiosa *in olive farms*

The occurrence of *X. fastidiosa* in the sampled olive tree leaves was assessed using real-time PCR (Harper *et al*., 2010) following the procedures described by Modesti *et al*. (2017) and Scortichini *et al*. (2018). For these analyses, sampled leaves were taken from branches not showing disease symptoms (i.e., leaf wilting or twig dieback).

RESULTS

Soil and leaf analyses

In the soil of the farms located in the area where the first sign of OQDS was noticed (i.e., the Gallipoli area) there were low contents of some micronutrients. In particular, according to the indicated normal mean value content in soil of each element assessed (Hodgson, 1963; Alloway, 1995; McLennan and Taylor, 1999; Kaiser *et al*., 2005; Pendias, 2010; Reimann *et al*., 2014; Noulas *et al*., 2018), manganese and molybdenum contents were low in all the farms assessed (Table 1). The contents of all the other micronutrients were within the range of the mean values. In the leaves sampled from farms of this area, copper, boron and molybdenum contents were below the minimum levels required for a normal olive tree growth (Mahler, 2000; Tittarelli *et al*., 2002; Sanzani *et al*., 2012) (Table 2). The analyses for farms located in Galatone district, an area currently showing extensive tree diebacks, also showed general low soil contents of manganese and molybdenum. In addition, there were very low amounts of copper, boron and molybdenum, and, to a lesser extent, of manganese was found into the leaves (Tables 1 and 2). All the other farms located in areas close to Gallipoli, namely Alezio and Sannicola or in districts with olive trees showing the OQDS, namely Gagliano del Capo, Leverano, Presicce, and Trepuzzi, had low amounts of molybdenum in soil and of copper in the leaves. In most of the farms, boron and molybdenum were also in low amount in soil and leaves (Tables 1 and 2).

Soil pH was close to 7.0, or greater than 7.0 or 8.0, for all farms, except for two in Galatone area that had soil pH of approx. 6.0 (Table 1). Real-time PCR showed that *X. f.* subsp. *pauca* was present in all the sampled trees.

Table 1. Magnesium and micronutrient contents, and pH values for soils sampled in Gallipoli, Galatone and other districts of Lecce province, from farms with olive quick decline syndrome and infections by *Xylella fastidiosa* subsp. *pauca*. For each farm, means (± standard deviation) are presented for 12 samples collected in three different sites.

District	Mg (%)	Fe (%)	Mn (ppm)	Cu (ppm)	Zn (ppm)	B (ppm)	Mo (ppm)	pH (H_2O)
Gallipoli 1	0.41±0.03	3.58±0.30	400.89±4.99	48.61±1.44	49.58±0.29	25.00±0.19	0.064±0.004	7.42±0.21
Gallipoli 2	0.32±0.01	1.78±0.06	262.22±3.30	19.22±0.13	34.33±0.23	16.92±0.14	0.123±0003	8.26±0.11
Gallipoli 3	0.25±0.02	1.77±0.14	269.22±3.72	41.24±0.38	26.65±0.16	16.55±0.18	0.065±0.006	7.72±0.21
Gallipoli 4	0.35±0.02	2.39±0.19	294.32±4.51	37.84±0.30	31.24±0.21	20.09±0.11	0.046±0.007	8.13±0.13
Gallipoli 5	0.32±0.02	2.14±0.12	278.12±1.86	88.16±0.75	32.23±0.15	16.36±0.08	0.065±0.006	6.81±0.22
Gallipoli 6	0.30±0.02	2.74±0.19	395.33±5.47	21.48±0.16	34.05±0.21	23.49±0.21	0.033±0.003	7.92±0.16
Gallipoli 7	0.29±0.03	2.67±0.27	275.82±4.30	30.53±0.41	36.08±0.18	19.77±0.13	0.122±0.006	7.93±0.14
Gallipoli 8	0.28±0.02	1.98±0.19	239.39±3.41	32.67±0.25	24.81±0.12	17.20±0.14	0.113±0.006	8.25±0.20
Galatone 1	0.14±0,01	1.79±0.14	141.42±1.18	54.13±0.41	20.38±0.18	12.08±0.10	0.472±0.008	6.12±0.34
Galatone 2	0.45±0.02	4.06±0.20	372.40±5.05	30.62±0.25	47.99±0.29	26.89±0.21	0.476±0.009	6.23±0.24
Galatone 3	0.42±0.03	2.48±0.16	175.43±2.21	46.00±0.51	28.51±0.15	23.94±0.11	0.569±0.002	8.63±0.19
Galatone 4	0.36±0.02	2.64±0.14	335.86±3.13	63.14±0.36	38.14±0.28	21.25±0.18	0.688±0.004	8.04±0.21
Galatone 5	0.35±0.02	2.29±0.13	221.03±2.59	21.11±0.20	37.48±0.14	16.91±0.14	0.218±0.004	8.25±0.34
Galatone 6	0.40±0.01	2.66±0.05	312.99±5.12	30.03±0.25	41.70±0.15	18.33±0.10	0.317±0.009	8.48±0.28
Galatone 7	0.35±0.02	2.07±0.17	256.48±2.15	37.60±0.20	28.21±0.13	16.67±0.13	0.511±0.004	8.41±0.33
Trepuzzi 1	0.36±0.03	1.72±0.19	601.43±66.38	86.43±0.58	32.59±0.11	22.00±0.10	0.027±0.005	8.61±0.29
Trepuzzi 2	0.33±0.02	1.49±0.11	530.67±34.91	62.70±0.27	32.96±0.07	20.01±0.18	0.031±0.006	8.72±0.19
Leverano 1	0.42±0.03	3.14±0.24	1322.25±117.87	31.49±0.13	44.73±0.19	42.29±0.35	0.178±0.009	8.28±0.09
Leverano 2	0.33±0.03	3.52±0.40	1141.06±132.51	18.58±0.06	50.02±0.15	52.65±0.39	0.024±0.011	7.95±0.10
Alezio	0.34±0.04	2.98±0.35	899.04±104.26	24.15±0.13	40.99±0.16	43.70±0.44	0.037±0.001	7.64±0.13
Sannicola	1.06±0.08	3.62±0.31	813.66±74.71	27.28±0.11	59.44±0.18	49.90±0.46	0.302±0.012	8.14±0.16
Presicce	0.29±0.02	3.38±0.32	872.89±76.77	35.61±0.22	55.87±0.23	48.74±0.24	0.081±0.009	8.10±0.21
Gagliano	0.30±0.02	3.60±0.36	1660.86±164.49	43.47±0.37	65.50±0.27	49.38±0.54	0.173±0.008	6.78±0.20

Statistical and principal component analyses

For elements measured in the soil samples, multivariate statistical analysis (PCA) and the related Pearson correlation matrix were carried out. The first two principal components used for the PCA model and explained about 60% of the total variance (R^2 = 0.39 for t[1] and 0.21 for t[2]). A specific trend of clustering among the eight groups was observed. In the t[1]/t[2] PCA score plot (Figure 1a), samples from Trepuzzi and Gallipoli districts were almost clustered at positive values of t[1], while samples collected in Leverano, Alezio, Gagliano del Capo and Presicce districts were grouped at negative values of the same t[1] component. Despite the presence of some outliers, the samples collected in the Galatone district were an homogeneous cluster, located in the upper right of the PCA score plot. In this case, the study of the variables (loading plot of Figure 1b) responsible for the class distribution along the first and the second principal components explained the arrangement of the samples in the PCA score plot of Figure 1a. In particular, Fe, Zn and Mn characterized soil samples from Leverano, Alezio, Gagliano del Capo and Presicce districts, while high contents of Mg, Ca, Mo and Cu were observed in the samples collected in the Galatone district. The cluster of samples from the Gallipoli area showed lower contents of all the measured elements. Pearson correlation matrix for soil samples (Table 3) revealed some positive and negative correlations with a high level of significance ($P < 0.001$) for some nutrients (in particular B with Mn, Zn, Fe and Ca; Mn with Zn, Fe, Ca; Zn with Fe and Ca; and Fe with Ca).

For the leaf samples, PCA gave about 55% of total explained variance (R^2 = 0.37 for t[1] and 0.18 for t[2]). This model was weakly predictive but sufficiently descriptive, showing a degree of separation among groups. Despite the different number of samples for each group, the t[1]/t[2] PCA score plot (Figure 2a) highlighted some differences among the studied samples, coming from eight different sites, especially for the first principal component t[1]. In particular, leaves samples from Leverano and Gallipoli districts were clear and homogeneous clusters, grouped, respectively, at positive and negative values of t[1]. In contrast, samples from the Galatone district were a scattered group in the t[1]/t[2] score plot. The study of the variables (loading plot of

Table 2. Calcium, magnesium and micronutrient contents in olive leaves sampled in Gallipoli, Galatone and other districts of Lecce province, from farms showing the olive quick decline syndrome and infections by *Xylella fastidiosa* subsp. *pauca*. For each farm, data show the means (± standard deviation) of 12 samples collected from three different trees. Data are referred to leaf dry weight.

District	Ca (%)	Mg (%)	Fe (ppm)	Mn (ppm)	Cu (ppm)	Zn (ppm)	B (ppm)	Mo (ppm)
Gallipoli 1	1.07±0.13	0.108±0.001	63.40±0.49	24.69±0.20	8.71±0.05	19.33±0.10	8.65±0.05	0.182±0.003
Gallipoli 2	0.60±0.01	0.062±0.001	49.38±0.40	17.34±0.19	5.31±0.04	19.13±0.13	9.68±0.06	0.103±0.003
Gallipoli 3	0.80±0.01	0.079±0.001	61.71±0.34	17.21±0.14	5.38±0.03	12.28±0.07	10.91±0.09	0.106±0.003
Gallipoli 4	0.96±0.06	0.106±0.008	65.46±0.38	24.02±0.22	6.40±0.05	16.04±0.11	11.02±0.09	0.113±0.004
Gallipoli 5	1.12±0.07	0.109±0.006	70.71±0.73	32.71±0.37	4.99±0.07	14.28±0.09	10.32±0.15	0.143±0.006
Gallipoli 6	1.23±0.06	0.040±0.006	109.14±1.17	35.20±0.51	4.91±0.01	15.05±0.09	9.58±0.14	0.063±0.002
Gallipoli 7	0.99±0.04	0.101±0.006	73.45±0.96	24.18±0.40	5.65±0.03	15.40±0.09	8.72±0.12	0.098±0.003
Gallipoli 8	1.00±0.05	0.096±0.005	62.01±0.68	18.49±0.28	5.22±0.02	18.30±0.14	8.40±0.11	0.091±0.003
Galatone 1	0.93±0.04	0.121±0.007	110.10±0.76	48.09±0.38	5.11±0.03	31.93±0.12	10.51±0.05	0.092±0.003
Galatone 2	0.93±0.04	0.130±0.005	72.25±0.59	17.88±0.19	6.88±0.03	32.52±0.18	11.34±0.13	0.174±0.004
Galatone 3	0.94±0.05	0.107±0.006	86.28±0.66	24.03±0.18	5.27±0.04	21.36±0.06	10.40±0.09	0.121±0.002
Galatone 4	1.02±0.07	0.104±0.007	79.24±0.60	23.17±0.15	6.29±0.07	17.75±0.07	11.67±0.11	0.267±0.005
Galatone 5	1.93±0.09	0.171±0.007	106.86±0.54	26.98±0.13	19.96±0.10	32.39±0.18	9.87±0.06	0.128±0.004
Galatone 6	1.07±0.05	0.102±0.004	65.11±0.62	23.23±0.21	13.10±0.11	26.87±0.15	22.62±0.16	0.081±0.003
Galatone 7	1.12±0.06	0.108±0.004	68.96±0.69	17.92±0.18	5.20±0.04	17.30±0.11	10.33±0.11	0.147±0.003
Trepuzzi 1	1.37±0.12	0.134±0.001	74.99±0.49	41.13±0.36	6.37±0.02	24.93±0.14	18.50±0.08	0.219±0.005
Trepuzzi 2	0.11±0.02	0.116±0.001	66.86±0.43	53.99±0.57	7.81±0.04	22.97±0.10	18.11±0.11	0.230±0.003
Leverano 1	1.55±0.09	0.172±0.013	80.35±0.49	60.45±0.45	11.76±0.04	21.30±0.07	11.87±0.05	0.223±0.005
Leverano 2	1.08±0.09	0.157±0.011	84.34±0.43	80.85±0.95	18.22±0.07	13.60±0.04	11.74±0.04	0.176±0.003
Alezio	1.15±0.05	0.123±0.005	71.21±0.68	40.20±0.40	5.80±0.04	18.33±0.11	13.34±0.17	0.406±0.072
Sannicola	1.37±0.13	0.165±0.021	72.96±0.40	39.25±0.25	4.65±0.02	20.30±0.07	15.77±0.12	0.405±0.007
Presicce	1.57±0.11	0.149±0.012	97.72±0.64	45.57±0.26	6.53±0.04	12.80±0.04	11.18±0.07	0.073±0.001
Gagliano	1.32±0.03	0.130±0.004	127.56±0.86	44.76±0.37	5.75±0.05	27.25±0.12	10.06±0.05	0.120±0.003

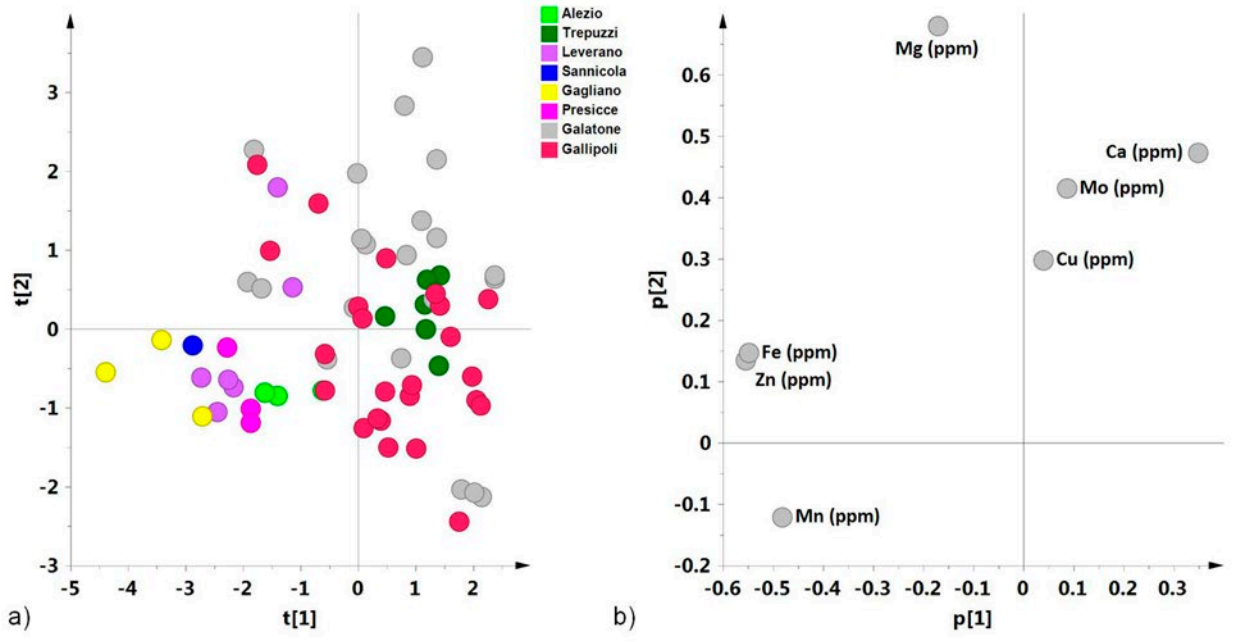

Figure 1. Principal component analysis for soil samples collected from different sites in the Salento area (Southern Apulia). t[1]/t[2] scoreplot **(a)** and corresponding p[1]/p[2] loadings plot **(b).** Two principal components (t[1]/t[2]) explained about 60% of total variance (R^2= 0.37 for t[1] and 0.18 for t[2]), with a predictability value of Q^2 = 0.097.

Table 3. Pearson correlation matrix among the variables for soil (A) and olive leaf (B) samples. *,**,*** indicate significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$.

	B ppm	Mo ppm	Zn ppm	Fe ppm	Ca ppm	Mg ppm	Cu ppm
A)							
Mn ppm	0.84***						
Zn ppm	0.81***	0.70***					
Fe ppm	0.74***	0.53***	0.81***				
Ca ppm	-0.43***	-0.33**	-0.34**	-0.51***			
Mg ppm	0.15	0.11	0.24*	0.17	0.17		
Cu ppm	-0.21	-0.14	0.02	-0.05	0.07	-0.05	
Mo ppm	-0.15	-0.19	-0.05	0.05	0.15	0.06	0.05
B)							
Mo ppm	0.28*						
Zn ppm	0.18	-0.01					
Fe ppm	-0.13	-0.05	0.20				
Ca ppm	0.15	0.22	0.22	0.50***			
Mg ppm	0.19	0.27*	0.27*	0.45***	0.80***		
Cu ppm	0.17	-0.09	0.29*	0.15	0.40***	0.42***	
Mn ppm	0.19	0.01	-0.03	0.31**	0.32**	0.55	0.34**

Figure 2b) responsible for the class distribution along the first and the second principal components explained the arrangement of the samples in the PCA score plot of Figure 2a. From the loadings plot, samples from the Gallipoli area had low amounts levels of all the measured elements, while samples from Alezio and Trepuzzi districts clustered together, with a greater contents of Mo and B. These two elements (Mo and B) also characterized samples from Sannicola, which, were mostly dispersed in the upper part of the graph. Samples collected in Leverano district were clustered in the right part of the PCA score-plot, with high contents of elements, in particular for Ca, Mg, Mn and Cu content. Pearson correlation matrix among the variables was used to evaluate the inter-element relationships in leaf samples (Table 3). Most of the elements in leaf samples were positively correlated with each other. In particular, Fe showed high positive correlation with Ca, Mg and Mn; Ca was characterized by highly significant correlation ($P < 0.001$) with Mg, Cu and Mn; Mg with Cu and Cu with Mn ($P < 0.01$), and B and Mo also showed correlation ($P < 0.05$).

DISCUSSION

These results indicate that in the Gallipoli area, where the OQDS was first noticed, as well as in districts similarly characterized by extensive damage to olive trees and the relevant occurrence of *X. f.* subsp. *pauca* (Boscia *et al.*, 2017), there is a general lack of some

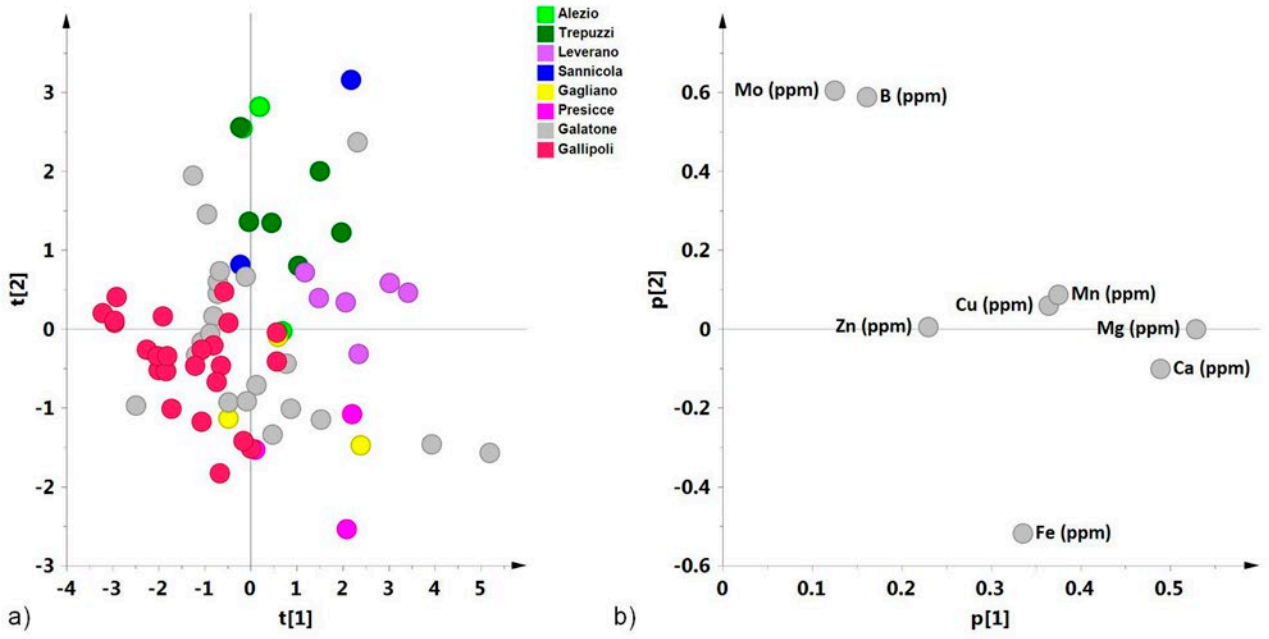

Figure 2. Principal component analysis for leaf samples collected from different sites in the Salento area (Southern Apulia). t[1]/t[2] score-plot **(a)** and corresponding p[1]/p[2] loadings plot **(b).** Two principal components (t[1]/t[2]) explained about 55% of total variance (R^2= 0.39 for t[1] and 0.21 for t[2]), with a predictability value of $Q^2 = 0.105$.

micronutrients (i.e., manganese and molybdenum) in the soils, and low contents of copper, molybdenum and boron in the leaves of olive trees. In the farms of Gallipoli, Galatone and Trepuzzi, manganese contents in the soils were low, although manganese in the leaves was within the range of normal content. This has been previously found in other studies, and emphasizes that low availability of manganese in soils characterized by a high calcium contents did not reduce amounts of this element in olive leaves (Chatzistathis and Therios, 2009).

No previous data can explain the low molybdenum content in the soil, its low content in many olive trees from the areas studied here where OQDS occurs. However, molybdenum availability should be favoured by pH greater than 5.5 (Kaiser *et al*., 2005; Bittner, 2014). This micronutrient is an essential part of nitrogenase, the enzyme catalizying nitrogen fixation of atmospheric nitrogen into ammonia (Hoffman *et al*., 2014). Low boron content within olive leaves is common for olive trees (Rodrigues *et al*., 2012), and its low bioavailability could be related to the calcareous soil matrix (Goldberg, 1997).

PCA confirmed that soil and leaf samples collected from the Gallipoli district had a lower amounts of all the measured elements compared with the other areas samples. In addition, most of the micronutrients in leaf samples showed positive correlations with each other. The significant relationships between these variables indicates similar sources of input (Islam *et al*., 2015; Moreira *et al*., 2016) for these micronutrients. However, detailed investigations in other areas where the OQDS is recorded are necessary to establish robust correlation between individual nutrients. For two farms of Galatone we found soil pHs of approx 6.0, values less than expected for calcareous soils. However, this area is characterized by a high soil salinity (Ancona *et al*., 2010), and that high concentrations of salts in calcareous soils can considerably reduce the pH (Lai and Stewart, 1990; Al-Busaidi and Cookson, 2003).

In agreement with a thorough survey performed on soils throughout Europe, including the Apulia region, and using the same technique herein applied (Ballabio *et al*., 2018), the present study also found adequate amounts of copper in the soils of the districts characterized by the occurrence of *X. f.* subsp. *pauca*. In contrast, the copper contents were low in leaves of olive trees infected by the bacterium. Low availability of copper within the olive leaves is unusual and could be related to the infection. The threshold that indicate low copper content in olive leaves varies according to different authors: Tittarelli *et al.* (2002), for Italian olive orchards, indicated 20 to 36 ppm, whereas De Andrés Cantero (2001) reported values ranging from 6 to 10 ppm. However, also considering the lower limit, the values observed in Salento in olive leaves infected by *X. fastidiosa*, especially in the Gallipoli district, were very often below these thresholds. In addition, leaf copper depletion is rarely found in olive and, apparently, it has never been reported in Italy (Tittarelli *et al*., 2002; Sanzani *et al*., 2012). A study carried out in an olive orchard free from infection of *X. fastidiosa* in Rende, Calabria, Southern Italy, found normal content of copper in soil and in the leaves, as well as normal concentration of molybdenum in soil and in leaves (Buttafuoco *et al*., 2016). The regular spraying of copper-based compounds to olive crowns to control pathogenic bacteria and fungi could explain the normal values of this micronutrient usually observed in Apulia soils (Provenzano *et al*., 2009), thus explaining the unusual observation for leaves in the present study.

Reduced copper content within highbush blueberry (*Vaccinium corymbosum* hybrids) leaves artificially infected by *X. f.* subsp. *multiplex* was found by Oliver *et al.* (2015). Similarly, *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial blight of rice, removes copper from host xylem, and the decreased concentration of this element leads to increased pathogen survival and virulence (Yuan *et al*., 2010). *Erwinia amylovora*, which causes fire blight, binds copper through exopolysaccharides, so decreasing *in planta* copper toxicity (Ordax *et al*., 2010).

Copper concentrations in the leaf may play an important role in *X. fastidiosa* infection. This pathogen accumulates copper when forming biofilms, a critical physiological state for its *in planta* survival (Cobine *et al*., 2013). When it is in the biofilm phase, *X. fastidiosa* has increased resistant to copper (Rodrigues *et al*., 2008). However, when the copper concentrations are greater than 200 µM, the pathogen loses capability to form biofilms for the relevant toxicity exerted by the element (Cobine *et al*., 2013). Our data indicate that *X. f.* subsp. *pauca* could decrease the copper contents within olive leaf xylem, thus explaining the general low content of this micronutrient found in olive trees infected by *X. f.* subsp. *pauca* in Salento. It is also known that subinhibitory doses of copper can promote the occurrence of *X. fastidiosa* persister cells (i.e., cells that neither grow nor die in the presence of bactericidal agents). These cells can repopulate the xylem after reduction of the stress condition (Muranaka *et al*., 2012). Also zinc leaf concentrations play an important role for *X. fastidiosa* aggressiveness. Zinc detoxification is required for commencement of host tissue colonization (Navarrete and De La Fuente, 2014). Similar to copper, zinc doses greater than

0.25 mM inhibit *X. fastidiosa* biofilm formation (Cobine *et al*., 2013).

The present investigation and previous experimental data corroborate the observed decrease in *X. f.* subsp. *pauca* cell densities and field OQDS symptoms in olive trees repeatedly sprayed with a biocomplex containing zinc (4%), copper (2%) and citric acid (Scortichini *et al*., 2018). Treatments to olive canopies this compound, characterized by a very effective capability to reach the host xylem networks, the leaf content of zinc and copper remained at greater amount than those that *X. f.* subsp. *pauca* can detoxify to increase its aggressiveness. Such a biocomplex also shows an *in vitro* antibacterial activity againsts *X. f.* subsp. *pauca* isolated from olive trees with OQDS in Salento (S. Loreti, N. Pucci, personal communication).

Restoration of the copper and zinc contents in olive trees foliage coupled with improved soil fertility, if performed during some years, could allow co-existence with *X. f.* subsp. *pauca* in orchards where the infections has not yet greatly reduced the crown of the tree. Restoration and possible maintenance of soil fertility contributes to the uptake of micronutrients for olive trees (Chatzisthatis *et al*., 2017). For an integrate disease management strategy aiming at reducing the spread of the bacterium, accurate removal of weeds from olive orchard during periods from the end of winter to spring to decrease the juvenile stages of the insect vector populations and the regular, light pruning of the tree crowns are very important. These strategies are about to be carried out on many olive farms of Salento.

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Current Topics

Grapevine trunk disease in European and Mediterranean vineyards: occurrence, distribution and associated disease-affecting cultural factors

Lucia GUERIN-DUBRANA[1,*], Florence FONTAINE[2], Laura MUGNAI[3]

[1] *Université de Bordeaux, ISVV, UMR1065 Santé et Agroécologie du Vignoble, Bordeaux Sciences Agro, F-33175 Gradignan, France*
[2] *Structure Fédérative de Recherche (SFR) Condorcet-FR CNRS 3417, Université de Reims Champagne-Ardenne, Unité de Recherche Résistance Induite et Bioprotection des Plantes EA 4707, BP 1039, (Cedex 2), F-51687 Reims, France*
[3] *Dipartimento di Scienze e Tecnologie Agrarie, Alimentari, Ambientali e Forestali (DAGRI) - Sez. Patologia vegetale ed Entomologia, University of Florence, Piazzale delle Cascine 28, 50144 Florence, Italy*
*Corresponding author: lucia.guerin@agro-bordeaux.fr

Abstract. A survey was carried out in 22 European and some non-European Mediterranean countries in 2015 and 2016, to gain insights into the main fungal grapevine trunk diseases (GTDs) in Europe. Information was obtained from 105 viticulture regions, representing approx. four million ha of vineyards. Vintage and table wines are the main industry products, followed by nursery plants and table grapes. Diverse scion cultivars are grown, with 13 cultivars providing 50% of survey respondent production, and Riesling Italico, Chardonnay, Cabernet Sauvignon and Merlot predominating. The five most used rootstocks are SO4, 110R, K5BB, 41B and 1103P, together representing about 50% of survey respondent production. Despite the diversity of grapevine training, Guyot and cordon remain the most widespread methods, and grapevines are mainly hand-pruned. The use of pneumatic shears and mechanical pruning is becoming more common in some countries. The survey questionnaire proposed a simplified set of symptoms or diseases occurring in mature vineyards [apoplexy, esca complex leaf symptoms (including Grapevine Leaf Stripe Disease, GLSD), dead cordon, Eutypa related dieback, Botryosphaeria or unidentified agent dieback, Phomopsis cane and leaf spot]. Apoplexy and esca/GLSD, defining the esca complex, were the most frequent and increasing syndromes in almost all countries, except Israel and the United Kingdom, where these diseases are not present or not recorded in the survey. Dead cordon and Phomopsis cane and leaf spot were mentioned as occurring in a large number of regions but not frequently. Within individual countries, the profile of GTDs varied according to the region, and to pedo-climatic and production conditions. GTDs on young vines were uncommon, but remain a subject of concern in some of European countries with large nursery production. This overview on spread and relevance of GTDs in Europe and related aspects will be a useful starting point for policy makers and for collaborative research on factors contributing to the increasing disease incidence of GTDs.

Keywords. Esca complex, Botryosphaeriaceae, *Phomopsis*, *Eutypa*, dieback.

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INTRODUCTION

Europe has the greatest concentration of vineyards in the world. In 2016, Continental European vineyards covered approx. 4 million ha, which is more than the half the world vineyard area (Fontaine *et al.*, 2016). Between 2009 and 2014, the average annual production of wine was 167 million hectolitres (hL), representing 65% of world production and 70% of global wine exports (https://ec.europa.eu/agriculture/wine/statistics_en). The world wine market, considered by the sum of exports for all producing countries, is 104.3 million hL, valued at 28.3 billion euro. These production data reflect the economic importance of the wine industry in Europe. European vineyards also embody strong cultural heritage, demonstrated by the diversity of grapevine cultivars and viticultural practices (Black and Ulin, 2013).

Increasing reports of damage caused by grapevine trunk diseases (GTDs) in the last 20 years have raised concerns about these diseases affecting the culturally and economically important grapevine industries. These diseases, causing decline and plant death, are one of the greatest threats to the wine industry (Fontaine *et al.*, 2016). Increased disease incidence and severity are commonly attributed to different factors, including expanded planted area and increased productivity in the 1990s, and changes in cultural practices (Surico *et al.*, 2004). The ban of sodium arsenite in the beginning of this century for disease management in the European Union, has also been suggested as a factor, but it is well known that the upsurge of symptoms was recorded throughout Europe much earlier, at end of the 1980s (Graniti *et al.*, 2000; Surico *et al.*, 2004; Bertsch *et al.*, 2013).

GTDs cause reduced yields, they decrease the longevity of vineyards and reduce wine quality (Calzarano *et al.*, 2004, 2018; Lorrain *et al.*, 2012). In France, for example, about 13% of French vineyards are affected by wood diseases (Grosman and Doublet, 2012). In Sicily, Sidoti *et al.* (2000) reported a case where 15% of young vines in a vineyard showed signs of decline. Decline of young vines associated with black foot agents has been reported in different countries (Rego *et al.*, 2000; Halleen *et al.*, 2006, 2007; Cardoso *et al.*, 2013; Dos Santos *et al.*, 2014; Carlucci *et al.*, 2017; Úrbez-Torres *et al.*, 2014; Langenhoven *et al.*, 2018). Consequently, large disease management efforts are required to limit the spread and damage of these diseases, and to maintain the high levels of quality and productivity of European viticulture and wine production, as a major industry in Europe (Gramaje and Di Marco 2015; Gramaje *et al.*, 2018; Mondello *et al.*, 2018a).

Establishment of GTD control methods must consider the present socio-economic situation and new challenges. The increase in vineyard area in Europe is probably due to new European Union regulations making it easier to plant new vineyards (Reg. EU 1308/2013, AA.VV., 2013). Climatic changes also permit expanded geographical distribution of vineyards. The boundary of European viticulture has shifted northwards with new viticulture regions in the United Kingdom and Belgium (Mozell and Thach, 2014). In southern European countries, adapted cultural practices, such as irrigation, are developing to compensate water stress (Songy *et al.*, 2019).

Knowledge of the distribution of GTDs and the main factors associated with their development is essential to predict their spread, and to improve disease management. Data on some GTDs is sparse and incomplete through national or regional surveys, and a comprehensive inventory of GTD occurrence and frequency in Europe and Mediterranean regions is urgently required. Therefore, the European Cooperation in Science and Technology (COST) Action FA1303 "Sustainable Control of Grapevine Trunk Disease" (Fontaine and Armengol, 2014) created a network of multidisciplinary scientific institutes and companies to improve understanding of GTDs. A specific objective of this COST Action was to develop an overview of the occurrence of the different GTDs in each of the main viticulture regions of the European Union and neighbouring Mediterranean countries.

GTDs include several diseases, all defined as syndromes associated with or caused by pathogenic fungi growing in woody tissues of the vine.

Esca disease, previously called "apoplexy" or "folletage", was the first GTD reported in many European and Mediterranean countries (Viala, 1926; Mugnai *et al.*, 1999; Larignon, 2016). Esca was the original name given to a grapevine wood symptom, white rot, associated with symptoms in the crowns, causing sudden vine death (apoplexy). Typical leaf stripe symptoms were later associated with the same wood degradation. Consequently, the esca disease, was extended to the whole set of these symptoms, and from them, was described as chronic or mild forms and severe or acute forms, also named apoplectic forms or apoplexy. The leaf stripe symptom was characterized by multiple banding discoloration surrounding dry, brittle, light brown or red-brown necrotic tissues bordered by narrow red or yellow borders. These external foliar symptom were proposed by Surico (2009) to be treated as the external expression of a probably tracheomycotic disease, i.e. a separate disease from internal stem white rot ("esca", as originally proposed by French researchers). Surico (2009) defined the concept of the "esca complex", comprising different

diseases according to the stage of vine life and the types of symptoms. Among them, the "Grapevine Leaf Stripe Disease", corresponding to the external specific symptom "tiger stripe" not associated to the internal white rot (Edwards *et al.*, 2001), could be seen more clearly in young diseased vines than in older vines. When GLSD is associated with white rot in the same plant, the name of esca or esca proper is used. During the present study, external visible symptoms on vines with characteristic leaf stripe patterns and sudden wilting were considered under the names, respectively, esca/GLSD and apoplexy. Apoplexy corresponds to a sudden wilting of entire vines or of one vine arm, including drying, shrivelling and leaf fall, and this can be caused by many factors, included root rots, not all of which have been described. Apoplexy and leaf stripe symptoms can both occur from late spring to the end of the growing season.

The causes of the external symptoms in the esca complex remain to be fully clarified, and Koch's postulates for these symptoms have not been completed. Two Ascomycetes, *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* (syn. *P. aleophilum*), are the most common vascular pathogens in GLSD and related diseases (brown-wood streaking, Petri disease). Present in vines alone or in combination, these fungi produce localized necrosis in wood xylem, such as black spots, eventually surrounded by pink to brown wood discoloration, and produce a range of phytotoxins that can be translocated to the leaves (Andolfi *et al.*, 2011). On older vines in European and Mediterranean regions, beside the Ascomycete fungi, it is common to find lignin-degrading Basidiomycete fungi (usually *Fomitiporia mediterranea*, and occasionally other Basidiomycetes) responsible for white rot. Other species of Ascomycetes from the Botryosphaeriaceae or Diatrypaceae (i.e *Eutypa lata*) can also be isolated from wood necroses from vines exhibiting typical leaf stripe foliar symptoms (Bruez *et al.*, 2014) without direct relationships between their presence and the foliar symptoms.

Eutypa dieback, also called "eutypiosis" or "dying-arm disease" was first described on apricot, and then on grapevine in the 1970s (Carter, 1991). Eutypa dieback has been reported in numerous countries from European and Mediterranean vineyards (Péros and Berger, 2003, Berraf-Tebbal *et al.*, 2011). In spring, new vine shoots are stunted with short internodes, the leaves are small chlorotic with marginal necroses and sometimes have dead interveinal tissues with the development of small fruit clusters (Rolshausen *et al.*, 2014). Foliar symptoms are associated with sectorial wood necroses or wedge-shaped cankers in perennial vine organs. The causal agent of Eutypa dieback is mainly the Ascomycete fungus *Eutypa lata*. Since the early 2000s, many other species in the Diatrypaceae have been isolated from necroses associated with Eutypa dieback symptoms (Trouillas *et al.*, 2010; Luque *et al.*, 2012, Pitt *et al.*, 2013, Rolshausen *et al.*, 2014), but *E. lata* remains the most common and virulent fungus causing the typical necrosis and foliar symptoms (Trouillas and Gubler, 2010). This fungus enters vines through pruning wounds, and colonizes the xylem tissues. There it produces phytotoxic compounds that may be transported by transpiration stream towards the aerial vine parts, producing stunting of new shoots (Molyneux *et al.*, 2002).

Botryosphaeria dieback of grapevine includes different symptoms and involves various Botryosphaeriaceae species (Úrbez-Torrez, 2011). The main symptoms include wedge-shaped cankers, dark streaking of grapevine wood, elongated black lesions on the shoots (cane cankers), progressive bud-break failure and plant dieback, and fruit rot in some hosts. At least 21 Botryosphaeriaceae species have been associated with Botryosphaeria dieback of grapevines (Úrbez-Torres, 2011), including *Diplodia seriata, Neofusicoccum parvum* and *Botryosphaeria dothidea* which are the most frequently isolated fungi (Úrbez-Torres, 2011). These pathogens may be present in asymptomatic tissues as latent infections (Gonzalez and Tello, 2011; Bruez *et al.*, 2014). The transition from the endophytic latent infection to active pathogen colonization could be related to factors such as host water stress (Luque *et al.*, 2010). Since the early 2000s, research on Botryosphaeriaceae on grapevine increasingly showed that species in this family play important roles in decline of vines (Larignon *et al.*, 2015). An overview of this decline in Europe is needed to identify the risk factors associated with Botryosphaeriaceae as wood pathogens.

The final GTD included in the present study was Phomopsis cane and leaf spot caused by *Diaporthe ampelina*, often associated with other less virulent species, which can also induce grapevine canker. Vines with Phomopsis cane and leaf spot have characteristic black necrotic irregular-shaped lesions on the cane internodes, occurring in spring and winter, and bleaching and cane cankers and sometimes branch dieback (Ravaz and Verge, 1925; Bugaret, 1984). Rachis necrosis and brown, shrivelled berries on fruit bunches occur near to harvest. The disease, still frequently named ''excoriose'' in many European viticulture regions, is caused by *Diaporthe* spp., particularly *Diaporthe ampelina* (previously called *Phomopsis viticola*) (van Niekerk *et al.*, 2005; Guarnaccia *et al.,* 2018). In internal wood tissue, *Diaporthe* spp. may cause perennial cankers and vascular discoloration similar to that observed in Botryospha-

eria and Eutypa diebacks (Úrbez-Torres *et al.*, 2013; Baránek *et al.*, 2018).

On young grapevines, Petri disease and black-foot are the most damaging wood diseases (Rego *et al.*, 2000; Agustí-Brisach and Armengol, 2013; Carlucci *et al.*, 2017). The foliar symptoms of both diseases are similar, including typical decline symptoms such as leaf chlorosis with necrotic margins, stunting, budbreak delay, decreased growth and vigour, and wilting of leaves or shoots. In longitudinal stem section, vines affected by Petri disease have brown or black streaking in the xylem vessels, often with necroses at the graft unions. Various fungi have been isolated from the altered wood tissues, including *P. chlamydospora* and, less frequently, various species of *Phaeoacremonium* (Mugnai *et al.*, 1999; Gubler *et al.*, 2015) and *Cadophora* (Gramaje *et al.*, 2010).

Black-foot also manifests as necrotic root lesions, rootstock wood necroses, and gradual decline leading to vine death. The fungi causing black-foot are mainly *Cylindrocarpon*-like species belonging to the genera *Ilyonectria, Neonectria, Campylocarpon* in the Nectriaceae (Agusti-Brisach and Armengol, 2013). Decline of young vines may also be caused by other fungi or nematodes, alone or in pathogen complexes, including Botryosphaeriaceae, *Phomopsis*, *Pythium, Phytophthora*, which have also been isolated from declining vines in young vineyards (Halleen *et al.*, 2007; Agustí-Brisach and Armengol, 2013).

The above descriptions of the main GTDs shows the diversity of syndromes and associated pathogenic agents that are involved. For each GTD, incidence and symptom profiles may greatly vary according to regional or local factors such as climate, soil and cultural practices.

The species composition of grapevine trunk pathogen communities may be affected by environmental factors. Climate plays an important role in pathogen distribution (Merrin *et al.*, 1995; van Nierkerk *et al.*, 2011). For instance, geographical distribution of Botryosphaeriaceae species is strongly linked to climatic conditions. *Lasiodiplodia theobromae* is tolerant to high temperatures, while some species, such as *Diplodia seriata*, are very adaptable to different temperatures (Úrbez-Torres, 2011; Songy *et al.*, 2019). In Europe and Mediterranean regions, various pedo-climatic conditions, "terroirs", are associated with a large number of local varieties of *Vitis vinifera* subsp. *vinifera*, cultivated for mainly for wine, but also for fresh fruit and juice production. Vine training and cultural practices are also diverse, and these result from long grapevine-growing traditions.

Considering the wide diversity of viticulturists, the present study addressed the following questions on GTDs: (1) What is the distribution of each GTD disease on young or mature vines in European and Mediterranean countries? (2) Can information be obtained on the most proliferating or economically important GTDs? and (3) Can GTD occurrence and frequency be explained by regional or local factors? To provide answers to these questions, a large survey was carried out using a qualitative questionnaire. The survey included sets of questions to gather information on the occurrence of GTDs, cultural practices and terroir characteristics.

MATERIALS AND METHODS

Survey questionnaire

The questionnaire was designed to gather information about the occurrence and frequency of the main symptoms associated to GTDs on mature and young vines, and data on grapevine production and agronomic practices that could be related to disease occurrence, from the main grape production regions of European and Mediterranean countries. The questionnaire comprised two parts to obtain information on: 1) GTDs occurring in mature and young vineyards at regional scale; 2) vineyard production and agronomic practices at regional scale; 3) regional climate type in the seasons influencing disease and pathogen cycles, i.e. spring and summer; and 4) reference person(s) who collected the data in each surveyed country/ region.

The questionnaire included open and closed questions. For occurrence of GTDs on adult vines, six types of syndromes were included: (1) apoplexy, (2) esca/ GLSD, ''dead cordon'', (i.e. the result of dieback caused by wood canker agents observed in vineyard. Dead cordon is an non-specific symptom that may be related to (3) Eutypa dieback; (4) Botryosphaeria dieback; and (5), when the causal agent of cankers and dieback was not identified "dead cordon agent not identified" was reported. A sixth possible syndrome was also listed as Phomopsis cane and leaf spot.

On young vines, three types of declining symptoms were proposed: (1) Petri disease, (2) black foot and (3) decline caused by undetected agent, physiological factor or other agents. For each described syndrome, a scale that rated the frequency of the syndrome was defined as: 0, not present or not recorded (with 'not recorded' indicated with a code); 1, present but not frequent; or 2, frequent. Three other closed questions on the perception of importance were included: Is the syndrome perceived to increase in the region? Is the syndrome perceived to be worrying in the region? What is the susceptibility of the main cultivars? The questionnaire also included knowledge about the amount of GTD investi-

gation in the country: Did laboratory or extension services carry out GTD diagnoses and detection? If yes, is this at regional or national level? The second part of the survey included questions on the range of production types within region or country, including: vintage wine, table wine, table grapes, grapevine nursery. Information was also requested on the main scion and rootstock cultivars, and on the relevant cultural practices linked to GTDs, including details of the common vine training, trellising systems, and pruning methods applied at regional scale.

Questionnaire dissemination and analysis

The questionnaire, formalized as tables on Excel sheets, was distributed in 2015, by email to each National corresponding researcher, as members of the COST Action FA1303 group. The COST Action included all the European countries with relevant grapevine cultivation plus some representative Mediterranean countries. In each country a reference person selected representative grape cultivation areas through collaboration with the most competent respondents in the area, and co-ordinated the data gathering from each respondent. These groups included researchers, extension staff and field technicians. The returned questionnaires with missing data or non-adapted responses were sent again. Focus respondents were surveyed in order to obtain feedback on the response accuracy and to validate some questionnaire responses. All participant responses were gathered in a database filing system. The descriptive analysis of the data was set up using simple calculations of proportions, rates and variable distributions. The analysis of contextual data developed a general view of the characteristics of the surveyed viticulture regions.

RESULTS

General information on grape production and grapevine growing methods

The questionnaire responses were received from 22 countries, including 19 European countries, one Maghreb country (Algeria) and two Middle East countries (Lebanon and Israel) (Table 1). Data from 105 viticulture regions, corresponding to the main regions of the surveyed countries, were recorded. The number of regions by country varied from one (England) to 12 (Italy). The vineyard areas in the surveyed countries totaled about 4 million ha, and varied greatly according to country, from 1,000 ha (England) to approx. one million ha (Spain). Among the surveyed countries, Spain, Italy, France and Turkey represented the main producers, with approx. 75% of the total vineyard area. Among the different types of production, Quality Wine ["Quality Wines Produced in Specified Regions" (QWPSR)] and Table Wine (TW) represented, respectively, the first and second ranges in almost all the surveyed countries, for 87 and 79% of reports (Table 2). Only in Algeria and Turkey Table Grape (TG) was reported as the main production. Table grape production was the second or third type of production in eight countries when rated for relevance, totaling 33 regions (Tables 1 and 2). Dried Raisin (DR) production was not commonly mentioned within the surveyed countries, but represents a major grape production component in Algeria and Turkey. Significant nursery plant production was reported in 16 countries, and generally this was rated as the third most important sector (77% of reports). Nursery production was reported as the 1st or 2nd type of production in importance only in six regions of five countries: Bulgaria, France, Hungary, Italy and Romania.

Eighty-six different scion cultivars were reported in the questionnaire responses. Eighteen were reported from at least four regions in different countries, including 66% of the reports (Figure 1). However, Chardonnay, Cabernet Sauvignon and Merlot were the most frequently grown varieties in the surveyed regions. Table 3 lists the other varieties recorded, as major ones in particular regions. The diversity of rootstock varieties reported in the regions surveyed was much less than that for scion variety. Twenty-four rootstock varieties were mentioned by respondents as the main rootstock planted varieties, but only eight rootstock varieties were mentioned in 89% of the reports (Figure 2). These were dominated by S04, 110R and K5BB. Three main training methods were reported, as Guyot (42% of reports), Cordon (33%) and Gobelet (14%). Three other training methods reported were: Pergola, especially in Italy, Lebanon and Algeria; Chablis, in France; and Sylvoz, in Hungary, Italy and Germany. The respondents generally noted that grapevines were mostly hand-pruned, but that pneumatic pruning shears for hand pruning and mechanical pruning were becoming increasingly used in some countries.

Distribution and frequency of GTDs

In 18 of 22 countries, GTD diagnoses and detection were carried out by laboratory or extension services at national (in ten countries) or regional (eight countries) levels (Table 1).

Table 1. General characteristics of grapevine production in surveyed European and Mediterranean countries.

Country	Country code	No. of surveyed vine cultivation regions	Vineyards area (× 1,000 ha)[b]	Type of production (in order of importance)[a]	Wine production (× 1,000 hL)[b]	GTD investigation diagnosis and detection (yes or no), regional or national level
European Union Mediterranean Countries						
Austria	AT	4	45	QW/TW/N	1,999	No
Bulgaria	BG	5	63	QW-TW/TG/N	745	No
Croatia	HR	2	29	QW/TW/N	842	Yes, national
Czech Republic	CZ	3	17	QW/TW/N	536	Yes, regional
France	F	7	789	QW/TW/N	46,534	Yes, regional
Germany	DE	4	102	QW/TW/N	9,202	Yes, regional
Greece	EL	3	110	QW/TG/N	2,800	Yes, regional
Hungary	HU	6	62	QW/TW/N	2,427	Yes, regional
Italy	IT	12	690	QW/TW/TG/N	44,229	Yes, national
Montenegro	ME	2	9	QW/TW/N	161	Yes,national
Portugal	PT	5	224	QW/TW/N	6,206	Yes, regional
Romania	Ro	5	192	QW/TW/N/TG	3,750	Yes, national
Serbia	RS	4	54	TW/TG	2,332	No
Slovakia	SK	6	16	QW/TW/TG	258	Yes, national
Slovenia	SI	3	16	QW/TW/N	494	Yes, national
Spain	SP	7	975	QW/TW/N	39,494	Yes, national
Switzerland	S	6	15	QW/TW/N	934	Yes, national
United Kingdom	UK	1	2	QW/TW	47	Yes, national
Non-European Union Mediterranean Countries						
Algeria	AL	71	74	TG/R/QW/TW	507	No
Israel	Il	8	8	QW/TW/TG/N	246	Yes, national
Lebanon	Le	3	14	QW/TG/TW	80	Yes, national
Turkey	T	6	502	TG/R/QW/TW	615	Yes, regional
Total	22	105	4066	-	1644,483	-

[a] QW (Quality wine); TW (Table wine); TG (Table grape), R (raisin), N (Nursery)
[b] 2014 data in OIV, 2014.

Table 2. Estimates of the relevance of grapevine production types, based on frequency of reports from the all viticultural regions surveyed.

Production type range	Quality wine (QW)		Table wine (TW)		Table grape (TG)		Nursery plants (N)		Raisins (R)		Total for production range
	No. Reports[a]	%[b]	No. Reports	%	No. Reports	%	No. Reports	%	No. Reports	%	
1st Production type	80	87.1	14	17.3	6	15.4	3	5.1	3	75	106
2nd Production type	11	11.8	64	79	19	48.7	3	7.7	1	25	98
3rd or 4th Production type	1	1.1	3	3.7	14	35.9	35	89.4	0	0	53
Total for type of production	93		81		39		41		4		258

[a] Number of reports over 105 regions surveyed.
[b] Percentage over total No. of reports for each production type.

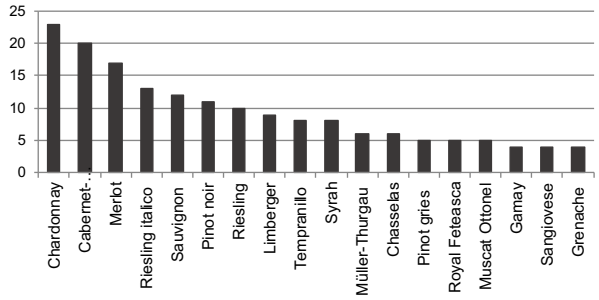

Figure 1. Numbers of reports of the main scion cultivars recorded in 105 surveyed regions.

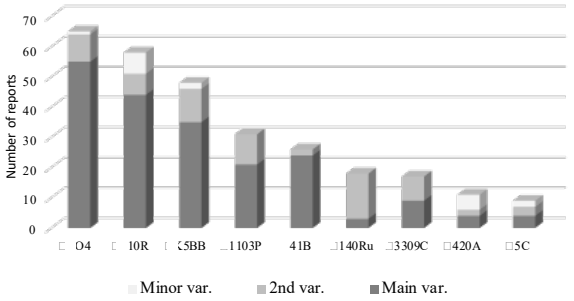

Figure 2. Number of reports of occurrence of the main rootstock varieties and their range in the regions where the survey was carried out.

Table 3. Grapevine scion varieties infrequently identified in survey questionnaire responses, with one to three mentions.

Scion variety	Report No.	Scion variety	Report No.
Furmint	3	Loureiro	1
Gamaret	3	Malvasia istriana	1
Grüne Veltliner	3	Mavrodaphni	1
Macabeu	3	Melon	1
Touriga Nacional	3	Meunier	1
Zweigelt	3	Montepulciano	1
Bobal	2	Montepulciano	1
Colombard	2	Montonico	1
Gewurztraminer	2	Muscat d'Alexandrie	1
Harslevelu	2	Negoska	1
Pinot blanc	2	Nuragus	1
Primitivo	2	Parellada	1
Yellow muscat	2	Passerina,	1
Agiorgitiko	1	Pecorino,	1
Airén	1	Plavac mali	1
Alfrocheiro preto	1	Portugais bleu	1
Aligoté	1	Prokupac	1
Alvarinho	1	Rebula	1
Arvine	1	Refosco	1
Baga	1	Rkatziteli	1
Bombino bianco	1	Saint-Laurent	1
Cabernet franc	1	Tamnjanika	1
Calabrese	1	Tinta Amarela	1
Carignan	1	Touriga Franca	1
Castelão	1	Trebbiano	1
Catarratto	1	Trebbiano romagnolo	1
Chenin	1	Treixadura	1
Dimiat	1	Ugni blanc	1
Fernão Pires	1	Vermentino	1
Garganega	1	Vernaccia	1
Glera	1	Vranac crni	1
Glera	1	Xarello	1
Grillo	1	Xinomavro	1
Jaen N	1	Zizak	1

GTDs on mature vines

Data on GTD occurrence in all the surveyed regions were combined. This revealed different frequency profiles for the different GTD syndromes reported in adult vines (Figure 3). The records of apoplexy and esca/GLSD showed similar profiles with a greater proportion of records for ''frequent'' occurrence , reaching 59% for apoplexy and 55.2% for esca/GLSD. These two syndromes were the most widespread and the only diseases reported in all the surveyed countries. They were present in most of the regions (apoplexy in 91% and esca/GLSD 86% over the 105 regions) (Table 4). In contrast, the frequency profiles of the three types of ''dead cordon", caused by canker agents, Eutypa dieback, Botryosphaeria dieback, or "agent not identified", showed less occurrence, and were reported as frequent, respectively, in only 20, 30.5 and 27.6% of the regions. These diseases were not observed (or not reported), respectively, from 22, 27.6 and 22% of the regions. Phomopsis cane and leaf spot was present in 60.9% of the regions, but was usually not frequent.

The syndromes surveyed had different occurrences in different countries, although all syndromes highlighted in the questionnaire were widespread in Continental European and Mediterranean countries (Table 4). There was a similar frequency profile for both syndromes, apoplexy and esca/GLSD within a country, except in Hungary, where esca/GLSD is more frequent than apoplexy and in Romania and Serbia, where apoplexy is more frequent than esca/GLSD. Dead cordon, caused by Eutypa dieback, was always reported less frequently than apoplexy and esca/GLSD. It was actually reported as frequent only in 7 countries, even if it was present in all countries except Israel. Dead cordon caused by other pathogens (Botryosphaeriaceae or not identified agent) were frequently recorded in almost all countries, except from Austria, probably because of lack of observation.

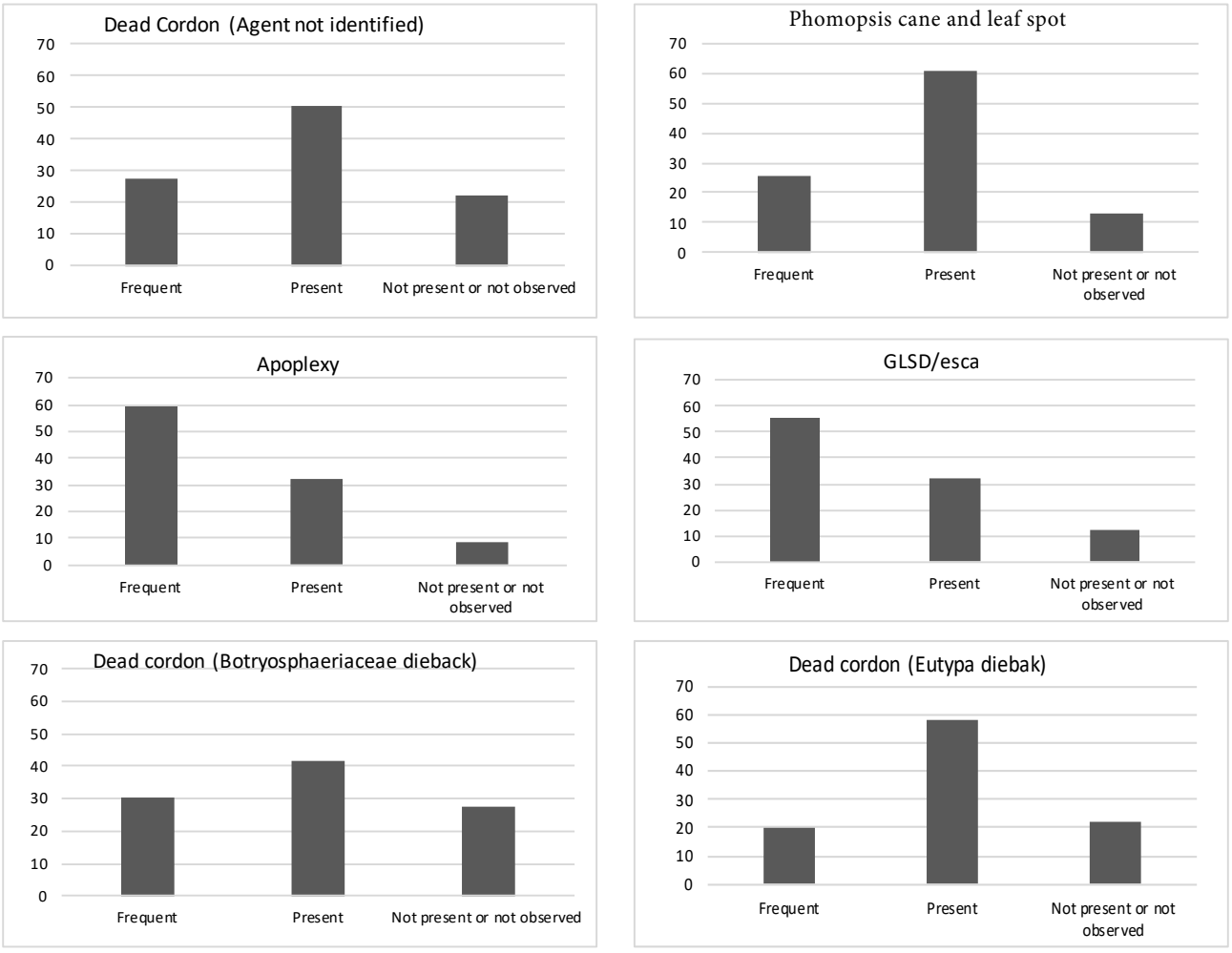

Figure 3. Reports (%) for all surveyed countries of the different GTDs on adult vines.

The level of occurrence of both of these syndromes varied according to the country and the region.

Cabernet Sauvignon was the most frequently recorded of the 18 main reported varieties (Figure 4) as the most susceptible variety to esca complex. Fourteen other varieties were cited at least once. Fewer reports concerned the susceptibility to the other GTDs. Some varieties like Ugni Blanc, Cabernet, Cabernet Sauvignon and Chasselas were reported as susceptible to Eutypa dieback in France and Germany. In Portugal, the varieties Touriga National, Loureiro, Alvarinho, Aragonez, Touringa Franca and Syrah were reported as susceptible to both esca complex disease and Botryosphaeria dieback. In Turkey, the variety Sultana Seedless was also noted as susceptible to these two disease syndromes.

GTDs on young vines

The occurrence of GTDs on young vines (Petri disease, black foot and general decline) is shown in Figure 5. The three forms on young vines were reported less frequently than the syndromes on adult vines. Decline diseases on young vines caused by Petri disease or unknown agent were mainly reported as "present but not frequent" (Petri disease, 44% and unknown agent, 61%). Petri disease was mentioned as frequent in nine regions from five countries (Hungary, Spain, Slovenia, Italy and Slovakia) (Table 5), and present in 14 countries, of the 22 surveyed countries. In seven other countries (Czech Republic, Romania, Austria, France, Lebanon, Algeria, Serbia), this decline was not observed (or not recorded). Black foot was mentioned as "frequent" only in two countries: Hungary and Spain, while it was noted "present", but not frequent, in nine other countries.

Table 4. Reported level of occurrence of Grapevine Trunk Diseases on adult vines in each surveyed European or Mediterranean country.

Country	No. of regions surveyed	Apoplexy/esca acute form						Esca/GLSD[a]						Dead cordon (Eutypa dieback)					
		Frequent		Present		Not present or not observed		Frequent		Present		Not present or not observed		Frequent		Present		Not present or not observed	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Algeria	6	3	50	0	0	3[b]	50	3	50	0	0	3[b]	50	3	50	0	0	3[b]	50
Austria	4	4	100	0	0	0	0	4	100	0	0	0	0	4	100	0	0	0	0
Bulgaria	5	0	0	5	100	0	0	1	20	4	80	0	0	0	0	5	100	0	0
Croatia	2	2	100	0	0	0	0	2	100	0	0	0	0	2	100	0	0	0	0
Czech Republic	3	1	33.3	2	66.7	0	0	0	0	3	100	0	0	0	0	3	100	0	0
England	1	0	0	1	100	0	0	0	0	1	100	0	0	0	0	1	100	0	0
France	7	6	85.71	1	14.29	0	0	7	100	0	0	0	0	1	14.29	6	85.71	0	0
Germany	4	3	75	0	0	1[b]	25	3	75	0	0	1[b]	25	0	0	3	75	1[b]	25
Greece	3	0	0	3	100	0	0	0	0	3	100	0	0	3	100	0	0	0	0
Hungary	6	2	33.3	4	66.7	0	0	5	83.3	1	16.7	0	0	0	0	5	83.3	1	16.7
Israel	5	0	0	5	100	0	0	0	0	5	100	0	0	0	0	0	0	5	100
Italy	12	10	83.3	2	16.7	0	0	11	91.7	1	8.3	0	0	0	0	11	91.7	1	8.3
Lebanon	3	3	100	0	0	0	0	3	100	0	0	0	0	0	0	3	100	0	0
Montenegro	2	1	50	1	50	0	0	2	100	0	0	0	0	0	0	1	50	1	50
Portugal	5	5	100	0	0	0	0	3	60	2	40	0	0	0	0	1	20	4	80
Romania	5	5	100	0	0	0	0	0	0	5	100	0	0	3	60	0	0	2	40
Serbia	5	4	80	1	20	0	0	0	0	1	20	4	80	5	100	0	0	0	0
Slovakia	6	0	0	6	100	0	0	0	0	6	100	0	0	0	0	6	100	0	0
Slovenia	3	3	100	0	0	0	0	3	100	0	0	0	0	0	0	3	100	0	0
Spain	6	6	100	0	0	0	0	6	100	0	0	0	0	0	0	6	100	0	0
Switzerland	6	4	66.7	2	33.3	0	0	4	66.7	2	33.3	0	0	0	0	6	100	0	0
Turkey	6	0	0	1	16.7	5[b]	83.3	1	16.7	0	0	5[b]	83.3	0	0	1	16.7	5[b]	83.3
Total	105	62		34		9		58		34		13		21		61		23	
%	100	59		32.4		8.6		55.2		32.4		12.4		20		58.1		21.9	

(Continued)

Table 4. (Continued).

Country	No. of regions surveyed	Dead cordon (Botryosphaeria dieback)						Dead cordon (agent not identified)						Phomopsis cane and leaf spot					
		Frequent		Present		Not present or not observed		Frequent		Present		Not present or not observed		Frequent		Present		Not present or not observed	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Algeria	6	1	16.7	0	0	5[b]	83.3	3	50	0	0	3[c]	50	0	0	3	50	3[b]	50
Austria	4	0	0	0	0	4	100	0	0	0	0	4	100	0	0	4	100	0	0
Bulgaria	5	1	20	1	20	3	60	2	40	3	60	0	0	0	0	5	100	0	0
Croatia	2	2	100	0	0	0	0	0	0	2	100	0	0	2	100	0	0	0	0
Czech Republic	3	0	0	3	100	0	0	3	100	0	0	0	0	0	0	3	100	0	0
England	1	0	0	1	100	0	0	0	0	1	100	0	0	1	100	0	0	0	0
France	7	1	14.3	1	14.3	5	71.4	2	28.6	4	57.1	1	14.3	0	0	7	100	0	0
Germany	4	0	0	3	75	1[b]	25	0	0	3	75	1[b]	25	0	0	3	75	1[b]	25
Greece	3	0	0	3	100	0	0	0	0	3	100	0	0	3	100	0	0	0	0
Hungary	6	3	50	2	33.3	1	16.7	3	50	3	50	0	0	0	0	4	66.7	2	33.3
Israel	5	0	0	5	100	0	0	0	0	5	100	0	0	0	0	5	100	0	0
Italy	12	6	50	3	25	3	25	5	41.7	5	41.7	2	16.	3	25	9	75	0	0
Lebanon	3	3	100	0	0	0	0	3	100	0	0	0	0	0	0	2	66.7	1	33.3
Montenegro	2	1	50	0	0	1	50	1	50	1	50	0	0	1	50	1	50	0	0
Portugal	5	3	60	2	40	0	0	0	0	1	20	4	80	1	20	4	80	0	0
Romania	5	0	0	4	80	1	20	3	60	0	0	2	40	5	100	0	0	0	0
Serbia	5	5	100	0	0	0	0	1	20	4	80	0	0	4	80	1	20	0	0
Slovakia	6	0	0	6	100	0	0	0	0	6	100	0	0	0	0	6	100	0	0
Slovenia	3	0	0	3	100	0	0	0	0	3	100	0	0	0	0	3	100	0	0
Spain	6	5	83.3	1	16.7	0	0	3	50	2	33.3	1	16.7	1	16.7	3	50	2	33.3
Switzerland	6	0	0	6	100	0	0	0	0	6	100	0	0	5	83.3	1	16.7	0	0
Turkey	6	1	16.7	0	0	5[b]	83.3	0	0	1	16.	5[b]	83.3	1	16.7	0	0	5[b]	83.3
Total	105	32		44		29		29		53		23		27		64		14	
%	100	30.5		41.9		27.6		27.6		50.5		21.9		25.7		60.9		13.3	

[a] Grapevine Leaf Stripe Disease.
[b] Not surveyed region.

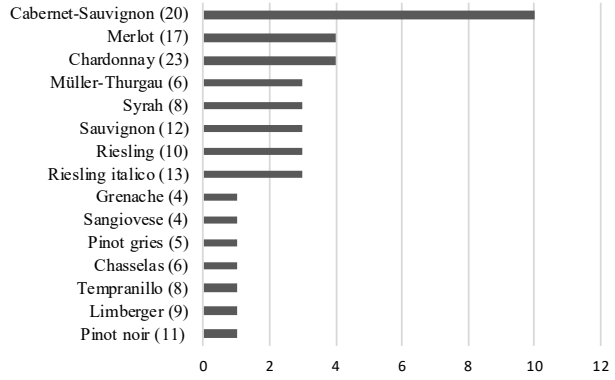

Figure 4 Numbers of survey response reports on cultivar susceptibility to esca complex (Total report number in brackets).

Focus on five sample countries

In order to give more details in specific situations, GTD distribution was analysed within five countries: Hungary, Bulgaria, Portugal, France and Italy.

Hungary

The profile of GTDs on adult vines varied according to the wine region (Table 6A). Apoplexy was recorded as "frequent and increasing" in two regions (Pécsi and Villány), characterized by dry spring and dry and hot summer weather, which may correspond to a sub-Mediterranean climate. The GTD profile in the Balat wine region differed from the other regions, with few recorded diseases or low levels of occurrence of GTD symptoms, such as apoplexy, esca/GLSD and dead cordon, in comparison with the five other regions. The Balaton area is characterised by the proximity of Lake Balaton and volcanic soils that create a peculiar environment. In the Egri region, the respondent reported frequent and increasing esca/GLSD and dead cordon caused by Botryosphaeria dieback. Dead cordon related to Eutypa dieback and Phomopsis cane and leaf spot were mentioned less frequently. They were recorded as "occasionally present" in three regions and "not recorded" in five regions.

Bulgaria

Contrasting GTDs profiles on adult vines were observed according to region (Table 6B). Esca/GLSD was recorded as "frequent" in the only region of the "sub-Balkan" area, the Rose Valley region. All the other disease syndromes on mature vines were recorded as

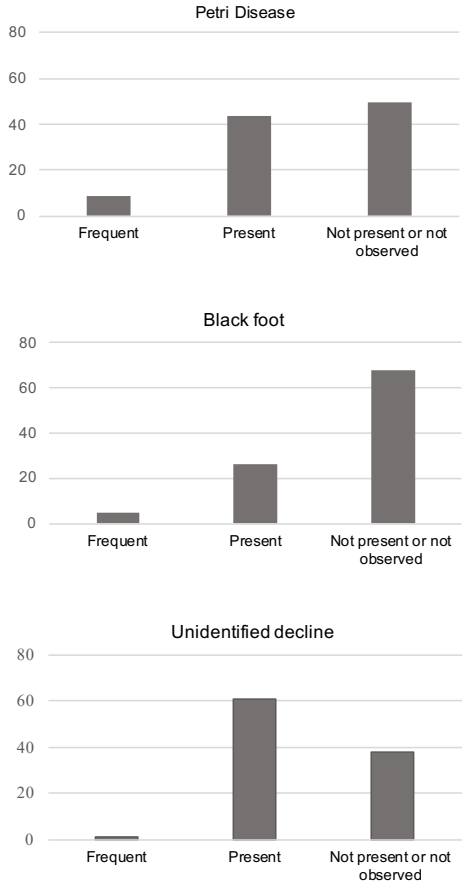

Figure 5. Proportions (%) of survey reports from all countries of different grapevine decline syndromes on young vines.

"present" or "frequent" in this region. In the Northern region, characterized by a wet and cold spring weather, dead cordon related to Botryosphaeria dieback or to an unidentified canker agent were recorded as "frequent". In contrast, only in the South-Western region were all GTDs on mature vines rarely or never observed.

Portugal

Apoplexy, esca/GLSD and dead cordon related to Botryosphaeria dieback and Phomopsis cane and leaf spot were recorded as "frequent" in Portugal (Table 6C). In contrast, dead cordon related to Eutypa dieback was recorded as "absent". In Vinho verde, in the northern Portugal, apoplexy and Phomopsis cane and leaf spot were recorded as "frequent and worrying". The climate of that region is characterized by cool and wet springs and a cool summers. In Dao and Alentejo, apoplexy and esca/GLSD were recorded as "frequent and increasing"

Table 5. Reported levels of occurrence of GTD on young vines in surveyed European and Mediterranean countries.

Country	Region No.	Petri disease						Black foot[a]						Unidentified decline[b]					
		Frequent		Present		Not present or not observed		Frequent		Present		Not present or not observed		Frequent		Present		Not present or not observed	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Algeria	6	0	0	0	0	6[c]	100	0	0	0	0	6[c]	100	0	0	0	0	6[c]	100
Austria	4	0	0	0	0	4	100	0	0	0	0	4	100	0	0	4	100	0	0
Bulgaria	5	0	0	5	100	0	0	0	0	3	60	2	40	0	0	5	100	0	0
Croatia	2	0	0	2	100	0	0	0	0	0	0	2	100	0	0	0	0	2	100
Czech Repu-blic	3	0	0	0	0	3	100	0	0	0	0	3	100	0	0	3	100	0	0
England, UK	1	0	0	1	100	0	0	0	0	1	100	0	0	0	0	1	100	0	0
France	7	0	0	0	0	7	100	0	0	3	42.9	4	57.1	0	0	2	28.6	5	71.4
Germany	4	0	0	3	75	1[c]	25	0	0	3	75	1[c]	25	0	0	3	75	1[c]	25
Greece	3	0	0	3	100	0	0	0	0	3	100	0	0	0	0	3	100	0	0
Hungary	6	2	33.3	2	33.3	2	33.3	2	33.3	2	33.3	2	33.3	0	0	5	83.3	1	16.7
Israel	5	0	0	5	100	0	0	0	0	0	0	5	100	0	0	5	100	0	0
Italy	12	1	8.3	8	66.7	3	25	0	0	0	0	12	100	0	0	9	75	3	25
Lebanon	3	0	0	0	0	3	100	0	0	1	33.3	2	66.7	0	0	3	100	0	0
Montenegro	2	0	0	2	100	2	100	0	0	0	0	2	100	0	0	1	50	1	50
Portugal	5	0	0	4	80	1	20	0	0	5	100	0	0	1	20	4	80	0	0
Romania	5	0	0	0	0	5	100	0	0	0	0	5	100	0	0	1	20	4	80
Serbia	5	0	0	0	0	5	100	0	0	0	0	5	100	0	0	5	100	0	0
Slovakia	6	1	16.7	0	0	5	83.3	0	0	0	0	5	83.3	0	0	1	16.7	5	83.3
Slovenia	3	2	66.7	1	33.3	0	0	0	0	0	0	3	100	0	0	3	100	0	0
Spain	6	3	50	3	50	0	0	3	50	2	33.3	1	16.7	0	0	5	83.3	1	16.7
Switzerland	6	0	0	6	100	0	0	0	0	4	66.7	2	33.3	0	0	0	0	6	100
Turkey	6	0	0	1	16.7	5[c]	83.3	0	0	1	16.7	5[c]	83.3	0	0	1	16.7	5[c]	83.3
Total	105	9		46		52		5		28		71		1		64		40	
%	100	8.6		43.8		49.5		4.8		26.7		67.6		0.9		60.9		38.1	

[a] Ascertained presence of *Cylindrocarpon*-like spp.
[b] Undetected agent, physiological and other agents or cause excluded.
[c] Not surveyed regions.

and/or "worrying". The spring and summer climate in Dao is wet-warm and in Alentejo is dry-hot. Apoplexy, esca/GLSD and dead cordon related to Botryosphaeria dieback were recorded as "frequent and increasing" in Bairrada, located in the western Portugal, an area with mild climate with abundant rainfall (Vinho verde, Bairrada).

France

The seven surveyed regions showed contrasting profiles of GTD occurrence on mature vines (Table 6D). Apoplexy and esca /GLSD were reported as "frequent" in almost all the regions, and were considered as "increasing" and/or "worrying" in five of of seven regions. In contrast, Eutypa dieback and Phomopsis cane and leaf spot were recorded in all the regions but not as frequently occurring. Eutypa dieback was recorded as "frequent" in Charente, a region with Oceanic climate and where the susceptible cultivar Ugni Blanc (used for distilled wines) is widespread. In the Languedoc Roussillon area, GTDs were reported as "present" or "not observed", except for esca/GLSD. Dead cordon related to Botryosphaeria dieback was reported as "present" or "frequent" only in two regions, Champagne and Rhône.

Italy

In Italy, esca/GLSD and apoplexy have high and increasing importance in all regions (Table 6E). Eutypa dieback is also widespread, being reported as "present" in all regions except Sicily (probably linked to low

Table 6. Reported levels of occurrence of GTD on adult grapevines in surveyed regions in five selected countries: Hungary (A), Bulgaria (B), Portugal (C), France (D), Italy (E). i, increasing incidence; w, worrying presence; iw, increasing and worrying P, present; PO, present occasionally. Legend: light grey : present but not frequent; medium grey: frequent; dark grey: absent or not recorded.

A. Hungary

Spring climate[a]	Summer climate[b]	Region	Apoplexy	Esca /GLSD	Dead cordon (Eutypa dieback)	Dead cordon (Botryosphaeria dieback)	Dead cordon (causal agent not identified)	Phomopsis cane and leaf spot
uf	DHsu	Tokaj			PO			
WCsp	DHsu	Balat	PO	PO				
WWsp	DHsu	Egri		i	PO	i		PO
Dsp	DHsu	Pécsi	i				PO	
WWsp	DHsu	Neszmélyi						PO
Dsp	DHsu	Villány	i				PO	PO

B. Bulgaria

Spring climate[a]	Summer climate[b]	Region	Apoplexy	Esca /GLSD	Dead cordon (Eutypa dieback)	Dead cordon (Botryosphaeria dieback)	Dead cordon (causal agent not identified)	Phomopsis cane and leaf spot
WCsp	DHsu	Northern region (Danube plain)			PO			
WWsp	DHsu	Black sea region		PO	PO			PO
WWsp	DHsu	Sub-Balkan region (Rose valley)			PO	PO		
WWsp	DHsu	Southern region (Thracian Lowland)				never	PO	
WWsp	DHsu	South-west region (Struma River Valley)	PO	PO	PO	never	PO	PO

C. Portugal

Spring climate[a]	Summer climate[b]	Region	Apoplexy	Esca /GLSD	Dead cordon (Eutypa dieback)	Dead cordon (Botryosphaeria dieback)	Dead cordon (causal agent not identified)	Phomopsis cane and leaf spot
WWsp	DHsu	Dão	iw	i				PO
WWsp	Csu	Bairrada	i	i				PO
WCsp	Csu	Vinho Verde		PO				w
WCsp	DHsu	Douro			PO		w (PO)	
WWsp	DHsu	Alentejo	w	i				

D. France

Spring climate[a]	Summer climate[b]	Region	Apoplexy	Esca /GLSD	Dead cordon (Eutypa dieback)	Dead cordon (Botryosphaeria dieback)	Dead cordon (causal agent not identified)	Phomopsis cane and leaf spot
WCsp	Csu	Bourgogne	w					
WCsp	Csu	Champagne	i	iw				
WCsp	Csu	Pays de Loire	iw	iw			iw	
WCsp	DHsu	Charentes	i	i				
WCsp	DHsu	Bordeaux						
WWsp	DHsu	Languedoc-Roussillon						
WCsp	WHsu	Rhône	w	w				

(Continued)

Table 6. (Continued).

E. Italy

Spring climate[a]	Summer climate[b]	Region	Apoplexy	Esca /GLSD	Dead cordon (Eutypa dieback)	Dead cordon (Botryosphaeria dieback)	Dead cordon (causal agent not identified)	Phomopsis cane and leaf spot
WWsp	DHsu	Sardinia				i		
WWsp	Csu	Friuli Venezia Giulia	w	w			w	
Dsp	DHsu	Sicily		i				
WWsp	DHsu	Apulia		w		w		
WWsp	DHsu	Tuscany		w				
WWsp	WHS	Veneto		w				
WCsp	DHsu	Abruzzi	w	w				
WWsp	DHsu	Romagna						
WWsp	DHsu	Emilia		w				
WWsp	DHsu	Lombardy		w				
WCsp	DHsu	Piedmont						
WWsp	Csu	Trentino		w				

[a] Wet and cool spring (WCsp), wet warm spring (WWsp), dry spring (Dsp), uncertainty factor (uf).
[b] Dry and hot summer (DHsu),Wet and Hot summer (WHsu), cool summer (Csu).

amounts of rain, which is important for Eutypa inoculum spread. Eutypa dieback was responsible for limited damage. Cankers leading to dead cordon were also recorded in all regions, but were reported as "highly relevant" only in Sardinia, Friuli, Sicily, Apulia, Tuscany, Lombardy and Trentino. Phomopsis cane and leaf spot was also reported from all regions, was more widespread in Apulia, Veneto and Piedmont than elsewhere.

Increasing and/or "worrying" GTD occurrence

The GTDs were recorded as "increasing" and/or "worrying" by the correspondents in numerous countries in Europe and some of the Mediterranean area (Table 7). These diseases were reported as more important in old than in young vineyards, mainly due to the spread of apoplexy and esca complex/GLSD, respectively, in ten and 11 surveyed countries ("increasing" or/and "worrying" occurrence), particularly in the main grape-producing countries (France, Italy, Spain and Turkey). This represents about a third of recorded regions. Dead cordon related to Botryosphaeria dieback was the third disease problem, reported to be "increasing" and/or worrying in seven countries, and 16 of the 105 surveyed regions.

Dead cordon related to Eutypa dieback was recorded as "increasing" and/or "worrying" in ten regions of Serbia, Romania and Algeria. Dead cordon caused by unknown agents was "increasing" or "worrying" in five countries. Like Eutypa dieback, Phomopsis cane and leaf spot had more local importance, being reported as "increasing" or "worrying in eight regions within Romania, Portugal, Turkey and Montenegro.

The presence of decline diseases is perceived as a problem even in young vineyards: as "increasing" (three reports) or "increasingly worrying" (five reports) in some regions, particularly in Hungary and Spain.

On young vines, black foot was reported as "increasing" and/or "worrying" only in three regions of Spain, and Petri disease was considered as "increasing" in two regions of Spain and two regions of Hungary.

DISCUSSION

This study reports the first large-scale survey of GTDs in European and closeby Mediterranean countries, which aimed to determine the spread and severity of the main grapevine wood diseases, i.e. the disease syndromes associated with, or caused by, pathogenic fungi colonizing the woody tissues of grapevines. The diseases included in the survey are summarized in the Introduction. Some are included in "esca complex", i.e. a complex of interrelated diseases differently designated according to the stage of vine life when they occur and the type of symptom they cause.

The surveys carried out showed that apoplexy and esca/GLSD (i.e. often esca proper, as white rot in wood is frequently present with GLSD leaf symptoms) occurred in all the surveyed countries, and were

Table 7. Number of the surveyed regions in European and Mediterranean countries where alarming situations of GTD spread was reported.

Disease	Increasing OR worrying			Increasing AND worrying			Total	
	Surveyed Region		Country	Surveyed Region		Country	Surveyed Region	
	No.	%		No.	%		No.	%
Symptoms on adult vine								
Apoplexy	30	28.6	Hungary, Spain, Switzerland, Romania, Portugal, France, Italy, Algeria, Serbia, Germany	4	3.8	Portugal , France, Algeria	34	32.4
Esca /GLSD	29	27.6	Hungary, Portugal, France, Italy, Algeria,Turkey, Spain, Germany, Lebanon, Montenegro, Romania	11	10.5	Portugal, Czech, Spain, Austria	40	38.1
Dead cordon (Eutypa dieback)	8	7.6	Serbia, Romania	2	1.9	Algeria	10	9.5
Dead cordon (Botryosphaeria dieback)	14	13.3	Hungary, Romania, France, Italy, Spain, Serbia	2	1.9	Portugal, Spain	16	15.2
Dead cordon (causal agent not identified)	7	6.7	Algeria Romania Serbia, Portugal	2	1.9	Algeria, France	9	8.6
Phomopsis cane and leaf spot	8	7.6	Romania, Portugal, Turkey, Montenegro	0	0	-	8	7.6
Symptoms on young vine								
Petri disease	4	3.8	Hungary, Spain, Portugal	0	0	-	4	3.8
Black foot [a]	3	2.7	Spain, Portugal	0	0	-	3	2.7

[a] Ascertained presence of *Cylindrocarpon*-like spp.

generally recorded as the most frequently occurring GTDs. Both syndromes are related to esca complex, and they were also noted as increasing and worrying in many countries, in a large vineyard area in Europe. In mature vines, the other disease syndromes such as dead cordon related to *E. lata*, Botryosphaeriaceae or to unknown origin, and Phomopsis cane and leaf spot, were also recorded in almost all the surveyed countries. These diseases were often recorded as not frequent but increasing in several surveyed countries. In contrast, decline of young vine was less widespread and less important than decline of adult vines. Only a few viticulture regions were concerned about Petri disease or black foot, reporting it as frequently present only in a few countries.

Apoplexy and esca/GLSD are both very easily identifiable syndromes due to their characteristic symptoms, respectively, of sudden wilting and leaf stripe. The extensive occurrence of esca complex in the surveyed regions supports the endemic behaviour of this GTD, and its increasing presence, since the end of the 20th Century (Fischer and Kassemeyer, 2003; Surico, 2009). In contrast, if all responses on dead cordon syndromes are pooled, these occur as frequently as the esca complex. Among the diseases leading to dead cordon, Eutypa dieback was the most studied, and this disease is also easily detectable when foliar symptoms are visible (Munkvold *et al.*, 1994; Dubos, 1999). The situation is similar for Phomopsis cane and leaf spot, with typical symptoms on the canes (Úrbez-Torres *et al.*, 2013). Mixed infection makes it difficult to sharply separate the different dieback diseases. GTD diagnoses in vineyards may be difficult without specific foliar symptom expression, as is the case for Botryosphaeria dieback. In this survey, reports of Botryosphaeria dieback, probably based on previous etiological studies, were very likely to be underestimated due to the lack of specific surveys in many regions and countries. The significant number of reports "dead cordon" "agent unidentifed" supports this finding. Numerous pathogenic Botryosphaericeae species, presenting

different life traits, may be involved in vine decline in European and Mediterranean countries (van Niekerk *et al.*, 2006; Bellée *et al.*, 2017). Their distribution and prevalence are related to many factors, such as climatic and agronomic influences. Some fungi are widespread in Europe, such as *Diplodia seriata*, reported in Spain (Armengol *et al.*, 2001; Martin and Cobos, 2007), Portugal (Phillips, 2002; Rego *et al.*, 2009), Hungary (Kovács *et al.*, 2017), Italy (Cristinzio, 1978; Burruano *et al.*, 2008; Spagnolo *et al.*, 2011**,** Carlucci *et al.*, 2009, 2015), France (Larignon *et al.*, 2001), Turkey (Akgül *et al.*, 2014), Germany (Fischer and Kassemeyer, 2003), Croatia (Kaliterna and Miličević, 2014), and Bulgaria (Nikolova, 2010). Similarly, *Neofusicoccum parvum* is reported from Croatia (Kaliterna *et al.*, 2013), in Turkey (Akgül *et al.* 2014), Portugal (Phillips, 2002), Spain (Luque *et al.*, 2009), Czech Republic (Baránek *et al.*, 2018) and Italy (Spagnolo *et al.*, 2011). Another virulent pathogen, *Lasiodiplodia theobromae*, was frequently isolated in Southern European and Mediterranean regions (El-Goorani and El Meleigi, 1972; Burruano *et al.*, 2008, Carlucci *et al.*, 2009), and recently was isolated from declining vines in France (Comont *et al.*, 2016).

The results of the survey showed that dead cordon related to Botryosphaeria dieback is increasing and worrying in more countries than those reporting Eutypa dieback. This could be related to the broad range of the Botryosphaeriaceae pathogenic species, characterised by various traits (endophytic, saprophytic, pathogenic) in numerous hosts. Furthermore, water stress has been reported to increase the severity of Botryosphaeria dieback on grapevine (van Nierkerk *et al.*, 2011; Amponsah *et al.*, 2014; Lawrence *et al.*, 2017). Incidence of disease caused by Botryosphaericeae in various plant hosts has increased in Europe during the last decades (Desprez-Loustau *et al.*, 2006; Slippers and Wingfield, 2007; Fabre *et al.*, 2011; Piškur *et al.,* 2011; Mehl *et al.*, 2014), and this may be related with climatic evolution towards more variable conditions, with increasing incidence and intensity of droughts over continental Europe and the Mediterranean region (Spinoni *et al.*, 2017). Further research on the group of pathogens implicated in grapevine decline is needed to better assess the distribution of the different pathogen species in European and Mediterranean countries, and to determine their life traits in relation with grapevine physiological status.

Results from this questionnaire also demonstrated occurrence of Phomopsis cane and leaf spot disease. This is not a wood disease and so not usually included among the Grapevine Trunk Diseases. Nevertheless, its main agents, *Diaporthe ampelina* and other *Diaporthe* species, are also recognized causes of cankers and dieback on grapevine (Úrbez-Torres *et al.*, 2013; Dissanayake *et al.*, 2015; Guarnaccia *et al.*, 2018). In the survey, the reports of Phomopsis cane and leaf spot, based on the typical easily visible symptoms on the basal internodes of canes, gave information on the disease spread in Europe. This highlights the need for accurate pathogen identification to analyse the involvement of *Diaporthe ampelina* (=*Phomopsis viticola*) and other *Diaporthe* species in perennial cankers and vine dieback in European vineyards.

Eutypa dieback was considered a menacing and spreading disease in Europe at the end of the 20th Century in Europe (Munkvold *et al.*, 1994; Dubos, 1999). However, the present survey has shown this diease is present, but not frequent, in a majority of regions. Increased knowledge of Eutypa dieback epidemiology and on efficient disease management (infected wood removal, wound protection and trunk renewal) may have contributed to decreased incidence of the disease (Dubos, 1999; Lecomte *et al.*, 2006). Nevertheless, Eutypa dieback is still present in 20% of the monitored region and was recorded as increasing or worrying in three countries, Serbia, Romania and Algeria.

In addition to the questions on the main GTDs diseases on vines, the survey also included an open question on other causes of decline observed in vineyards. Beside virus and phytoplasma diseases, that are widespread in Europe, *Verticillium* and *Fusarium* diebacks were also mentioned a few times.

The reports of increase of esca complex and other forms of decline on adult plants reported for numerous surveyed countries corroborated previous observations. Several hypotheses were advanced for this increase. In the 1960s–1970s there was a large increase in grapevine planted area, which often required intensive plant production methods, due to the large demand for of low-priced mass market grape products. This significant production led to increased vulnerability of vines to fungal agents of wood diseases (Surico *et al.*, 2004; Gramaje and Armengol, 2011). Domestic grape varieties well-adapted to local pedoclimatic conditions were still grown for inexpensive table or wine production, but were largely replaced by easily available international commercial varieties that better meet commercial needs. Cultural practices also changed, including the training systems, which have direct impacts on incidence of wood diseases incidence (Lecomte *et al.*, 2018), and use of mechanical pruning tools. Another factor leading to the increase in GTDs spread may be climatic change, which would affect plant disease prevalence and have impacts on the complex interactions between pathogens and hosts (Gregory *et al.*, 2009). Account should be taken of the increased awareness of these diseases,

which leads to increased numbers of reports. The reported GTDs increasing in the surveyed viticulture regions matched the new wave of forest decline observed in European countries and around the world (Surico et al, 2004; Allen *et al.*, 2010). For instance, the results of this survey showed an increase in the apoplexy observed in different countries that might be related to the summer climatic conditions more favourable for expression of this syndrome, such as drought during summer coming after rain (Dubos, 1999, 2002; Surico *et al.*, 2000). Some studies have considered the relationships between GTDs and climate (Marchi *et al.*, 2006; van Niekerk *et al.* 2011; Calzarano *et al.*, 2018). The present survey results indicated the need for further study on these relationships, and Bois *et al.* (2017) proposed a methodology to address the relationships between grapevine pests and diseases and climate. The present survey has shown variation in GTDs occurrence among the regions within individual country. This variation could be explained by different climatic regimes. For instance, in Hungary, a higher level of occurrence for apoplexy was reported in two regions characterized by dry springs and hot/dry summers, compared to other regions of this country. Similarly, in Bulgaria, "Rose Valley" region showed a higher level of occurrence for esca complex/GLSD in comparison with other regions. This region is characterized by frequent rain during spring over a long period (pers. comm.), quite different from the south-eastern region (Struma), characterized by a continental Mediterranean climate, where GTDs were observed only occasionally. However, beyond these two examples, the data from this survey showed that climate did not always drive the occurrence of GTDs in a region.

Other factors, such as soil type, cultural practices, the rootstock and variety used and vine age may influence the host plant physiology, and hence the level of vulnerability to GTDs. Systematic monitoring and increased GTD research on epidemiology and host/pathogen interactions are needed to better predict the distribution and incidence of GTDs, in the context of climatic changes. Widespread monitoring has been instigated in many regions in Europe. For instance, in France, the National Observatory of GTDs shows large variations in disease incidence among particular grapevine plots (Bruez *et al.*, 2013).

The results of the present survey showed great diversity of grapevine varieties planted in the European and Mediterranean countries, probably reflecting only a small part of the diversity of cultivated varieties. However, this provides evidence of a long wine-producing tradition in these countries. The homogenization given by widespread planting of the same international varieties co-exists with increasing interest in renewal of vintage varieties that are well-adapted to local environments, and also new population variability selected to enlarge genetic backgrounds. In this survey, among the recorded varieties, the six most frequently cited (Cabernet Sauvignon, Merlot, Chardonnay, Riesling, Sauvignon and Pinot Noir) correspond to those commonly grown in the European vineyards (OIV, 2017). Among these varieties, Cabernet Sauvignon was the most reported as susceptible to esca. Cabernet Sauvignon has also been reported in the literature as susceptible to other GTDs, such as Botryosphaeria dieback (Larignon *et al.*, 2001, Quaglia *et al.*, 2009, Bruez *et al.,* 2013) and Eutypa dieback (Dubos, 1999). However, the cv. Merlot, also one of the main reported cultivars in the survey, was indicated as moderately susceptible, presenting less disease incidence in vineyards than Sauvignon blanc and Riesling (Murolo and Romanazzi, 2014), or than Cabernet Sauvignon (Christen *et al.*, 2007). From the National French GTD vineyard survey, Bruez *et al.* (2013) also showed that Merlot presented low esca complex incidence. This cultivar was considered to be tolerant to Eutypa dieback (Dubos, 1999). Regarding Chardonnay, Andreini *et al.* (2014) observed lower percentage of GLSD ("esca symptoms") on Chardonnay vines than on Cabernet Sauvignon or Trebbiano vines, and Bruez *et al.* (2013) reported different level of incidence according to the surveyed region. The number of studies on Chardonnay was less than those on Merlot and Cabernet Sauvignon. For the four other predominating cultivars, Riesling Italico, Pinot noir, Limberger and Riesling, few studies of their susceptibilities to GTDs have been reported. From a mature vineyards survey of 64 cultivars, Murolo and Romanazzi (2014) reported the greatest esca incidence for Riesling, and no symptoms present for Limberger. Pinot noir has been evaluated as moderately affected by Botryosphaeria dieback (Larignon *et al.,* 2001). Trebbiano and Sangiovese were reported as less susceptible to GLSD ("esca") than Cabernet Sauvignon (Andreini *et al.*, 2014), while Sangiovese showed lower percentage of esca symptomatic vines in comparison with Trebbiano in a separate study.

The behaviour of a particular cultivar may vary according to the environmental conditions. Li *et al.* (2017) showed for Cabernet Sauvignon vines of similar age that disease incidence varied from 0.10 to 11.7 in vineyards from one region. The cultivation of resistant or tolerant grapevine cultivars to diseases has been suggested as a major challenge for the 21st Century, to decrease pesticide use against foliar pathogens. Results of the present survey show it is also relevant to select cultivars less susceptible to GTDs. Evaluation of the dif-

ferent responses of the cultivars in particular environments remains essential.

The main varieties of rootstocks used for grafted vines reported in this study were SO4, 110R, K5BB and 1103P. It was generally accepted that rootstock mother plants can also be a source of infections by pathogenic fungi associated to Petri disease and esca complex. Gramaje *et al.* (2010) compared the response to infection of different rootstock varieties to Petri disease pathogens. They found that the rootstocks 140 Ru and 110R were the most greatly affected. Both were among the commonly reported rootstocks in our survey, and 110R was the second most frequently reported. Liminana *et al.* (2009) reported that the 16 year-old trunks of rootstocks 140Ru, 11R, Fercal and 101-14MG presented the most extensive necrosis. The susceptibility of rootstocks to pathogenic fungi associated to GTDs may be considered as well as the susceptibility of scion varieties conferred by the rootstock used. For instance, in an Italian vineyard, Marchi (2001) showed that vines cv. Trebbiano grafted on rootstocks 1103P, 420A or K5BB expressed more esca symptoms than those grafted on S04 or 140RU. In contrast, when two other varieties (cv. Fiano and Sauvignon blanc) were grafted on S04 they had greater esca incidence compared with the same cultivars grafted on 1103P (Murolo and Romanazzi, 2014). These authors explained their results by the greater drought resistance of 1103P than other rootstocks. Environmental conditions must be considered to explain the results, as well as the roles of other internal or external factors (Marchi *et al.*, 2006; Calzarano *et al.*, 2018) to explain these differences.

GTDs on young vines were reported in the survey with a lower occurrence than for GTD on adult plants. GTDs were rarely reported as "increasing" and/or "worrying" on young vines. Petri disease and black foot diseases were only reported to be increasing in Spain, and Petri disease, in Hungary. These diseases are characterized by general decline symptoms, but the diseases were reported only where where plant pathologists have made specific observations and diagnoses to identify the pathogens involved. Therefore, the lack of monitoring of young vineyards, resulting in an underestimation of the problems, cannot be discounted for this result. To our knowledge, this survey was the first to evaluate occurrence of decline diseases in young vines on a large geographical scale.

The large number of survey responses, from a total of 105 viticulture regions in 22 countries, allowed us to draw an overall picture of GTDs spread in Continental European and Mediterranean countries. Some countries with relevant grapevine production were missing from the survey including Tunisia, Morocco, and some Balkan and Central European countries (e.g. Poland). The survey data were based on correspondent declarative responses and not on formally recorded information. Differences between countries may be important: in 15 countries out of 22, GTD surveys or observatories were established at regional or national levels.

In other cases, the responses may be based on expert knowledge on the phytosanitary state, depending on personal perceptions. For some countries, the lack of information or correspondent response may have led to underestimation of occurrence of the disease syndromes. Therefore, the results presented in this paper need to be taken with some caution. Nevertheless, the survey data showed that almost all the surveyed countries (except four), had laboratories or extension services based on GTD survey or/and diagnostic/detection at regional or national levels. That information favoured validity of the collected data, even if the etiological complexity of dieback diseases requires more detailed investigations, such the recording of data from plots and etiological evidence provided by identification of the involved pathogenic fungi, especially for the dead cordon syndrome.

CONCLUSIONS

Taking account of the results of this survey with caution, all findings from this large-scale European consultation, extended to several Mediterranean countries and producers, highlighted the important roles of GTDs, particularly esca complex, in vine decline in European and Mediterranean grape-producing regions. Furthermore, the survey has emphasized the need to accurately determine the occurrence and distribution of the causes of grapevine decline, such as the involvement of Botryosphaericeae and *Diaporthe* species in dieback and vine death. Etiological and epidemiological studies should be conducted to identify and quantify the environmental and agronomic factors associated with GTDs. Also urgent is the need to share and disseminate all nursery and vineyard management practices that have proved useful for GTD prevention, or for limiting the damage resulting from these diseases. The recently established European Winetwork project was set up to increase knowledge transfer on current and innovative strategies for grapevine disease management in Europe (Mondello *et al.*, 2018b, www.winetwork.eu). This initiative needs to be maintained and promoted.

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Research Papers

The potential for pesticide trunk injections for control of thousand cankers disease of walnut

Elisa DAL MASO[1], Benedetto Teodoro LINALDEDDU[2], Genny FANCHIN[2], Massimo FACCOLI[3], Lucio MONTECCHIO[2,*]

[1] *PAN/De Rebus Plantarum, Spinoff of the University of Padova, Viale della Navigazione interna 51, 35129 Padova, Italy*
[2] *Department of Land, Environment, Agriculture, and Forestry (TeSAF), University of Padova, Via dell'Università 16, 35020 Legnaro, Italy*
[3] *Department of Agronomy, Food, Natural Resources, Animals, and Environment (DAFNAE), University of Padova, Via dell'Università 16, 35020 Legnaro, Italy*
*Corresponding author: montecchio@unipd.it

Abstract. Thousand cankers disease, caused by the pathogen *Geosmithia morbida* vectored by the bark beetle *Pityophthorus juglandis*, has emerged as an important disease of walnut trees in Europe. The present study was performed to evaluate the efficacy of trunk injections of four commercial fungicides and one insecticide for control of the fungus and its vector. Laboratory tests indicated that fungicides containing prochloraz + tetraconazole were the most effective. Field trials on non-infected trees allowed for the selection of a mixture containing prochloraz and tetraconazole (Binal Pro), the insecticide abamectin (Vertimec EC) and the adjuvant 2-(2-ethoxyethoxy) ethanol (Carbitol™) as having rapid host uptake. Injections of this formulation in naturally infected black walnut trees reduced the presence of *G. morbida*, supporting trunk injection as an efficient and low impact technique to manage fungal damage on infected trees.

Keywords. Prochloraz, tetraconazole, abamectin, *Geosmithia morbida*, *Pityophthorus juglandis.*

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INTRODUCTION

Widespread dieback and mortality of black walnut (*Juglans nigra* L.) has occurred in the United States of America since the mid-1990s (Kolařík *et al.*, 2011; Utley *et al.*, 2013). The causal agents were determined in 2008 to be a combination of infestation by the bark beetle vector *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae, Scolytinae) and infection by the fungus *Geosmithia morbida* M. Kolarik, E. Freeland, C. Utley & N. Tisserat (Kolařík *et al.*, 2011).

Adults of *P. juglandis* carry spores of *G. morbida* on their bodies and infect host trees via the galleries they create in the bark of host tree branches and trunks. *Geosmithia morbida* then grows within and around the insect

Table 1. Commercial products, active ingredients, and concentrations tested for their fungicidal effects on colony growth of *Geosmithia morbida*.

Active ingredient	Commercial product	Active ingredient concentration (μg mL^{-1})	Manufacturer	Range of active ingredient tested (μg mL^{-1})
Thiabendazole	TECTO 20S	2.2×10^5	Syngenta Crop Protection s.p.a.	$7.44 \times 10^3 - 7.44 \times 10^{-1}$
Procloraz	SPORTAK 45 EW	4.5×10^5	BASF Italia s.p.a	$1.52 \times 10^4 - 1.52$
Allicin	CONQUER	5×10^3	Neem Biotech Ltd.	$1.69 \times 10^2 - 1.69 \times 10^{-2}$
Procloraz + tetraconazole	BINAL PRO	$2.3 \times 10^5 + 4.1 \times 10^4$	GOWAN Italia s.p.a.	$7.78 \times 10^3 - 7.78 \times 10^{-1}$ and $1.39 \times 10^3 - 1.39 \times 10^{-1}$

feeding sites and galleries. This combined damage/infection process was named "thousand cankers disease" (TCD) by Tisserat *et al.* (2009). Cankers developing from the numerous fungus introduction points gradually coalesce, compromising phloem transport efficiency (Tisserat *et al.* 2011). The disease symptoms include flagging leaves, and thinning and dieback of the host canopy. Over a period of some years, larger branches are progressively killed, and the disease often leads to tree death (Utley *et al.* 2013; Montecchio *et al.*, 2016; Hefty *et al.*, 2018).

In 2013, *P. juglandis* and *G. morbida* were detected for the first time in Italy and Europe, on black (*J. nigra* L.) and European (*J. regia* L.) walnuts (Montecchio *et al.*, 2014; Montecchio and Faccoli, 2014). The pest and pathogen were included in the EPPO A2 List (EPPO, 2018).

Effective TCD management options have been limited to sanitation efforts, based on cutting, chipping and burning infected trees (Haun *et al.*, 2010; Mayfield *et al.*, 2014). No information is available on effects of pesticide treatments to save infected trees or to protect healthy ones.

The aims of the present study were: 1) to evaluate and compare the antifungal activity of four commercial fungicides against *G. morbida*; 2) to formulate an injectable pesticide blend with sufficient uptake and activity against the pathogen and vector; and 3) to assay the efficacy of the pesticide blend against *G. morbida* and its vector in infected black walnut trees.

MATERIALS AND METHODS

Pathogen culture

The strain of *G. morbida* (designated LM13GMN) used in this study, selected for its pathogenicity, was originally isolated from a symptomatic *J. nigra* branch collected in May 2014 from an infected black walnut plantation (Santorso, Vicenza, 45°72′ N, 11°40′ E; Montecchio and Faccoli, 2014; Montecchio *et al.*, 2015). Pure cultures of the fungus were maintained on potato dextrose agar (PDA, Difco Laboratories) and stored at 8(±1)°C in the culture collection of the Department TeSAF, University of Padova, Italy. The ITS sequence of this isolate is available in GenBank (accession number MH503927).

In vitro experiment

Four commercial fungicide products - Tecto 20S (active ingredient (a.i.) thiabendazole; Syngenta Crop Protection), Sportak 45EW (a.i. prochloraz; Basf Italia), Conquer (neem, a.i. allicin) and Binal Pro (a.i. prochloraz + tetraconazole; Gowan Italia), (Table 1), were tested *in vitro* at a range of concentrations to determine the LC_{50} values (lethal concentrations for 50% of the colonies) for *G. morbida*.

Each product was diluted with sterile de-mineralized water (at 100%, 75%, 50%, 25%, 10%, 1%, 0.1% or 0.01%), and 0.35 mL of the unbuffered suspensions were evenly spread on the surfaces of 10 mL of PDA in 94 mm diam. Petri dishes (Dal Maso *et al.*, 2014), with 27 replicates per treatment and concentration. The range of the a.i. concentrations was 1.52×10^4 to 1.69×10^{-2} μg mL^{-1} (Table 1). In total, 972 plates were processed. Each PDA plate was inoculated centrally with a 5 mm diam. agar/mycelium plug taken from the margin of an actively growing *G. morbida* colony on PDA, with the aerial mycelium facing the inoculated agar surface (Aloj *et al.*, 1993; Secor and Rivera, 2012). After an incubation at 28±1°C in the dark for 3 days, plugs were transferred to untreated PDA and kept in the same conditions (Aharoni *et al.*, 1997; Allen *et al.*, 2004; Suleiman, 2010; Dal Maso *et al.*, 2014). The effects of the fungicides on subsequent fungal growth were checked weekly using a microscope (up to ×200 magnification) for 4 consecutive weeks. Growing colonies were classified as "viable", and those that failed to grow were classified as "in-active".

The fungus growth data were statistically analyzed using R cran with extension package *drc* (Ritz *et al.*, 2015; R Core Team, 2018). For each fungicide product, a regression curve was fitted using dose-response analyses for binomial outcomes. The best model function was chosen based on Akaike's information criterion (AIC), standard errors and residual analyses of the fitted models (Secor and Rivera, 2012). The LC_{50} values were then calculated and compared among the commercial fungicide products by means of one-way analysis of variance (ANOVA, $P < 0.05$), with the R extension package *rpsychi* (Cohen, 2002). Multiple comparisons were evaluated, and 95 % confidence intervals were computed for each active ingredient.

The products Sportak 45 EW and Binal Pro produced the least LC_{50}s, and were therefore selected for *in planta* experiments.

In planta experiments

Sportak 45 EW and Binal Pro are not formulated for trunk injection, so their solubility and uptake rates at different concentrations were evaluated in triplicate on asymptomatic 12-year-old *J. nigra* trees (N45°39', E11°32', Montecchio Precalcino, VI). The fungicide products were applied using a Bite® injection tool (Montecchio, 2013; Dal Maso *et al.*, 2014). A total of 28 formulations were tested, differing in concentrations of two adjuvant chemicals –[(2-(2-ethoxyethoxy) ethanol (Carbitol™) or acetic acid (1.2 %)], and one commercial insecticide [abamectin 1.84 % w/w, effective against bark beetles and registered for trunk injection (Vertimec EC, Syngenta Crop Protection)] (Table 2).

As uptake rate is known to be a limiting factor for tree trunk injection treatments (Dal Maso *et al.*, 2014), weekly tests (from the first week of May to the second week of September), were carried out to select the best formulation that, when injected at 25 cm from the ground, allowed for an uptake rate of 1 mL cm^{-1} of trunk circumference (at breast height) within 24 h.

According to the results obtained in the preliminary test, the formulation no. 21, containing Binal Pro, Vertimec EC and Carbitol™ (Table 2), was selected and used in a subsequent fungicide efficacy experiment. Twelve trees showing symptoms of thousand canker diseases, in a naturally infected 17-year-old black walnut plantation (N45°38', E11°39', Bressanvido, VI; Montecchio and Faccoli, 2014), were treated during the first week of September 2016. Six trees were injected with 1 mL cm^{-1} circumference of the no. 21 formulation, six trees were injected with the same volume of water, as in experimental controls (average of 100 mL per tree). Injections were each made through a single port 20 cm above-ground.

After 310 d from treatment (July 2017), two black walnut trees per treatment were randomly selected. Three twigs for each cardinal direction (N, E, S or W) were collected from each tree at 11-13 m above-ground. For each twig, four cankers were carefully debarked to detect *P. juglandis* insects or galleries. Each entire sample was then incubated under humid conditions for 2 weeks, at 24±1°C in the dark and observed each day. The proportions (percent) were recorded of necrosis from which hyaline mycelium developed, with conidiophores and conidia typical of *G. morbida* (Kolařík *et al.*, 2011). Fungus identity was confirmed by analysis of the internal transcribed spacer region (ITS1-5.8S-ITS2) of rDNA.

Percentages of samples positive for *G. morbida* were arranged by treatment in contingency tables, then Fisher's Exact Tests for count data were processed in R cran (R Core Team, 2018).

RESULTS

In vitro experiments

All the tested fungicides inhibited mycelium growth of *G. morbida*, with LC_{50} values ranging from 5.48 to 4.4×10^2 µg mL^{-1}. Analysis of variance showed significant differences among the four commercial products for efficacy to limit growth of *G. morbida* colonies ($F(968, 3) = 110.77$; $P < 0.01$). Tecto 20S (thiabendazole) was the least effective compound, with an LC_{50} of 4.4×10^2 µg mL^{-1}, followed by Conquer (allicin) with an LC_{50} of 1.5×10^2 µg mL^{-1}. Sportak 45 EW (prochloraz) and Binal Pro (prochloraz + tetraconazole) gave the greatest inhibition of *G. morbida*, with LC_{50} values of, respectively, 5.48 and 5.84 µg mL^{-1}.

In planta experiments

The field tests showed that, on asymptomatic trees, the formulation with the most rapid uptake rate was the no. 21 (containing 1.90×10^4 µg mL^{-1} prochloraz, 3.4×10^3 µg mL^{-1} tetraconazole, 0.9×10^3 µg mL^{-1} abamectin, and 8.38×10^5 µg mL^{-1} Carbitol™: Table 2), with the greatest uptake rate detected the first week of September 2016 (16 weeks after injection).

The percentage of samples showing cankers 310 d after treatment, from which *G. morbida* developed, were evenly distributed among replicates in the equivalent treatment classes ($P > 0.05$), and among for the water treated plants ($P > 0.05$).

Table 2. Average volumes of solutions injected into *Juglans nigra* trees during 60 min, for 28 different formulations tested on at atmospheric pressure or manually applied external pressure, as obtained in the pesticide uptake rate test. a = Sportak 45 EW commercial product; b = Binal Pro commercial product.

Formulation No.	Procloraz μg mL^{-1}	Tetraconazole μg mL^{-1}	Carbitol™ μg mL^{1}	Abamectin Mg mL^{-1}	Acetic acid mg mL^{-1}	Injection method	Injection speed mL/60 min
1	2.25×10^{5} [a]	0	0	0	0	Atmospheric pressure, 101325 Pa	0
1	2.25×10^{5} [a]	0	0	0	0	External pressure, 111377 Pa	0
2	2.25×10^{5} [a]	0	0	0	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
2	2.25×10^{5} [a]	0	0	0	1.26×10^{4}	External pressure, 111377 Pa	0
3	2.25×10^{5} [a]	0	2.42×10^{5}	0	0	Atmospheric pressure, 101325 Pa	0
3	2.25×10^{5} [a]	0	2.42×10^{5}	0	0	External pressure, 111377 Pa	0
4	2.25×10^{5} [a]	0	2.42×10^{5}	0	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
4	2.25×10^{5} [a]	0	2.42×10^{5}	0	1.26×10^{4}	External pressure, 111377 Pa	0
5	2.25×10^{5} [a]	0	4.83×10^{5}	0	0	Atmospheric pressure, 101325 Pa	0
5	2.25×10^{5} [a]	0	4.83×10^{5}	0	0	External pressure, 111377 Pa	0
6	2.25×10^{5} [a]	0	4.83×10^{5}	0	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
6	2.25×10^{5} [a]	0	4.83×10^{5}	0	1.26×10^{4}	External pressure, 111377 Pa	0
7	4.5×10^{4} [a]	0	5.80×10^{5}	1.8×10^{3}	0	Atmospheric pressure, 101325 Pa	0
7	4.5×10^{4} [a]	0	5.80×10^{5}	1.8×10^{3}	0	External pressure, 111377 Pa	0
8	4.5×10^{4} [a]	0	5.80×10^{5}	1.8×10^{3}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
8	4.5×10^{4} [a]	0	5.80×10^{5}	1.8×10^{3}	1.26×10^{4}	External pressure, 111377 Pa	0
9	4.5×10^{4} [a]	0	7.74×10^{5}	1.8×10^{3}	0	Atmospheric pressure, 101325 Pa	0
9	4.5×10^{4} [a]	0	7.74×10^{5}	1.8×10^{3}	0	External pressure, 111377 Pa	0
10	4.5×10^{4} [a]	0	7.74×10^{5}	1.8×10^{3}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
10	4.5×10^{4} [a]	0	7.74×10^{5}	1.8×10^{3}	1.26×10^{4}	External pressure, 111377 Pa	0
11	3.82×10^{4} [b]	6.8×10^{3} [b]	5.16×10^{5}	1.8×10^{3}	0	Atmospheric pressure, 101325 Pa	0
11	3.82×10^{4} [b]	6.8×10^{3} [b]	5.16×10^{5}	1.8×10^{3}	0	External pressure, 111377 Pa	0
12	3.82×10^{4} [b]	6.8×10^{3} [b]	5.16×10^{5}	1.8×10^{3}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
12	3.82×10^{4} [b]	6.8×10^{3} [b]	5.16×10^{5}	1.8×10^{3}	1.26×10^{4}	External pressure, 111377 Pa	0
13	3.82×10^{4} [b]	6.8×10^{3} [b]	7.1×10^{5}	1.8×10^{3}	0	Atmospheric pressure, 101325 Pa	0
13	3.82×10^{4} [b]	6.8×10^{3} [b]	7.1×10^{5}	1.8×10^{3}	0	External pressure, 111377 Pa	0
14	3.82×10^{4} [b]	6.8×10^{3} [b]	7.1×10^{5}	1.8×10^{3}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
14	3.82×10^{4} [b]	6.8×10^{3} [b]	7.1×10^{5}	1.8×10^{3}	1.26×10^{4}	External pressure, 111377 Pa	0
15	2.25×10^{4} [a]	4.01×10^{3} [b]	7.74×10^{5}	9×10^{2}	0	Atmospheric pressure, 101325 Pa	0
15	2.25×10^{4} [a]	4.01×10^{3} [b]	7.74×10^{5}	9×10^{2}	0	External pressure, 111377 Pa	0
16	2.25×10^{4} [a]	4.01×10^{3} [b]	7.74×10^{5}	9×10^{2}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
16	2.25×10^{4} [a]	4.01×10^{3} [b]	7.74×10^{5}	9×10^{2}	1.26×10^{4}	External pressure, 111377 Pa	0
17	2.25×10^{4} [a]	4.01×10^{3} [b]	8.7×10^{5}	9×10^{2}	0	Atmospheric pressure, 101325 Pa	0
17	2.25×10^{4} [a]	4.01×10^{3} [b]	8.7×10^{5}	9×10^{2}	0	External pressure, 111377 Pa	0
18	2.25×10^{4} [a]	4.01×10^{3} [b]	8.7×10^{5}	9×10^{2}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
18	2.25×10^{4} [a]	4.01×10^{3} [b]	8.7×10^{5}	9×10^{2}	1.26×10^{4}	External pressure, 111377 Pa	0
19	1.91×10^{4} [b]	3.4×10^{3} [b]	7.42×10^{5}	9×10^{2}	0	Atmospheric pressure, 101325 Pa	0
19	1.91×10^{4} [b]	3.4×10^{3} [b]	7.42×10^{5}	9×10^{2}	0	External pressure, 111377 Pa	0
20	1.91×10^{4} [b]	3.4×10^{3} [b]	7.42×10^{5}	9×10^{2}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
20	1.91×10^{4} [b]	3.4×10^{3} [b]	7.42×10^{5}	9×10^{2}	1.26×10^{4}	External pressure, 111377 Pa	0
21	1.91×10^{4} [b]	3.4×10^{3} [b]	8.38×10^{5}	9×10^{2}	0	Atmospheric pressure, 101325 Pa	0
21	1.91×10^{4} [b]	3.4×10^{3} [b]	8.38×10^{5}	9×10^{2}	0	External pressure, 111377 Pa	2.1 (1.4 - 4.2)
22	1.91×10^{4} [b]	3.4×10^{3} [b]	8.38×10^{5}	9×10^{2}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
22	1.91×10^{4} [b]	3.4×10^{3} [b]	8.38×10^{5}	9×10^{2}	1.26×10^{4}	External pressure, 111377 Pa	0
23	9.54×10^{3} [b]	1.7×10^{3} [b]	1.26×10^{5}	4.5×10^{2}	0	Atmospheric pressure, 101325 Pa	0
23	9.54×10^{3} [b]	1.7×10^{3} [b]	1.26×10^{5}	4.5×10^{2}	0	External pressure, 111377 Pa	0
24	9.54×10^{3} [b]	1.7×10^{3} [b]	1.26×10^{5}	4.5×10^{2}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0

(Continued)

Table 2. (Continued).

Formulation No.	Procloraz μg mL^{-1}	Tetraconazole μg mL^{-1}	Carbitol^{TM} μg mL^{1}	Abamectin Mg mL^{-1}	Acetic acid mg mL^{-1}	Injection method	Injection speed mL/60 min
24	9.54×10^{3} [b]	1.7×10^{3} [b]	1.26×10^{5}	4.5×10^{2}	1.26×10^{4}	External pressure, 111377 Pa	0
25	9.54×10^{3} [b]	1.7×10^{3} [b]	9.03×10^{5}	4.5×10^{2}	0	Atmospheric pressure, 101325 Pa	0
25	9.54×10^{3} [b]	1.7×10^{3} [b]	9.03×10^{5}	4.5×10^{2}	0	External pressure, 111377 Pa	0
26	9.54×10^{3} [b]	1.7×10^{3} [b]	9.03×10^{5}	4.5×10^{2}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
26	9.54×10^{3} [b]	1.7×10^{3} [b]	9.03×10^{5}	4.5×10^{2}	1.26×10^{4}	External pressure, 111377 Pa	0
27	0	0	9.67×10^{5}	0	0	Atmospheric pressure, 101325 Pa	0
27	0	0	9.67×10^{5}	0	0	External pressure, 111377 Pa	0
28	0	0	0	0	0	Atmospheric pressure, 101325 Pa	0
28	0	0	0	0	0	External pressure, 111377 Pa	0

Statistically significant differences were found among cardinal directions ($P < 0.05$), with greater numbers of positive necroses (average = 37.5%) detected in the twigs collected from the direction opposite to the injection points, compared with those in the trees treated with formulation no. 21 (average = 9.7 %).

Although all sampled cankers showed scolytid exit holes and galleries, the proportions of necroses positive for the pathogen was significantly less in trees injected with formulation no. 21 (16.7%) than in control trees (42.7 %; Fisher's Exact Tests, $P < 0.01$).

Live *P. juglandis* was recorded in only one experimental control tree.

DISCUSSION

The main goal of the present study was to provide a preliminary evaluation of control of *Geosmithia morbida* through trunk injections of commercial pesticides.

Among the four products tested *in vitro* at eight different concentrations, Sportak 45EW (containing prochloraz) and Binal Pro (prochloraz + tetraconazole) demonstrated the lowest LC_{50} values for colony growth of *G. morbida*. Due to their impacts on ergosterol biosynthesis (Cabras *et al.*, 1998), fungicides belonging to the imidazole and triazole classes are used worldwide for control of many plant pathogens, including as *Fusarium* spp., *Colletotrichum musae*, *Nigrospora* spp., *Hymenoscyphus fraxineus*, *Magnaporthe oryzae*, *Penicillium italicum* and *Rhynchosporium secalis* (El-Goorani *et al.*, 1984; Johanson and Blazquez, 1992; Kendall *et al.*, 1993; Yan *et al.*, 2011; Dal Maso *et al.*, 2014; Fan *et al.*, 2014).

Although demonstrating some fungicidal effect, Conquer (allicin-based) was less effective than prochloraz and prochloraz + tetraconazole mixture products tested in this study. Nevertheless, the LC_{50} obtained (1.5×10^{2} μg mL^{-1}) was in line with those previously recorded for *H. fraxineus* (Dal Maso *et al.*, 2014). Tecto 20S (thiabendazole), known to be fungitoxic at low concentration against a wide range of Ascomycetes (Allen and Gottlieb, 1970; D'Aquino *et al.*, 2013; Zouhair *et al.*, 2014), was active against *G. morbida* only at high concentrations.

Due to their *in vitro* fungicidal efficacy against *G. morbida*, Sportak 45EW and Binal Pro were selected for the formulation of 28 injectable preparations, to determine the formula with the greatest uptake rate for use in the trials on infected trees. The selection of appropriate trunk injection compounds is key to the successful application of trunk injection strategies. Ten months after infected black walnut trees were injected with the formulation no. 21, the percentage of cankers positive for *G. morbida* was significantly less compared to the proportion of positive cankers for the experimental control trees. However, the efficacy changed with injection direction, with reduced effects in the parts of the tree canopies opposite to the injection ports. This was probably due to irregular distribution of the active ingredients within the canopies, as was previously reported by Tanis *et al.* (2012) and Aćimović *et al.* (2014), and this suggests that multi-port injections to individual trees could produce better infections reductions.

The insect vector *P. juglandis* was observed in only one experimental control tree, while insect emergence holes and breeding tunnels were frequently found in the sampled twigs and branches of the symptomatic trees. In Southern Europe, *P. juglandis* usually has two partially overlapping generations each year, from mid-May to late October (Faccoli *et al.*, 2016). Therefore, the emergence holes and the breeding tunnels found in infected walnut trees are probably caused by colonizations that occurred before the chemical treatment carried out in September 2016. This would explain the occurrence of cankers

on trees chosen as symptomatic before the trunk injection with fungicides, as the insect had already infected these hosts. The difficulty finding active insects in the tree branches sampled in July 2017, in the middle of the reproductive season of *P. juglandis*, suggests that the insecticide treatment provided good plant protection against new insect bark colonizations.

Despite the preliminary nature of the research described here, this study has demonstrated that endotherapy of walnut trees can slow the development of *G. morbida* for at least 1 year. However, further investigations are required to fully assess the efficacy of azole fungicides for protection of walnut trees from *G. morbida* infections (Fan *et al*., 2014; Parnell *et al*., 2008).

Because the trial was performed with technical limitations (numbers of trees to inject, injection ports to open), to avoid value losses of timber, more comprehensive trunk injection trials in larger numbers of trees could elucidate important practical details. The could include the number of injection ports necessary to obtain homogeneous distribution of fungicides in the tree crowns, the efficiency of different injection methods, and the potential for applications that prevent thousand canker disease.

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Research Papers

Population structure of the faba bean blight pathogen *Ascochyta fabae* (teleomorph, *Didymella fabae*) in Tunisia

Noura OMRI BEN YOUSSEF[1,*], Lydie KERDRAON[2], Lucie MIEUZET[3], Imen HALILA[1], Noura JAMMEZI[1], Ahlem MBAZIA[1], Mohamed KHARRAT[1], Christophe LE MAY[3,4,*]

[1] *Laboratoire des Grandes Cultures, Institut National de la Recherche Agronomique de Tunisie, Université de Carthage, Rue Hédi Karray, EL Manzah, 1004, Tunis, Tunisie*
[2] *INRA, UMR, UMR BIOGER, INRA-AgroParisTech, Avenue Lucien Brétignières BP 01 78850 Thiverval-Grignon, France*
[3] *INRA, UMR 1349 IGEPP, Domaine de la Motte, 35653 Le Rheu, France*
[4] *Agrocampus Ouest, UP ESP, 65 rue de Saint Brieuc, 35043 Rennes, France*
*Corresponding authors: lemay@agrocampus-ouest.fr; noura.mori@gmail.com

Abstract. Ascochyta blight, caused by *Ascochyta fabae* (teleomorph: *Didymella fabae*) has decreased faba bean production in Tunisia and worldwide. The teleomorph has recently been observed in Tunisia, raising new questions of how to control this major disease. Isolates (317) of the pathogen were obtained between 2011 and 2013, from four geographical regions in Tunisia (Beja, Bizerte, Jendouba and Tunis). The 240 isolates obtained in 2012 were compared for mating type frequency and genetic variation by using ten polymorphic SSR markers. Of these isolates, MAT1-2 was more common (2:1) in Tunisia than MAT1-1, but this ratio can change according to population. Low to high genetic variation was detected between locations and among pathogen populations. Beja showed the greatest genotypic richness (R = 0.42), followed by Tunis (R = 0.25), Bizerte (R = 0.13), and Jendouba (R = 0.11). Indices of association (I_A) and *Rd* were significantly different from 0 in all the populations, suggesting high multilocus linkage disequilibrium and confirming clonal populations. Population structure of the isolates was inferred using Bayesian analyses, Principal Component Analysis (PCA), and Minimum Spanning Networks, which all revealed that the populations from each location were not distinct. Evaluating changes in seasonal genetic diversity showed low to high variances of F_{ST} values between the two cropping seasons in all regions. However, the PCA analysis failed to separate the *A. fabae* isolates sampled during the two successive seasons into two groups, indicating that these populations did not constitute distinct genetic groups. These results suggest that gene flow was limited among populations, even those separated by short geographic distances. Future studies should enlarge the number of samples of representative populations, to overcome the limitations of a small sample size and to provide a more accurate assessment of *A. fabae* population structure.

Keywords. Ascochyta blight, *Ascochyta fabae*, faba bean, genetic differentiation, population genetics.

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INTRODUCTION

The faba bean disease, Ascochyta blight, caused by *Ascochyta fabae* Speg. (anamorph) and its teleomorph *Didymella fabae* Jellis & Punith, is a widespread disease, particularly in temperate regions, including Canada, Argentina, Europe, the Mediterranean region, the Middle East, Australia, New Zealand, Japan, Korea and China (Maurin *et al*., 1990). This is also one of the most important foliar diseases affecting faba beans in Tunisia (Kharrat *et al*., 2006). The fungus attacks all aerial parts of host plant (leaves, stems, pods and seeds) causing necrotic lesions, and leads to faba bean yield and quality damage. In Western Europe, depending on weather conditions during the cropping seasons, Ascochyta blight causes averages of 10 to 30% yield losses (Hanounik and Robertson, 1989). In Tunisia, yield losses are estimated to 15%, and may reach 95% in disease favourable conditions (Kharrat *et al*., 2006).

Ascochyta fabae is an haploid heterothallic pathogen that can survive during intercropping seasons on infested seeds and plant debris. Seeds constitute the major source of primary inoculum, and seed exchanges between and within countries are considered the main sources of introduction and mechanism of disease expansion or dispersal (Kaiser *et al.* 1997). *Ascochyta fabae* is maintained on crop stubble as asexual and sexual forms for at least one season (Rubiales and Trapero-Casas, 2002; Omri Ben Youssef *et al.,* 2012). Due to the stage of maturation of pseudothecia, the release of ascospores may overlap with the vegetative development of faba bean (Rubiales and Trapero-Casas, 2002).

Although applying fungicides to control *A. fabae* on seeds and faba bean plants gives satisfactory results, plant resistance remains the best way to control this disease. Considerable efforts have been made to identify sources of resistance in faba bean germplasm, particularly in Syria, Egypt, Canada, Morocco, France and Tunisia (Hanounik and Robertson, 1989; Rashid *et al.* 1991; Maurin and Tivoli, 1992; Kharrat *et al*., 2006). Partial host resistance has contributed to reducing chemical intervention to control the disease (Avila *et al*., 2004; Kharrat *et al*., 2006).

Pathogen variability represents a major challenge for the development and use of resistant cultivars. In general, new virulent isolates of pathogens emerge through selection imposed by their hosts. These shifts in virulence necessitate the adoption of disease control strategies that target pathogen populations rather than individuals (McDonald and Linde, 2002). Studying plant pathogen diversity principally aims to identify which forces will heavily influence the evolution of pathogen populations, and to assess their ability to evolve (Milgroom and Peever, 2003; de Meeûs *et al*., 2007). Several studies have evaluated population diversity of some *Ascochyta* spp. using different molecular tools, in particular for *A. rabiei* (Bayraktar *et al*., 2007; Ali *et al*., 2012; Atik *et al*., 2013) and *A. pinodes* (Le May *et al*., 2012; Padder *et al*., 2012; Laloi *et al*., 2016; Le May *et al*., 2017). Studies conducted on populations of *A. rabiei* collected in several countries showed high diversity levels that were attributed to genetic flow provided by the exchange of seeds between farmers (Nourollahi *et al*., 2011). For *A. fabae* populations, very few studies evaluated the genetic diversity of this fungus. A study using Random Amplified Polymorphic DNA (RAPD) markers, which included 36 isolates taken from different countries (Tunisia, Morocco, Italy, France, Spain, Australia and Algeria), showed high genotypic diversity, with 26 different genotypes observed within these isolates (Rouaissi *et al*., 2001). More recently, Ozkilink *et al.* (2011, 2015), using a set of 18 Simple Sequence Repeat (SSR) markers, showed that *A. fabae* populations from Syria displayed a high level of polymorphism. They showed high diversity but no differentiation, and rejected a null hypothesis of random mating in the studied population, which is indicative of predominating asexual reproduction.

Ascochyta blight has decreased faba bean production in Tunisia, a country characterized by variable weather conditions, diverse crop areas, various seed origins (produced by farmers or otherwise), and cultural practices. Since *D. fabae* has been observed recently in Tunisia (Omri Ben Youssef *et al*., 2012), it has become especially important to study the genetic diversity of the pathogen populations in the main faba bean growing areas affected by Ascochyta blight. The present study was undertaken to: (i) collect information about the genetic diversity of *A. fabae* populations in the various areas of faba bean production in Tunisia; (ii) assess the mating type distribution in this country using mating-type (MAT) specific primers; and (iii) determine whether the diversity of *A. fabae* populations evolves from one cropping season to another.

MATERIALS AND METHODS

Fungal isolates

A total of 317 *A. fabae* isolates were used in this study to analyze genetic variability. All isolates were collected between 2011 and 2013, from several faba bean fields in four different geographical locations of Tunisia, including Beja (three to nine fields, 122 isolates), Bizerte (two to seven fields, 87 isolates), Jendouba (three to eight

Table 1. Origin of *Ascochyta fabae* isolates (location, sampling date) used in the study.

Location	Sampling date	Number of populations	Number of isolates collected
Beja	2011	3	13
	2012	9	109
Bizerte	2012	2	71
	2013	7	16
Jendouba	2011	8	28
	2012	3	39
Tunis	2012	2	21
	2013	2	20

fields, 67 isolates), and Tunis (two fields, 41 isolates) (Table 1). All isolates were collected from infected host leaves, and as single-conidium isolates. For each isolation, approx. 5 mm² of diseased leaf tissue was surface sterilized for 1 min in 70% ethanol, rinsed three times in sterile water, placed on sterile filter paper to remove excess water. The tissue piece was then cultured on a V8 medium (99 mL V8 vegetable juice (Campbell), 35 g agar, and 901 mL distilled water, autoclaved at 105°C for 30 min) in Petri dishes for 14 d. Pycnidiospores from resulting cultures were spread on 2% malt agar and incubated for 12h, as described by Onfroy *et al.* (1999). Single germinating pycnidiospores were transferred (with dissecting microscope magnification) to fresh potato dextrose agar (PDA) plates, and cultures were incubated at 20°C with a 12h photoperiod under cool white fluorescent lamps. These single-spore cultures were then maintained on malt agar slants and stored in the dark at 4°C.

DNA extraction

Each isolate was grown in 75 mL of Tripton (LT) liquid medium supplemented with streptomycin (1.5 g) and penicillin (0.75 g). Each culture was raised from four pieces (each approx. 1 cm²) cut from the margin of an actively growing culture on malt agar. Inoculated vials were incubated, under agitation, for 14 d at 20°C, with a 12h photoperiod under cool white fluorescent lamps. Mycelia were harvested by vacuum filtration through two layers of sterilized Miracloth (Calbiochem CN Biosciences, Inc.), rinsed twice in sterile water, and then stored at -80°C until lyophilized. DNA was extracted from mycelium and isolated using Nucleospin Plant II Kit (Macherey-Nagel) according to the manufacturer's instructions.

Mating type assays

The mating type of all the 240 *A. fabae* isolates collected in 2012 was determined using the multiplex MAT-specific PCR assay (Cherif *et al.,* 2006). Primer combinations AL2p2SeqF4 (5'GCAACATCCTAGCATGATG3') specific to MAT1-1, AL1p1SeqF5 (5'CTGTCTCACCCAAGGCAAAC3') specific to MAT1-2, and ACom1A1AvAfAp (5'CACATCACCCCACAAGTCAG3') were used, specific to an aligning flanking 3′ region of *A. lentis, A. viciae-villosae, A. fabae* and *A. pisi*. Single PCR was carried out in 25 µL containing 10 ng of genomic DNA, 1× PCR buffer (containing 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 1 unit of *Taq* DNA polymerase (Promega) and 0.2 µM each of the primers. Amplification was performed in a BioRad Cycler thermal cycler (Bio-Rad Laboratories), and cycling conditions consisted of an initial denaturation at 95°C for 3 min followed by 35 cycles, each of 94°C for 20 sec, 58°C for 20 sec, and 72°C for 40 sec, with a final extension at 72°C for 10 min. DNA amplicons were separated in 1.5% ethidium bromide-stained agarose gels, and were visualized under UV light on a gel documentation system ChemiDOCTM XRS (Bio-Rad). Amplicon size was estimated using a DNA ladder (Hyperladder II).

SSR amplification and analyses

Ten SSR loci and their primer sequences (Table 2) were obtained from a published report on *A. fabae* (Ozkilink *et al.*, 2011). The M13-tailed primer was used to detect genetic diversity of all *A. fabae* isolates of the collection. The M13 sequence (CACGACGTTGTAAAACGAC) was added to the 5′ end of the forward primers, which were synthesized by Sigma-Genosys Ltd. PCR was performed in a 12 µL reaction mixture containing 0.2 mM dNTP, 1× PCR buffer, 1.5 mM $MgCl_2$, and 1 unit of *Taq* DNA polymerase (Promega), with the addition of 1ng genomic DNA, 0.24 µL (10 nM) of the forward primer labeled at the 5′ end with M13 sequence, 0.3 µL (10 nM) of the reverse primer and 0.06 µL (10 nM) of the fluorescent dye FAM (Applied Biosystems). Following an initial denaturation step of 2 min at 94°C, PCR was performed for a total of 50 cycles, each of 20 sec at 94°C, 25 sec at 58°C, and 23 sec at 67°C, with a final extension for 10 min at 72°C. For analysis on the genetic analyzer, 3 µL of the 25 µL diluted and pooled PCR products were mixed with 9.9 µL of formamide and 0.1 µL of LIZ-445 size standard (Applied Biosystem) in a 96 well PCR plate (GeneMate). The mixture was heated for 5 min at 95°C then chilled on ice and analyzed with the DNA analyzer.

Table 2. Primer sequences of the ten SSRs markers used for genetic study of *Ascochyta fabae* isolates.

Locus	Repeating motif	Primer sequences (5' to 3')
CAA46	F: CTA CAT TTC CCG TGC CTG AC R: GGC AGC CAG AGT TTG AGA AC	(CAA)17
CAA28	F: GAG TCA GTG GCG AGT GTG G R: GTC CGT TGC CCG TCT TTC	(GTGTAGT)3N(94) (TGT)8
AFCAA12	F: TCT TGG ACG CGT CTC TCT TG R: GCC AGT CTG GTT CAT CTA CC	(GTT)8N(32) (CACTG)6
AFCAA13	F: TTC GGC AGC ACA TCC TTC AG R: TGA GCA ATC TGA GCG GTT GG	(GCA)6N(13) (CAGCAACAA)3N(27) (CAA)4N(15)(CAA)(16)
AFCAA5	F: ATC ATC GCG TAC GTC GAC AC R: AAG ATG CTG GAG GGT GTC AG	(CTGCCACTGACACAGCTA)6
CA31	F: GAGCGTACCCAAACGCTATC R: GCTTCTTCGGCCTCAGTATG	(TGTGAGCG)11
AFCAA1	F: TCT ACT GAC GAT GCA TAG CG R: TAC CCA AAC GCT ATC GAA GC	(TTG)8
CA10	F: GCT TGT GCT TGT GCT TGT TC R: ACA TTC GTC CAT TGC ACC TT	(GTGTTGTGC)3N(103) (TGTGT)3
CA3	F: AGC AAC AAC AAG ACG CAG TG R: AGC TTG GGA TCT GCT TCC TT	(GTGTGCAGTGTGTA)7
CAA57	F: GAG CGT ACC CAA ACG CTA TC R: GCT TCT TCG GCC TCA GTA TG	(TGTGAGCG)9

Data analyses

As indicated in Table 1, isolates from the various locations were sampled over several seasons. Two different analyses were performed on the collection of *A. fabae* isolates. These were: i) gene diversity, genetic differentiation and genetic diversity, carried out for 240 isolates obtained in 2012; and ii) seasonal population differentiation, carried out for the isolates (317) sampled during the following seasons in the four locations (Beja, Bizerte, Jendouba and Tunis).

Gene diversity and genetic differentiation

SSR data were used to define MultiLocus Genotypes (MLGs), and were checked for repeated MLGs. The number of repeated MLGs (G) was identified using GENCLONE 2.0 (Arnaud-Haond and Belkir, 2007). Genotypic evenness was evaluated using the index R= (G-1)/ (N-1), with G as the number of distinct multilocus genotypes and N the number of isolates (Grünwald *et al*., 2003). Genetic diversity was estimated by allelic richness (*Ar*) using POPULATIONS 1.2.32 software (Langella, 1999). *Ar* was corrected for unequal sample size in each datasetby standardizing allelic richness to the smallest sample size, set at six individuals. Expected heterozygosity (H′) was computed using Arlequin software 3.1 (Excoffier *et al*., 2005). Clonality was assessed with the index of association (I_A) and the R_d statistic, a measure of the multilocus linkage disequilibrium, and was calculated using Multilocus software version 3.1b (Agapow and Burst, 2001). The association between the scored alleles was estimated by comparing the variance of the genetic distances in the data set to the mean variance of 1,000 artificial re-sampled datasets. The R_d statistic is much less dependent on the number of loci than the index of association (Montarry *et al*., 2010). I_A and R_d values are low for recombining population, and high for clonal or selfed populations (Burt *et al*., 1996).

Population structure analyses

Partition of molecular diversity among and within regions, as well as among and within populations, was studied for the 240 isolates collected in 2012, using an analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992; Lynch and Milligan, 1994). AMOVA was performed using Arlequin 3.1 (Excoffier *et al*., 2005). Pairwise F_{ST} values were calculated with Arlequin software, for sub-populations of *A. fabae* sampled in the different locations. A Bonferroni correction (adjusted alpha = 0.00316; 15 comparisons) was applied to take into account multiple testing.

A principal component analysis (PCA) was performed using the procedure available in the package ADEGENET (Jombart *et al*., 2008) for the statistical

freeware R version 3.1.1. PCA has an important advantage over other methods, such as the Bayesian clustering algorithm implemented in STRUCTURE (Pritchard *et al*., 2000), because it does not require strong assumptions about an underlying genetic model, such as the Hardy-Weinberg equilibrium or the absence of linkage disequilibrium between loci (Jombart *et al*., 2008).

STRUCTURE software version 2.2 was also used without any assumptions about population structure or assigning individuals to populations. The analysis was performed using 5×10^5 burn-in replicates and a run length of 1×10^6 Markov chain Monte Carlo (MCMC) replicates, adopting the admixed model and the correlated allele frequencies option. The number of genetic groups (K value) was estimated using the model developed by Evanno *et al.* (2005), which provides an estimate of the posterior probability of the data for a given K, Pr(X/K) (Perrier and Jacquemoud-Collet, 2006). The height of the modal value of the distribution was used as an indicator of the strength of the signal detected by STRUCTURE software. Five independent runs were performed for each analysis in order to verify the convergence of parameter estimates, and isolates were classified by location in order to determine if the same genotypes were present in the different populations and locations.

A Minimum Spanning Network (MSN) is an excellent way to visualize relationships among individuals. By using the package Poppr from R statistical software, version 3.1.2 (©2014, The R Foundation for Statistical Computing), MSN was calculated using Nei's distance for the different *A. fabae* populations. Multilocus genotypes (MLGs) were collapsed to multilocus haplotypes, represented by circles each containing the number of associated isolates and sized in proportion to haplotype frequency. Haplotype information could lead to more powerful tests of genetic association than single-locus analyses, but it is not easy to estimate haplotype frequencies from genotype data due to phase ambiguity. The challenge is compounded when individuals are pooled to save costs or to increase sample size. By collapsing the total allele frequencies of each pool suitably, the maximum likelihood estimates of haplotype frequencies based on the collapsed data can be calculated very quickly regardless of pool size and haplotype length (Kuk *et al*., 2013).

Seasonal population differentiation

Pairwise comparisons were carried out between populations collected over two successive seasons, independently for each location (Beja, Bizerte, Jendouba, and Tunis). Multilocus genotype variability (N_T) and allelic richness (R) were estimated (described above), and pairwise F_{ST} values were calculated using Arlequin software. APCA was also performed to detect the distributions of isolates collected in the two successive growing seasons.

Mating type frequency analyses

Chi-square was used to test mating type ratios for all the locations sampled in the 2012 growing season. The ratios were expected to be 1:1 for randomly mating populations (Milgroom, 1996). Mating type ratios were considered significantly different from 1:1 at $P < 0.01$.

RESULTS

Population diversity of Ascochyta fabae *in Tunisia*

The genetic diversity and structure of Tunisian *A. fabae* populations were investigated using ten SSRs and mating type markers. The multiplex PCR easily differentiated two mating types, and the isolates were unambiguously assigned to one of those types. Of the 240 Tunisian isolates sampled in 2012, MAT1-2 was more common in Tunisia than MAT1-1 with a ratio of 2:1 (Table 3). At Beja and Tunis, the two mating types occurred in a 1:1 ratio ($P = 0.925$). For the other two locations, all the populations showed skewed mating type distributions, indicating that MAT1-2 was more common in all these populations (Table 3). The overall distribution showed statistically significant deviation from the 1:1 ratio in only two locations: Bizerte and Jendouba ($P < 0.001$).

All ten microsatellite loci were found to be polymorphic for the 240 isolates. The greatest heterozygosity were obtained for AFCAA13 and CAA28. Low levels of genetic diversity were detected among and within the locations, as populations from different locations shared many common alleles at all the SSR loci (percentage of common alleles greater than 35%) (Table 4). Narrow genetic diversity was found within each of the sixteen

Table 3. Isolates of *Ascochyta fabae* collected from different locations of Tunisia in the 2012 cropping season, and their mating types (MAT 1-1 and MAT 1-2) as determined by PCR.

Location	Number of isolates	*MAT 1-1*	*MAT 1-2*	c^{2e}	P^f
Beja	109	55	54	0.008	0.925
Bizerte	71	15	56	28.928	< 0.001
Jendouba	39	1	38	38.348	< 0.001
Tunis	21	10	11	0.154	0.694

Table 4. Distribution of private (P) and common (C) alleles among populations of *Ascochyta fabae* sampled in 2012 from four main locations of faba bean production in Tunisia (Beja, Bizerte, Jendouba, Tunis).

Location		Loci										Total
		CAA46	CAA28	AFCAA13	AFCAA12	AFCAA5	CA31	AFCAA1	CA10	CA33	CAA57	
Beja	C	21	7	7	10	8	11	12	15	12	11	114
	P	3	1	0	1	0	0	2	0	3	1	11
	Total	24	8	7	11	8	11	14	15	15	12	125
	%P	*12.5*	*12.5*	*0.0*	*9.1*	*0.0*	*0.0*	*14.3*	*0.0*	*20.0*	*8.3*	8.8
Bizerte	C	4	4	4	3	3	3	3	4	4	3	35
	P	4	1	1	3	0	4	7	2	2	4	28
	Total	8	5	5	6	3	7	10	6	6	7	63
	%P	*50.0*	*20.0*	*20.0*	*50.0*	*0.0*	*57.1*	*70.0*	*33.3*	*33.3*	*57.1*	*44.4*
Jendouba	C	2	3	4	3	3	2	2	3	2	2	26
	P	5	2	0	1	0	0	1	1	4	1	15
	Total	7	5	4	4	3	2	3	4	6	3	41
	%P	*71.4*	*40.0*	*0.0*	*25.0*	*0.0*	*0.0*	*33.3*	*25.0*	*66.7*	*33.3*	*36.6*
Tunis	C	4	4	4	3	4	3	3	3	5	3	36
	P	0	0	0	0	0	0	1	0	0	1	2
	Total	4	4	4	3	4	3	4	3	5	4	38
	%P	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*25.0*	*0.0*	*0.0*	*25.0*	*5.3*

populations, reflecting abundant intraregional gene flow. At a location level, the percentage of common alleles shared among populations ranged from 55.6% to 94.7% (Table 4). Isolates from Beja had four loci (AFCAA13, AFCAA5, CA31 and CA10) that were common among the isolates of the different populations sampled at this location. At Bizerte, only one locus (AFCAA5) was common between the isolates. At Jendouba, three loci were only found among the isolates sampled in the different fields at this location (AFCAA13, AFCAA5, and CA31). At Tunis, of the ten loci, only two alleles were shared among isolates of the two populations sampled (AFCAA1, CAA57) (Table 4).

Low to high genetic and genotypic diversity was detected, depending on the location and the population (Table 5). GENCLONE analysis detected 67 different multilocus genotypes (MLG) of *A. fabae* among all the isolates sampled in 2012, with only six genotypes present in more than one location. Twenty genotypes were detected only once in the overall population. No MLG was detected in all four locations (Table 5). MLGs 28, 4, 60, and 62 represented 12.4%, 7.8%, 6.6% and 5.4% of the studied isolates, respectively. MLGs 27 and 42 represented, 3.9% and 3.1% of the isolates, respectively. The other MLGs included less than 2.7% of isolates. By location, genotypic richness (R) ranged from 0.11 (Jendouba) to 0.42 (Beja). Genotypic diversity was significantly greater than 0 ($P < 0.05$) in all four locations. Isolates from Beja showed the greatest genotypic diversity (D = 0.982), followed by those from Bizerte (D = 0.809), Tunis (D = 0.803) and Jendouba (D = 0.726) (Table 5). At Beja, the frequency of the most common genotype (MLG 27) represented 8.8% of all the isolates sampled at that location. At Bizerte, the main MLG (MLG 28) represented 39.7% of all the isolates. At Jendouba and Tunis, the main MLGs (MLG 4 and MLG 42) represented 43.5 and 30.8% of all the isolates sampled at those two locations (Table 5). Different MLGs were recorded at different locations, respectively (Table 5). MLGs 13, 28, and 62 were detected at Beja and Bizerte, MLG 56 at Beja and Tunis, MLG 42 at Jendouba and Tunis, and MLG 60 was detected at Bizerte and Jendouba.

Genetic diversity indices also showed low to high variability among isolates within populations at the different locations studied (Table 6). Genotypic richness (R) ranged from 0 to 1 within the different populations studied. Isolates from Beja showed the greatest genotypic richness (R = 0.42), followed by Tunis (R = 0.25), Bizerte (R = 0.13) and Jendouba (R = 0.11). Allelic richness (*Ar*) was greatest at Beja (*Ar* = 5.84), followed by Bizerte (*Ar* = 3.95), Tunis (*Ar*= 3.35) and Jendouba (*Ar* = 1.63). As the populations studied were of unequal sample size, *Ar* was corrected for each dataset. This showed that Beja still had the greatest allelic richness (*Ar* = 5.04), followed by Tunis (*Ar* = 3.2), Bizerte (*Ar*= 3.15) and Jendouba (*Ar* = 1.49). Statistics on the Index of association (I_A) and *Rd* were significantly different from 0 at all the locations (I_A ranged from 1.827 ($P < 0.001$) to 7.223 ($P < 0.001$); *Rd* ranged from 0.182 ($P < 0.001$) to 0.642 ($P < 0.001$)). This

Table 5. Occurrence of multilocus genotypes (MLGs) among sixteen populations of *Ascochyta fabae* sampled at four locations (Beja, Bizerte, Jendouba, Tunis) in Tunisia, during the 2012 cropping season.

MLG	Beja	Bizerte	Jendouba	Tunis	Total occurence
MLG1-MLG2	X				3; 2
MLG3		X			1
MLG4-MLG5			X		20; 1
MLG6-MLG12	X				1; 2; 3; 1; 3; 2; 2
MLG13	X	X			2; 1
MLG14-MLG18	X				4; 4; 3; 2; 1
MLG19-MLG20			X		2; 1
MLG21-MLG22		X			3; 7
MLG23				X	3
MLG24		X			7
MLG25				X	2
MLG26-MLG27	X				1; 10
MLG28	X	X			3; 29
MLG29	X				1
MLG30		X			7
MLG31-MLG37	X				1; 1; 5; 3; 2; 1; 2
MLG38		X			4
MLG39-MLG40	X				4; 1
MLG41			X		1
MLG42				X	8
MLG43-MLG47	X				1; 2; 1; 4; 1
MLG48			X		4
MLG49-MLG50	X				1; 3
MLG51			X		1
MLG52-MLG55	X				2; 3; 1; 1
MLG56	X			X	1; 3
MLG57	X				3
MLG58				X	3
MLG59	X				5
MLG60		X	X		1; 16
MLG61				X	7
MLG62	X	X			1; 1"
MLG63-MLG67	X				2; 2; 4; 2; 3; 13
Ni	109	71	39	21	
Nu	48	10	8	6	
Fm	8.8%	39.7%	43.5%	30.8%	
D	0.982	0.809	0.726	0.803	

Nu = Number of unique multilocus genotypes in each population.
Fm = Frequency of the most common genotype
D = Genotypic diversity (Pielou, 1969).
Null hypothesis of no genotypic diversity was tested by comparing *D*-values obtained from 1000 randomized data sets to those estimated from the observed data set. *0.01 < *P* < 0.05, **0.001 < *P* < 0.01, ***$P < 0.001$

Table 6. Genetic diversity, multilocus gametic disequilibrium, and test for random mating within *Ascochyta fabae* populations from Tunisia sampled during the 2012 cropping season.

Location	Populations	N	G	R	*Ar*	*Ar* (n=6)	H'	I_A	R_d
Beja	Bej1	8	8	1	5.5	5.3	0.886	0.241	0.029
	Bej2	7	6	0.83	4.1	4.1	0.742	1.531	0.174
	Bej3	12	11	0.91	6.0	5.3	0.850	1.464	0.171
	Bej4	12	10	0.82	6.7	5.7	0.841	2.971	0.349
	Bej5	16	11	0.67	6.5	5.1	0.800	2.677	0.304
	Bej6	6	6	1	4.4	4.4	0.867	1.360	0.175
	Bej7	14	12	0.85	6.8	5.4	0.809	2.192	0.252
	Bej8	25	19	0.75	7.7	5.4	0.806	1.735	0.198
	Bej9	9	8	0.86	4.9	4.7	0.792	2.274	0.271
	Total	*109*	*48*	*0.42*	*5.84*	*5.04*	*0.821*	*1.827*	*0.182*
Bizerte	Biz1	16	4	0.20	3.5	2.9	0.495	7.376	0.820
	Biz2	55	6	0.10	4.4	3.4	0.589	7.071	0.786
	Total	*71*	*10*	*0.13*	*3.95*	*3.15*	*0.542*	*7.223*	*0.641*
Jendouba	Jen1	6	2	0.20	1.9	1.9	0.300	8.000	1.000
	Jen2	22	3	0.10	1.1	1.1	0.089	-	0.511
	Jen3	11	1	0	2.0	1.5	0.018	2.996	-
	Total	*39*	*5*	*0.11*	*1.63*	*1.49*	*0.135*	*5.498*	*0.603*
Tunis	Tun1	15	6	0.36	3.7	3.4	0.480	4.789	0.534
	Tun2	6	4	0.60	3.0	3.0	0.613	4.251	0.476
	Total	*21*	*6*	*0.25*	*3.35*	*3.2*	*0.630*	*4.520*	*0.483*

N = number of isolates.
G = number of distinct multilocus genotypes.
R = genotypic richness.
Ar=, allelic richness
Ar (n=6), allelic richness corrected for samples size
H': expected heterozygosity without bias (Nei, 1978).
I_A = association index.
Rd statistic is a measure of the multilocus linkage disequilibrium (Agapow and Burt, 2001)

indicates high multilocus linkage disequilibrium and clonal populations (Table 6).

Structure of Ascochyta fabae *populations in Tunisia*

Partition of molecular diversity among and within locations, as well as among and within populations, was studied using an analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992; Lynch and Milligan, 1994). AMOVA revealed that 74.34% ($P < 0.001$) of the total genetic variance was partitioned within populations. A relatively low proportion of genetic variability was attributable to differences among populations within locations (14.52 %, $P < 0.001$)) and among locations (11.14 %, $P = 0.0146$).

High variances of F_{ST} values were detected, ranging from -0.055 to 0.982. Pairwise F_{ST} s were greater between

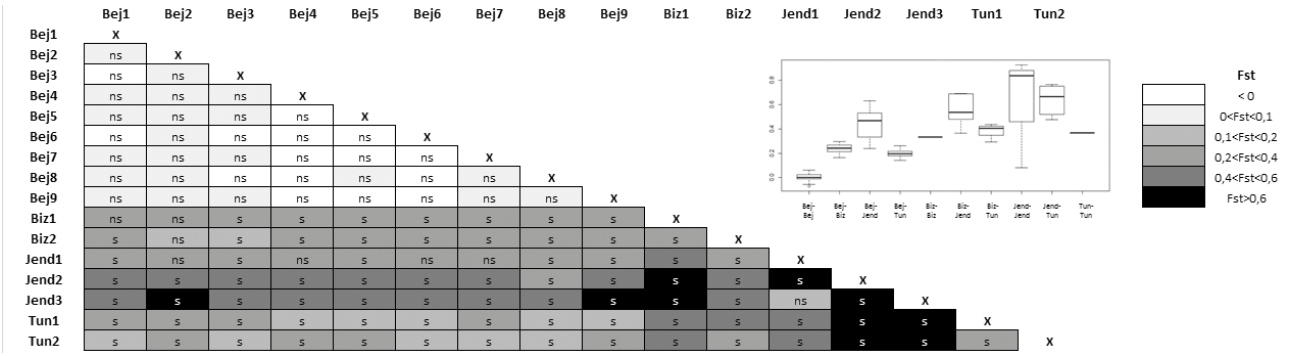

Figure 1. Matrix of pairwise F_{ST} among *Ascochyta fabae* sub-populations sampled in 2012. "ns" indicates non-significant differentiation between two populations (p: sub-population) from different locations (Bej = Beja, Biz = Bizerte, Jend = Jendouba, Tun =: Tunis). The distribution of pairwise F_{ST}s among each sub-population is indicated by the box plots.

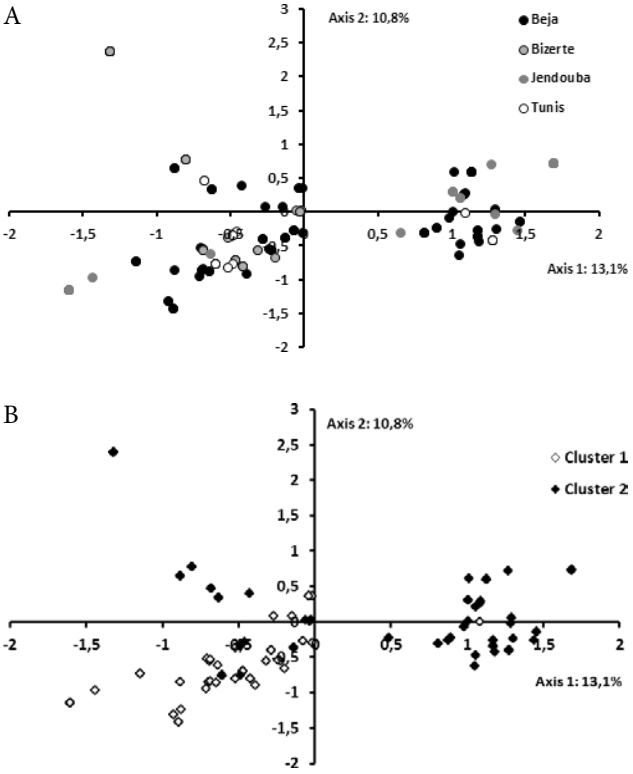

Figure 2. Principal component analysis (PCA) realized on the *Ascochyta fabae* sub-populations sampled from the five locations in Tunisia in 2012. **A**: isolates were assigned to their respective location (Beja, Bizerte, Jendouba, Kef, or Tunis), **B**: isolates were assigned to their respective clusters (C1, C2, or C1/C2) estimated with STRUCTURE (k = 2, deltak = 10).

locations than between populations within specific locations (Figure 1). The isolation for distance test did not show significant correlation (data not shown). The F_{ST} value was not correlated to the geographic distance separating two populations.

Lack of genetic differentiation of *A. fabae* populations was confirmed by the STRUCTURE and PCA analyses (Figure 2). PCA failed to separate the *A. fabae* isolates of the different populations sampled at Beja, Bizerte, Jendouba and Tunis into different groups (Figure 2A). The low percentage of genetic diversity explained by the two principal axes of the PCA (respectively, 13.1% and 10.8%) suggests that all the isolates belonged to the same population. The STRUCTURE analysis also failed to separate the isolates from the different locations into different groups (k = 2, *deltaK* = 10) (Figure 2B). The Bizerte populations were homogenic compared to the other locations.

A minimum spanning network (MSN) using Nei's distance for the different populations of *A. fabae* was calculated. As with the two other analyses, the MSN failed to clearly classify and structure the different populations (Figure 3). Except for the isolates from the population Jen 2 that constituted a single node, all others were distributed throughout the network, and the isolates were not grouped according to origin.

Seasonal genetic structure change in different Tunisian locations

Seasonal change of genetic diversity was monitored at the four locations (Beja, Bizerte, Jendouba and Tunis), and with the 317 isolates. Except for Tunis, all the locations showed different genotypic richness (R) in the two seasons. R values were greater in the second season than in the first (0.40 to 0.83 at Beja, 0.14 to 0.47 at Bizerte, and 0.16 to 0.30 at Jendouba) (Table 7). Multilocus genotypes occurred in from four to 46 isolates for the different populations. Only Beja and Jendouba showed common genotypes between the two successive seasons (Table 7).

Low to high variances of F_{ST} values were detected between the two successive seasons at the different loca-

Table 7. Genetic differentiation, test for random mating (I_A, R_d), and pairwise genetic comparison F_{ST} between two successive seasons at four Tunisian locations.

	Beja		Bizerte		Jendouba		Tunis	
	Season 1	Season 2	Season 1	Season 2	Season 1	Season 2	Season 1	Season 2
N[a]	13	109	71	16	28	39	21	20
N_T	11	46	11	8	9	8	6	4
R	0.83	0.40	0.14	0.47	0.30	0.16	0.20	0.16
N_C	2		-		3		-	
I_A	1.473***		4.797***		4.929***		4.541***	
R_d	0.167***		0.535***		0.548***		0.506***	
F_{ST}	0.0177 ns		0.1882***		0.0645*		0.3269***	

A = Number of isolates analyzed.
N_T = Number of MLG.
N_C = Number of common MLG.

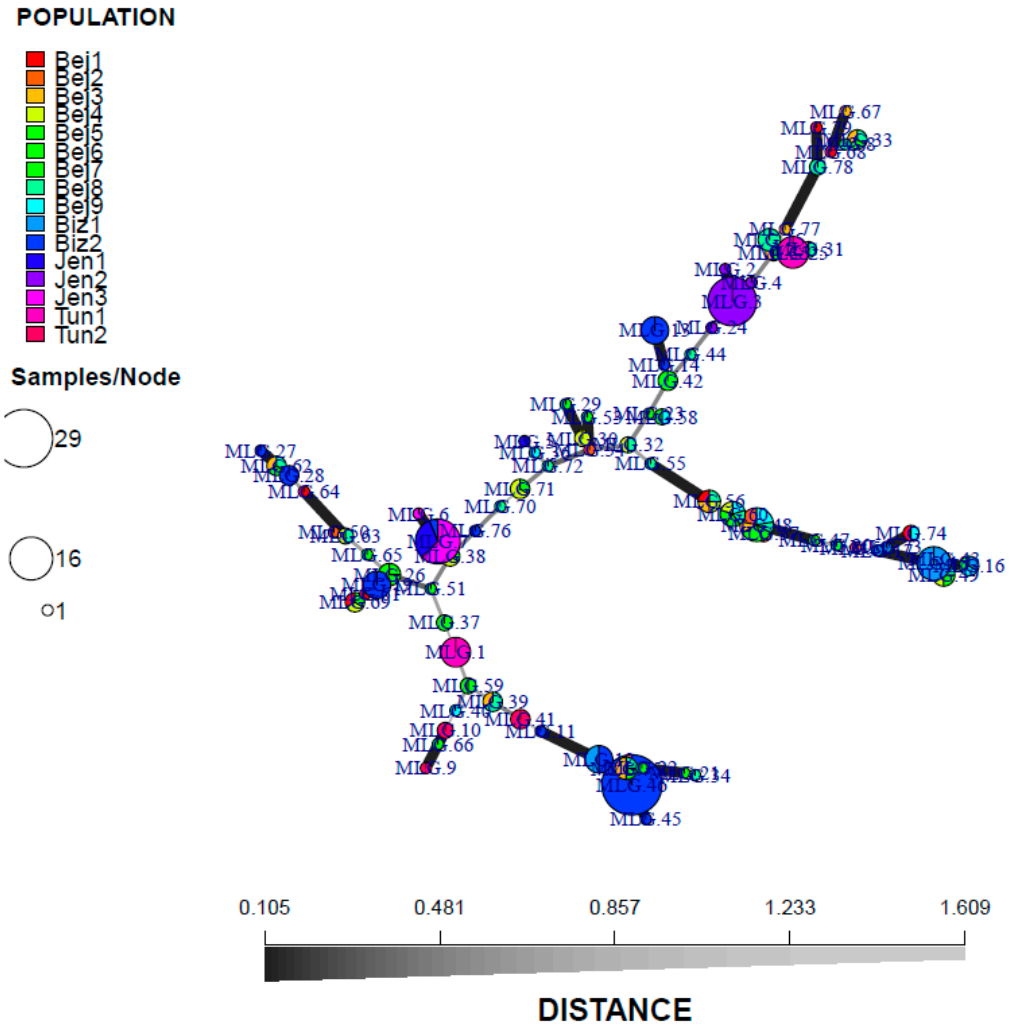

Figure 3. Minimum spanning network (MSN) of 67 haplotypes detected in the *Ascochyta fabae* collection of 2012. Each circle represents a unique haplotype and the different colours represent the sampling fields. The circle sizes represent the haplotype frequencies and the numbers of isolates is indicated in circles. Line widths and the shading represent relatedness of the haplotypes, based on Nei's genetic distance.

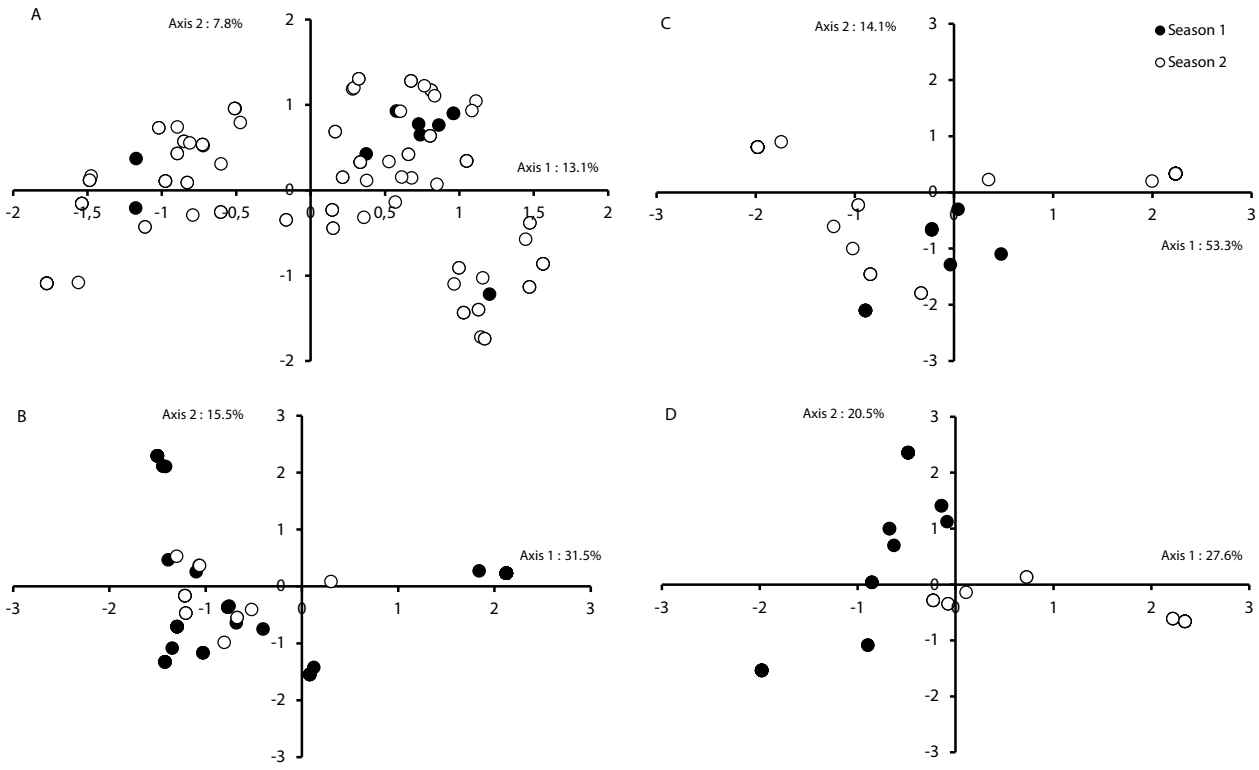

Figure 4. Principal component analysis (PCA) realized on the *Ascochyta fabae* sub-populations sampled during two successive cropping seasons at four locations in Tunisia. (A = Beja; B = Bizerte; C = Jendouba; D = Tunis).

tions. Pairwise F_{ST} were significantly different between the two seasons in Tunis (F_{ST}= 0.3269), Bizerte (F_{ST}= 0.1882) and Jendouba (F_{ST}= 0.0645) (Table 7). The PCA failed to separate the *A. fabae* isolates sampled in the two seasons from each location into two different groups, with the exception of the isolates from Tunis (Figure 4). The STRUCTURE analysis was also performed on these data, but failed to separate isolates from the different seasons into different groups (data not shown).

DISCUSSION

This study is only the second published report of examination of the diversity of *A. fabae* populations. Each of the ten pairs of SSR primers produced a single amplicon for each locus of each isolate with a high level of polymorphism, as established by Ozkilink *et al.* (2011). Among the 240 Tunisian isolates sampled in 2012, MAT1-2 was more common in Tunisia than MAT1-1, and mating type results showed that Tunisian populations have skewed distribution (1:2 ratio) for MAT1-1:MAT1-2. Of the four locations studied, two (Beja and Tunis) gave a 1:1 distribution while for the other two locations, MAT1-2 was more common than MAT1-1. This skewed distribution was confirmed by a chi-square test, suggesting that sexual reproduction may not occure at these two locations. These results were also confirmed by statistics on the Index of association (I_A) and *Rd,* both of which were significantly different from 0. This indicates that multilocus gametic disequilibrium and clonal (genotypic) diversity analyses did not provide evidence for randomly mating populations.

Our results were consistent with those of Ozkilinc *et al.* (2015) for the number of MLGs observed in *A. fabae* populations, but not for the distribution of mating types. The occurrence of sexual reproduction in the life cycle of Tunisian *A. fabae* populations is not likely to be regular, and ascospores may not be important primary inoculum, unlike for other Ascochyta blight diseases (Tivoli and Banniza, 2007). This conclusion should be confirmed, as the sample sizes of the populations in the present study were unequal , and therefore insufficient for the appropriate statistical tests. The observed disequilibrium could be due to sampling phenomena, particularly small sample sizes.

In their study, Ozkilinc *et al.* (2011) sampled *D. fabae* from chickpeas in southeastern Turkey and Israel

between 2004 and 2007. They showed high gene diversity, but with gametic disequilibrium associated with small sample sizes. This result suggests that the gametic disequilibrium observed could be caused by admixtures of genetically distinct populations and/or sampling error, as all populations were assigned to a single genetic population in the STRUCTURE analysis, PCA and MNS analyses (Milgroom, 1996).

Despite the lack of differentiation *into* populations, many rare genotypes and a significant number of private alleles were detected. Only five genotypes of the pathogen were present in more than one location. This contradiction may be explained by the characteristics of the used marker, and suggests that new introductions of the pathogen had been made, either through mutation as microsatellite loci mutate rapidly or through migration or recombination. Between 2003 and 2012, Tunisia annually imported an average of 92.6 tons of faba bean seeds, for either multiplication by seed companies or for farmer use (ONAGRI, 2015). These imports, from countries such as Syria, may be a potential source of new pathogen genotypes. Faba beans imported for consumption average approx. 392 tons annually (ONAGRI, 2015). They are less rigorously controlled for diseases and could be a potential introduction source of new genotypes.

The limited dispersal of *A. fabae* isolates within fields may also lead to gametic disequilibrium. Even if random mating had occurred within restricted genotype profiles, this would have resulted in population admixture, which is one of the causes of gametic disequilibrium (Milgroom, 1996). The absence of common MLGs to all four locations suggests that populations from different locations may originate from multiple population sources. This hypothesis is supported by the F_{ST} values significantly different from zero. Migration could take place through seed movement. Farmers' use of their self-produced seeds may lead to genotypic differentiation at the field scale within the same region according to the values of F_{ST} calculated for pairs of fields; this appeared particularly at Bizerte and Jendouba. The hypothesis is plausible given that agricultural systems in Tunisia are characterized by the predominance of small-holder farmers relying on subsistence farming. In addition, lack of appropriate disease management strategies can result in pathogen multiplication and spatial dispersion through rain episodes and favourable temperatures (Tivoli and Banniza, 2007).

Furthermore, the greater genetic diversity detected at Beja could also be due to climatic conditions being more conducive to the pathogen in this region. *Ascochyta fabae* is favoured by low temperatures, and severe rainfall episodes that result in splash dispersal of pycnidiospores (Maurin *et al*., 1990). The different locations had similar temperatures during the cropping season (Beja; Tmean = 14.3°C, Tmin = 8.9°C, Tmax = 19.6°C: Bizerte; Tmean = 14.9°C, Tmin = 10.5°C, Tmax = 19.3°C: Jendouba; Tmean = 14.6°C, Tmin = 8.6°C, Tmax = 20.7°C: Tunis; Tmean = 15.0°C, Tmin = 10.1°C, Tmax = 19.9°C). However, differences in rainfall were observed. Beja recorded annual rainfall of 479 mm, compared to 293 mm at Tunis, 345 mm at Jendouba, and 368 mm at Bizerte. This greater higher rainfall at Beja provided a conducive environment for *A. fabae* to spread and rapidly reproduce (Pritchard *et al*., 1989; Tivoli and Banniza, 2007).

Selection of *A. fabae* by the host may be ruled out as having any significant impact. Research was carried out in Tunisia attempting to identify sources of resistance (Kharrat *et al*., 2006). Some faba bean cultivars were released, but none are currently sold in their pure original states at seed markets. Due to the mixing of seed types, cultivars lose their distinctive characteristics after a few years, and the pathogen would face a host population characterized by high levels of genetic diversity. This would not necessarily expose the pathogen to directional selection, but would likely maintain pathogen diversity (McDonald and Linde, 2002).

Monitoring seasonal changes of the genetic structure of *A. fabae* at Beja, Bizerte, Jendouba and Tunis showed that new genotypes were detected in the second season in each region. This was unlikely to be derived from an event of sexual reproduction, since the allelic richness did not correlate with the number of MLGs identified in each region. A high level of inbreeding, revealed by significant values of F_{ST} in the two seasons, as well as the absence or scarcity of common genotypes, support the hypothesis that genotypic variation may be the result of genotype migration through seed exchange between different regions, as reported by Kaiser *et al.* (1997), and/or differential genotype adaptive potential to environmental conditions. As shown in the case study of Frenk el *et al.* (2010) from wild and domesticated *Cicer* species, a summer cropping system can act as a climatic factor for diversification of *A. rabiei* populations. These findings weaken any hypothesis of sexual reproduction in the different regions, but strengthen that of involvement of other evolutionary forces of diversity such as genotypic migration. This emphasizes the important role of seeds in disease dispersion at a regional scale (Tivoli and Banniza, 2007; Ali *et al*., 2012).

In conclusion, our study has shown that neither the genetic diversity observed within the Tunisian populations of *A. fabae*, nor multilocus gametic disequilibrium, provide evidence for randomly mating populations of

the pathogen in Tunisia. Lack of differentiation could be attributable to migration or mutation. The role of sexual reproduction should be confirmed by extending the sampling of isolates in these different locations, by calculating the P_{sex} index (GENETIX) and by evaluating the locus compatibility parameters (Arnaud-Haond *et al*., 2005). The extent of asexual compared to sexual reproduction can affect disease management.

Sexual reproduction plays a role in the overwintering/adaptability of *A. fabae* by generating pathogen variability and facilitating long-distance dispersal via airborne ascospores (Kaiser, 1992; Trapero-Casas and Kaiser, 1992; Kaiser *et al*., 1997). This can shape the structure and change genetic variability in populations. Despite recent advances, the role of gene flow (and of its two mains triggers, reproduction and dispersal) in the local evolution of pathogen populations is still poorly assessed. Many fungal plant pathogens alternate cycles of asexual multiplication with single annual episodes of sexual reproduction (Barrès *et al*., 2012). The number of cycles of asexual multiplication has demographic impact as this corresponds mostly to the epidemic phase of a disease, and will also affect the genetic characteristics of pathogen populations.

Some studies of population genetics of airborne plant pathogens have used nested hierarchical sampling strategies. Gobbin *et al*. (2005) explained the arrival of new genotypes and the erosion of clonal structure in *Plasmopara viticola* populations by the continual input of sexual spores. For *A. fabae*, no data of changes in clonal structure during epidemic seasons is available. Studying the temporal changes in genes and genotypic diversity between the beginning and the end of an epidemic would be informative for the different epidemiological processes involved (Prugnolle and De Meeûs, 2010). This could provide insights into the balance between auto- and allo-infection processes.

To fully determine the degree of variability contributed by sexual recombination in pathogen populations, sampling on small spatial, but large temporal scales would help to address this question. Furthermore, analyzing progeny from naturally occurring pseudothecia for segregation would further indicate variability due to recombination (Milgroom, 1996). Studying the biology (temperature optima) of the two mating types and their fitness may further clarify this point. Migration of clones driven by seed movement at national and international levels may play a key role in obtaining high levels of genotypic diversity (Kaiser *et al*., 1997). On the other hand, multiple cycles of asexual reproduction result in rapid increases of secondary inoculum and aggressive pathogen clones (Kaiser *et al*., 1997; Tivoli and Banniza, 2007).

Strategies to better control Ascochyta blight will prioritize a focus on seeds. Several rules will need to be developed, including: i) imposition of standards for certified faba bean seed production, and inciting farmers to use certified seeds (to reduce seed transmitted diseases), and to avoid the use of seeds sold in local markets; ii) establish a strict system to control imported faba beans for local consumption; and iii) advocating systematic fungicide seed treatments to avoid risks of disease transmission through seeds during growing seasons.

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Research Papers

Incidence of Fusarium foot and root rot of cereals under conservation agriculture in north west Tunisia

Samira CHEKALI[1,*], Samia GARGOURI[2], Moncef BEN HAMMOUDA[3], Hatem Cheikh M'HAMED[4], Bouzid NASRAOUI[5]

[1] *Laboratoire de Protection des Végétaux, Institut National de Recherche Agronomique de Tunisie, Pôle Régional de Recherche-Développement Agricole du Nord-Ouest Semi-Aride au Kef, Université de Carthage, BP 221, 7100, Le Kef, Tunisia*
[2] *Laboratoire de Protection des Végétaux, Institut National de la Recherche Agronomique de Tunisie, Université de Carthage, Rue Hédi Karray, 2049, Ariana, Tunisia*
[3] *Laboratoire de Physiologie de la Production des Céréales, Ecole Supérieure d'Agriculture du Kef, Université de Jandouba, 7119 Le Kef, Tunisia*
[4] *Laboratoire de Recherche de Science et Techniques Agronomiques, Institut National de Recherche Agronomique de Tunisie, Université de Carthage, Rue Hédi Karray, 2049, Ariana, Tunisia*
[5] *Laboratoire de Recherche des Bioagresseurs et Protection Intégrée en Agriculture, Institut National Agronomique de Tunisie, Université de Carthage, 43 Avenue Charles Nicolle, 1082, Tunis-Mahrajène, Tunisia*
*Corresponding author: samirachekali@gmail.com

Abstract. Conservation agriculture, based on direct drilling for crop establishment, has emerged in Tunisia since 1999/2000 as an alternative to conventional agriculture based on conventional drilling. The main objective of this approach is to ensure yield stability from crops and replenishment of soil organic matter. Previous research has demonstrated increased risks from pathogens favoured by mulching. The impacts of direct drilling on yields, and Fusarium foot and root rot of durum wheat, oat and barley, were studied over three successive growing seasons (2009/10, 2010/11, 2011/12) in northwest Tunisia. Disease incidence was estimated by the recovery frequency of *Fusarium* spp. isolates from stem bases and roots of plants of the three cereals. In addition, disease severity was assessed by occurrence of whiteheads that senesced prematurely, and the browning intensity on durum wheat stem bases. Grain yields were recorded at the ripening stages. *Fusarium culmorum* and *F. pseudograminearum* were isolated from the three cereals, with *F. culmorum* being the dominant pathogen. Direct drilling increased the incidence (60%) of these pathogens on stem bases and roots during the warmer seasons of 2009/10 and 2010/11, but less infection (37%) compared to conventional drilling was observed in the wetter season of 2011/12. Direct drilling increased the percentage of whiteheads of durum wheat (73%), but did not affect disease severity, which was estimated by the discolouration on stem bases and grain yield.

Keywords. Direct drilling, conventional drilling, Fusarium foot and root rot, cereals, grain yield.

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INTRODUCTION

Conservation agriculture technology is increasingly relevant for addressing the needs of resource-poor farmers, and the challenges of resource degradation, sustainability, food insecurity, poverty alleviation, climate change, labour shortages and high energy costs (Kassam *et al*., 2012). Direct drilling (hereafter abbreviated as DD) for crop establishment is practiced on about 111 million ha worldwide, and the proportional of adoption by farmers is 47% in South America, 38% in the United States and Canada, 12% in Australia,4% in New Zealand, and 4% in the rest of the world, including Europe, Asia and Africa (Derpsch *et al*., 2010). In North Africa, conservation agriculture systems have been promoted, particularly in Morocco and Tunisia. In Morocco, 4,000 ha of DD have been reported, despite long-term research on DD farming initiated in the early 1980s (Mrabet, 2012). In Tunisia, the promotion and development of these approaches was farmer-centered, and the area under DD increased from 27 ha in 1999 to nearly 6,000 ha in 2007 and 8,000 ha in 2008 (FAO, 2011). DD is now applied on 12,000 ha, which cover 0.8 % of cereal agricultural land, an area distributed among 200 farmers and operated by 102 direct drilling machines (ICARDA, 2016).

Under DD, the soil is left undisturbed from harvest to planting, except for narrow strips where the seeds are planted and the fertilizer is applied (Schillinger *et al*., 1999). Despite the benefits, DD may constitute potential risk for disease development since leaving infected plant residues on soil surfaces contributes to increasing pathogen inoculum. This may influence the incidence of diseases caused by pathogens which survive on crop debris. These include the complexes of species causing foot and root rot (Smiley *et al*., 2005). This pathogen complex includes *Fusarium pseudograminearum* (teleomorph *Giberella coronicola*), *F. culmorum*, *F. avenaceum* (teleomorph *Giberella avenacea*), *Bipolaris sorokiniana* (teleomorph *Cochliobolus sativus*), and *Microdochium nivale* (teleomorph *Monographella nivalis*). Under adopted conventional drilling (CD) in Tunisia, *F. culmorum* followed by *F. pseudograminearum* are the main pathogens isolated from cereals in the semi-arid regions (Gargouri, *et al*. 2001; Boughalleb *et al*. 2008). Both of these fungi survive in crop residues, but with different biologies. *F. culmorum* is can grow at lower temperatures than *F. pseudograminearum* (Doohan *et al*., 2003), and persists as hyphae within stubble residues and as chlamydospores in soil (Burgess, 2011).

These pathogens produce lesions on the coleoptiles, roots, and sub-crown internodes of affected plants, and cause browning of the stem bases. Damage to cereals is often unnoticed until white heads appear shortly before crop maturity, or as shriveled grain that is noted during harvest (Papendick and Cook 1974; Burgess *et al*., 2001). Infection is favoured by wet conditions shortly after sowing of crops, and disease severity increases and yield reductions become significant when infected plants are under water stress and/or exposed to high temperatures late in the growing season (Cook, 1981; Paulitz *et al*., 2002.; Smiley *et al*., 2005; Chekali *et al*., 2011). In Tunisia, losses in grain yields as much as 25% occur during the dry seasons (Chekali *et al*., 2013). Crop rotation is a major control method for these pathogens, reducing disease incidence in legume / fallow / cereals cropping systems (Chekali *et al*., 2016).

Reports of effects of DD on development of cereal foot and root rot diseases have been varied among regions. For DD adopted in Tunisia, there is no information on these diseases. The objective of the present study was to evaluate the impacts of DD on Fusarium foot and root rot development and yields of durum wheat, oat and barley subjected to different cropping sequences over 3 years in northwest Tunisia.

MATERIALS AND METHODS

Site description

A field trial was conducted during the 2009/10, 2010/11 and 2011/12 growing seasons, to compare DD to CD. The trial was located at the Experimental Station of Institut National des Grandes Cultures(INGC) located at Boussalem (36°36'35" N, 8°58'17" E), at an altitude of 124 m. The area has a warm temperate Mediterranean climate. The soil at the trial site contained 54% clay, 31% silt and 15% sand. The winter is the rainy season. According to Köppen(1936), the climate is classified as hot-summer Mediterranean climate with annual mean precipitation of 500–600 mm. This precipitation varied between 400 and 700 mm during the three growing seasons of this study (Table 1).

Experimental design and field lay-out

The trial was set up as a Split Plot Design. Four blocks were divided into two Main Plots, corresponding to CD and DD. These were each divided into five Sub Plots, each of 360 m^2 (30 m × 12 m), of the following rotations scenarios: durum wheat after oat, oat after durum wheat, durum wheat after barley, barley after durum wheat or durum wheat after faba bean. Prior to sowing, the soil was harrowed, and durum wheat var.

Table 1. Monthly rainfall (mm) recorded during the experimental period. The critical period for pathogen infection and disease development is highlighted in gray.

Growing seasons	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	Jun.	Jul.	Aug.	Total
2009/10	104.5	38.5	29.0	37.0	44.0	32.5	70.0	41.5	28.0	0.0	0.0	0.0	425.0
2010/11	14.5	64.0	88.0	42.0	55.0	102.0	42.0	420.0	26.5	49.0	4.0	0.0	529.0
2011/12	28.0	228.0	85.0	89.5	50.0	105.5	60.5	74.5	5.5	35.0	7.0	5.0	751.0

Table 2. Minimum and maximum temperatures recorded during the experimental period. The critical period for pathogen infection and disease development is highlighted in gray.

Growing seasons		Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	Jun.	Jul.	Aug.
2009/10	Max.	27.9	24.2	22.5	14.7	17.3	17.8	16.9	22.5	27.1	30.8	35.0	35.2
	Min.	17.4	12.6	7.5	7.6	5.0	5.1	6.4	11.4	13.0	15.9	20.8	21.1
2010/11	Max.	28.1	26.1	20.0	18.1	17.2	14.5	18.0	23.1	23.8	32.0	37.3	37.4
	Min.	17.4	13.0	10.5	4.9	5.2	4.5	7.8	9.0	13.5	15.7	20.4	19.7
2011/12	Max.	33.6	22.0	20.1	15.1	14.2	11.4	17.4	22.7	26.4	36.5	37.8	37.8
	Min.	18.3	13.2	12.0	7.0	5.3	4.7	8.1	10.6	11.2	19.1	21.7	20.3

'Rezzek', a local line of oat, barley var. 'Rihane'or fababean var. 'Sabeur' were sown. Ammonium nitrate fertilizer was added at different plant growth stages. Weeds, diseases (powdery mildew, Septoria blotch, rust) and insects (aphids) were controlled throughout the trial. Grain harvests were in June each season (Table 3).

Pathogen isolation and disease incidence

Forty mature plants of each of the three cereals were removed from individual plots using a zsampling pattern in each plot, and the plants were then thoroughly washed with tap water. Sections (each 2 mm length) of stem bases and roots were surface sterilized for 2 min in 6% NaClO and then for 10 s in 70% alcohol, followed by rinsing in sterile distilled water. These tissue pieces were then dried, and placed on 25% potato dextrose agar (¼ PDA) containing 100 mg L^{-1} streptomycin sulfate. Resulting fungal cultures were incubated for 5 d (12 h light/12 h dark) at 25°C. *Fusarium* spp. hyphae were transferred on carnation leaf agar (CLA) to favour conidium production, and were morphologically identified according to Leslie and Summerell (2006). *F. culmorum* and *F. pseudograminearum* identifications were confirmed by molecular techniques using the protocol of Möller *et al.* (1992) for DNA extraction. Primers and protocols used for PCR amplification of *F. culmorum* were those of Schilling and Geiger (1996), and for *F. pseudograminearum* those of Aoki and O'Donnell (1999). The incidence of infections was determined by the frequency of isolation of each *Fusarium* sp. from stem bases and roots.

Disease severity

Disease severity was assessed only for durum wheat, by determining prematurely senescing inflorescences (whiteheads) and the brownish discolourations on the stem bases. At the anthesis stage (Feekes 10.51), (late April), whiteheads were estimated visually three times in each treatment, and expressed as percent of total heads per plot. The brown colour that appeared between the first and the second internodes of stems was evaluated using a 0-3 scale (0 = no symptoms, 1 = light browning at the first internode (0-25%), 2 = clear browning extending to the second internode (25-50%), or 3 = dark browning extending to the second internode (75-100%)). Ratings were converted to severity indices using the following formula:

$$\text{Severity index} = \frac{((0) \times (0) + (1) \times (1) + (2) \times (2) + (3) \times (3))}{\text{Total number of observed plants}}$$

Yield estimates

Grain yields from different crop rotations were measured during each growing season.

Table 3. Technical itinerary adopted during the experimental period.

Growing season	2009/10		2010/11		2011/12	
	CD	DD	CD	DD	CD	DD
Soil management (from October to November)						
Harrowing number (0–15 cm deep)	3	0	2	0	2	0
Sowing rates (kg ha^{-1})						
Durum wheat	160	160	160	160	160	160
Oat	150	150	150	150	150	150
Barley	120	120	120	120	120	120
Fababean	120	120	120	120	120	120
Fertilizer applications (kg ha^{-1}) Ammonium nitrate (NH_4NO_3)						
- *Seedling stage (Feekes 1)*						
Durum wheat	120	120	100	100	100	100
Oat	100	100	100	100	100	100
Barley	100	100	100	100	100	100
- *Stem elongation stages (Feekes 7)*						
Durum wheat	100	100	130	130	100	100
Oat	100	100	130	130	100	100
Barley	100	100	130	130	100	100
Pesticide treatments (L ha^{-1})						
Herbicide:						
Before sowing - Glyphosate (as Round up+)	0	2	0	2	0	2
- *Seedling stage (Feekes 1)*						
- Durum wheat: Fénoxaprop-Ethyl, Iodosulfuron-Méthyl Sodium and Méfenpyr-diethyl	1	1	1	1	1	1
- Oat: 2-4-D-Acide, di Métosulam	1	1	1	1	1	1
- Barley: Pinoxadan, Cloquintocet-mexyl	1–0.8	1–0.8	1–0.8	1–0.8	1–0.8	1
- Fababean: Bentazone	1.25	1.25	1.25	1.25	1.25	1.25
Fungicide						
- Epoxiconazole	1	1	1	1	-	-
Insecticide						
- Deltamethrine	1	1	1	1	-	-

Statistical analyses

Data were analyzed using ANOVA to test the significance of main effects with four replicates, growing season, sowing method (DD *vs* CD) and crop sequences as main factors, using SPSS Statistics version 20 software published by IBM Crop 2011. Means comparisons was performed using Student's LSD tests ($P = 0.05$ or 0.01).

RESULTS

Identification of pathogens

During the three growing seasons, morphological identification of isolates recovered from 4,800 stem base fragments and 4,800 root fragments of the three cereals revealed that *F. culmorum* was isolated from 39.3% of stem bases and 16.2% from roots, and *F. pseudogramiearum was isolated from* 2.4% of stem bases and 0.9% of roots. Molecular identification using the specific primers OPT18 R and OPT18 F amplified 470 bp of *F. culmorum* fragments, and Fp1-1 and Fp 1-2 amplified 520 bp of *F. pseudograminearum* fragments.

Disease incidence

Data analyses revealed that growing season conditions significantly affected the incidence of infection by both *F. culmorum* ($P = 0.02$) and *F. pseudograminearum* ($P < 0.01$) on stem bases of the cereals. In addition,

Table 4. ANOVA of the effects of growing season (GS), sowing method (DD *vs* CD) and crop sequences (CS) on incidence of *Fusarium culmorum* (*Fc*) or *F. pseudograminearum* (*Fpg)* infections of durum wheat, oat or barley stem bases and roots during the experimental period.

SV	DF	*Fc*				*Fpg*			
		Stem base		Roots		Stem base		Roots	
		F value	Pr> F	F value	Pr> F	F value	Pr> F	F value	Pr> F
GS	2	3.910	0.024	0.007	0.993	11.53	0.000	2.050	0.136
DD *vs* CD	1	33.740	0.000	15.130	0.000	5.540	0.021	0.070	0.782
CS	4	0.930	0.446	1.220	0.309	2.620	0.042	0.680	0.602
CS × DT	2	0.610	0.545	0.790	0.457	5.977	0.004	0.370	0.964
GS × CS	8	1.500	0.170	0.870	0.543	2.301	0.029	0.480	0.864
DT × CS	4	1.990	0.120	0.630	0.638	3.533	0.011	0.430	0.786

there was a statistically significant incidence difference between the DD and CD treatments for *F. culmorum* on stem bases and roots (both $P < 0.01$) and for *F. pseudograminearum* only on stem bases ($P = 0.02$). However, no statistically significant effects were detected for the crop sequence effects, except for the incidence of *F. pseudograminearum* recovered from the stem bases ($P = 0.04$), and between the different tested factors (Table 4). Hence, combined data analyses were carried out for all of the crop sequences.

DD increased the incidence infection of *F. culmorum* on stem bases of durum wheat, oat and barley combined, compared to CD during the trial period. This effect was greater in the two dryer seasons, at 60%; ($P < 0.01$) in 2009/10, and 58% ($P < 0.01$) in 2010/11, than in the wetter season of 2011/12 (32%: $P < 0.01$). On roots, the incidence of infection by this pathogen (60%) was increased ($P < 0.05$) by DD only in 2010/11 (Figure 1).

Fusarium pseudograminearum was present in isolation only in the two growing seasons 2009/10 and

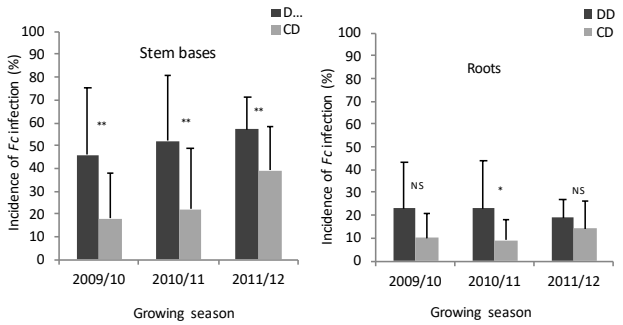

Figure 1. Mean incidence of *Fusarium culmorum* infections on stem bases and roots of durum wheat, oat or barley as affected by different sowing methods (DD *vs* CD) during three growing seasons. Error bars are 2 × standard deviations. NS: No significant difference ($P > 0.05$). *: significant difference ($P < 0.05$). **: highly significant difference ($P < 0.01$) according to Student's Test.

2010/11. Despite the low incidence on stem bases of the three cereals, DD significantly increased incidence of this pathogen (80%; $P < 0.02$) during the 2009/10 growing season, compared to CD (Figure 2).

Disease severity on durum wheat

DD affected occurrence of whiteheads only in the 2010/11 growing season. This drilling method greatly increased the percentage of durum wheat whiteheads (97%; $P < 0.01$; Figure 3), but had no effect on disease severity estimated by discoloration on stem bases.

Durum wheat yields, under DD vs CD and Fusarium foot and root rot

The average of grain yields of durum wheat estimated over the three growing seasons were not significantly different between DD and CD. The yields were estimated 2,700 kg ha^{-1} from DD, and 2,600 kg $ha^{-1}$from CD, in the 2009/10 and 2010/11 growing seasons. However, in the 2011/12 growing season, the durum wheat yields were 4,760 kg ha^{-1} from DD and 4,660 kg $ha^{-1}$from CD. No statistically significant interaction was detected between DD or CD and infection incidence or disease severity, or for occurrence of whiteheads.

DISCUSSION

Conservation agriculture and crop residue management may have impacts on development of soilborne pathogens and the diseases they cause, in medium or long term periods. In this study durum wheat, oat and barley were subjected to different crop rotations and DD or CD crop establishment methods, and assessed for dif-

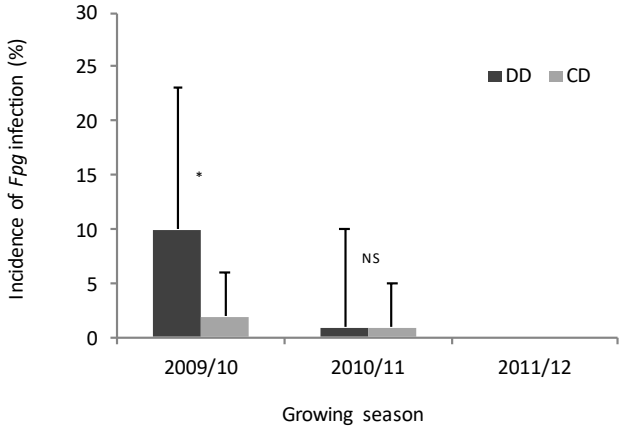

Figure 2. Mean incidence of *Fusarium pseudograminearum* stem base and root infections resulting from different cereal sowing methods (DD *vs* CD) for durum wheat, oat of barley during three growing seasons. Error bars are 2 × standard deviations. NS: No significant difference ($P > 0.05$). *: significant difference ($P < 0.05$).

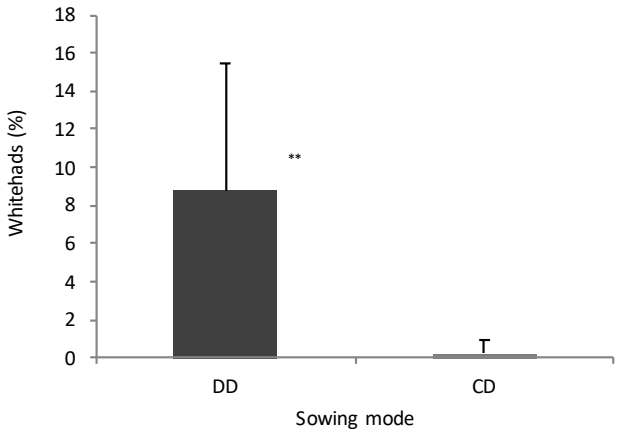

Figure 3. Mean proportions of whiteheads in durum wheat for different sowing methods (DD vs CD) during the 2010/11 growing season. Error bars are 2 × standard deviations. **: highly significant difference ($P < 0.01$) according to Student Test.

ferences in root and crown rot diseases caused by *Fusarium* spp. *Fculmorum* and *F. pseudograminearum* were frequently isolated from stem bases and roots of the three cereals, These results are in agreement with those of Gargouri (2003) and Boughalleb *et al.* (2008), who reported the dominance of *F. culmorum* in Tunisia.

The presence and distribution of each of these species varied in the three experimental years. Both pathogens were isolated as a complex during dry growing seasons (2009/10, 2010/11), while in the wet season (2011/12) only *F. culmorum* was isolated. Similar results were obtained by Lipps and Deep (1991), and Steinkellner and Langer (2004) in Austria. Precipitation and temperature occurring during the experimental period probably influenced the development of each species. Rainfall over the three growing seasons was 425 mm in 2009/10, 529 mm in 2010/11 and 751 mm in 2011/12, with considerable fluctuation during each season. Precipitation over the infection periods (January-February) was almost the same in 2010/11 and 2011/12, but less in 2009/10, and during the disease development period (March-April) was slightly greater in 2009/10 and 2011/12 than in 2010/11 (Table 1). In parallel, the mean recorded temperatures from infection to disease development (January-April) in 2009/10 (11.3–14.3°C) and 2010/11 (10.4–14.5°C) were greater than those in 2011/12 (8.9–14.7°C) (Table 2). The relatively low temperatures in January (9.8°C) and February (8°C) of 2012 (Table 2), in comparison with the other two seasons, probably favoured the development only of *F. culmorum*. These results indicate that this pathogen developed at low temperatures, as reported by Doohan *et al.* (2003), Gargouri (2003), Pitt and Hocking (2009) and Poole (2010). This indicates that *F. culmorum* is more adapted to cold regions than *F. pseudograminearum*, which develops better when it is warm. Some authors have suggested that the relationship between the two *Fusarium* species and DD depends on physical, nutritional and microbiological characteristics of soils. Smiley and Patterson (1996) demonstrated that diseases caused by these pathogens increased with amount of soil surface crop residues that occur, and they are directly correlated with amounts of soil organic nitrogen and carbon. However, under DD, more soil water is conserved than for CD, which could reduce the incidence of cereal foot rot, especially if caused by *F. culmorum* which is enhanced by plant water stress (Papendick and Cook, 1974). On the other hand, *F. pseudograminearum* becomes a serious problem on wheat both in low and high precipitation zones of the Pacific northwest of the United States of America, and was more dependent than *F. culmorum* on crop residues for survival (Paulitz*et al.*, 2002).

The present research showed that after only one year of the experiment, DD increased the incidence of infection by the two fungi, mainly on stem bases of the three different cereals. This effect was consistent with those reported by Windels and Wiersma (1992), Smiley and Patterson (1996) and Bailey *et al.* (2001); and those obtained by Cook (2001), Paulitz*et al.* (2002)and Schroeder and Paulitz (2006). These authors suggested that the main reasons for the increasing range and prevalence of root diseases on wheat and barley in the United States of America and many other cereal-growing areas of the world are the increased frequency of cereals in the crop rotations, and the use of minimum or

no-tillage. Other authors have expressed opposite views. Papendick and Cook (1974) explained that under DD, more water is conserved in soil, leading to reduced incidence of cereal foot rot, especially if caused by *F. culmorum*, which is enhanced by plant water stress. Govaerts*et al*. (2006) suggested that DD may decrease the effects of the disease by improving soil quality, water retention and microbial activity. In our research conditions, we suggest that both high temperature and water stress at flowering time both had major effects, increasing the incidence of the disease.

The severity of disease estimated by whiteheads appearing prematurely on durum wheat was greatly increased (97%) by DD in 2010/11, which is not in agreement with the results of Wildermuth *et al*. (1997). They found fewer whiteheads (4.3%) after DD,and more (19.3%) after reduced drilling and CD (12.2%) and *F. pseudograminearum* infection. Others have suggested that DD reduces the formation of whiteheads by inducing humid crop canopy atmospheres. Results from the present study can be explained by insufficient moisture at soil level, which was probably caused by high evapotranspiration in the high temperature recorded in the region.

DD did not increase disease severity, estimated by the brown discolourations on durum wheat stem bases. In addition, the grain yields were not affected.

CONCLUSIONS

Results of this study have shown that *F. culmorum* followed by *F. pseudograminearum* were the main pathogens recovered from stem bases and roots of the three cereals examined. The results also showed that for the three cereal types, DD increased incidence of *F. culmorum* infection by 58-60% in dry seasons, and by 32% in a wetter season. However, incidence of *F. pseudograminearum* infection increased by 80% in dry seasons under DD. In contrast to several other reports, DD increased disease severity by 97%, as expressed by whiteheads appearing in durum wheat prior to maturity. This may be attributed to the high temperatures and water stress at the experimental site.

There was no difference between DD and CD for disease severity, estimated by the discolouration of cereal stem bases.

In conservation agriculture, for cereal production, inputs are reduced, soil quality improves, and grain yields get close to, or slightly greater than, those from conventional agriculture. This is because there is more available water for the crop growth where conservation agriculture is applied. In the present study, no significant differences in grain yields from the three cereals were observed between DD and CD.

Given the high incidence of *F. culmorum* infections on durum wheat, oat and barley under DD observed in this study, further research is required at more sites over long periods, and in different farming systems. Furthermore, these studies should also consider host variety/species effects on diseases caused by *Fusarium* spp.

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Research Papers

Genetic variability, chemotype distribution, and aggressiveness of *Fusarium culmorum* on durum wheat in Tunisia

Safa OUFENSOU[1,2,*], Virgilio BALMAS[1], Barbara SCHERM[1], Domenico RAU[1], Salvatore CAMIOLO[1], Vanda Assunta PROTA[1], Mossadok BEN-ATTIA[2], Samia GARGOURI[3], Matias PASQUALI[4], Safia EL-BOK[6], Quirico MIGHELI[1,5]

[1] *Dipartimento di Agraria, Università degli Studi di Sassari, Via E. De Nicola 9, 07100 Sassari, Italy*
[2] *Laboratoire de Bio-surveillance de l'Environnement, Faculté des Sciences de Bizerte, Route de Tunis, 7021Zarzouna, Université de Carthage, Tunisia*
[3] *Laboratoire de Protection des Végétaux, Institut National de Recherche Agronomique de Tunisie, Rue Hédi Karray, 2049 Ariana, Tunisia*
[4] *DeFENS-Department of Food Environmental and Nutritional Sciences, Università degli Studi di Milano, Via Celoria,2, 20133 Milano, Italy*
[5] *Unità di ricerca-Istituto Nazionale di Biostrutture e Biosistemi, Università degli Studi di Sassari, 07100 Sassari, Italy*
[6] *Laboratoire de Biodiversité, Biotechnologies et Changements Climatiques, Faculté des Sciences de Tunis, Université Tunis-El Manar, 2092 Tunis, Tunisia*
*Corresponding author: safaoufensou@gmail.com

Abstract. *Fusarium culmorum* is the most commonly reported root rot pathogen in Tunisian durum wheat. Isolates of the pathogen from four durum wheat growing areas in the north of Tunisia were analyzed for their chemotypes. Two chemotypes were detected at unequal abundance (96% of 3-ADON and 4% of NIV). Distribution of a SNP mutation located at the position 34 bp after the first exon of the EF-1α partial sequence was analysed, to verify whether the haplotype was specifically associated to Fusarium root rot. A and T haplotypes were homogeneously distributed in three different Tunisian regions (Mateur, Beja and Bousalem) but not for the region of Bizerte, from which greatest number of A haplotype strains were detected. The isolates were tested for their virulence under glasshouse conditions, and a mean of 91% of crown and root infection was observed. Chemotype influenced virulence, but there was no significant influence of the geographical origin or haplotype on virulence. The distribution of three inter simple sequence repeats (ISSR) was examined, to better understand the structure of *F. culmorum* populations in Tunisia. A total of 27 fragments were obtained with eight polymorphic bands. Cluster analysis showed a high level of similarity between isolates. Analysis of molecular variance confirmed that there was little genetic differentiation among *F. culmorum* strains from different locations.

Keywords. *Fusarium* crown and root rots, population structure, trichothecenes.

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INTRODUCTION

Durum wheat is extensively grown in Tunisia, covering over 40% of the cereal-producing areas (Fakhfakh *et al.*, 2011). *Fusarium culmorum* is reported to be among the most prevalent pathogens responsible of foot and root rot (FRR) of durum wheat and other small grain cereal crops (Burgess *et al.*, 2001; Smiley *et al.*, 2005; Wagacha and Muthomi, 2007; Scherm *et al.*, 2013). FRR is particularly severe in areas affected by water stress, that are usually found in Tunisia (Gargouri *et al.*, 2001), in Southern Italy (Balmas *et al.*, 2006) and in Turkey (Tunali *et al.*, 2006), Iran (Eslahi, 2012) and Syria (El-Khalifeh *et al.*, 2009). *Fusarium culmorum* infects host plants at the initial growing stages, causing rotting of root and crown tissues. In some cases, lesions and browning of the coleoptiles or seedling death may occur. When *F. culmorum* infects wheat plants at later growing stages, brown spots on basal internodes can be observed. Under high humidity conditions, reddish-pink discolourations of the nodes appear due to the development of sporulating pathogen mycelium (Scherm *et al.*, 2013; Balmas *et al.*, 2015).

Fusarium culmorum causes serious problems since it causes yield reductions and mycotoxin accumulation. The pathogen produces type B trichothecenes (Smiley *et al.*, 2005; Miedaner *et al.*, 2008; Obanor *et al.*, 2010; Pasquali *et al.*, 2016). The compounds are harmful to humans and animals, resulting in cancer development and other generally irreversible effects (Bennett and Klich, 2003; Pestka and Smolinski, 2005). Type B trichothecenes are also considered as virulence factors through inhibition of host plant defence mechanisms (Wagacha and Muthomi, 2007; Scherm *et al.*, 2013). Increased virulence of *F. culmorum* strains is associated to their ability to contaminate plant tissues with high doses of trichothecenes, although this correlation has not always been confirmed (Gang *et al.*, 1998; Scherm *et al.*, 2011), or these compounds may have minor effects ith they are translocated from the basal portions of host plants (Winter *et al.*, 2013).

For the most important mycotoxins, the maximum permitted levels have been set, for grains and cereal-derived products used for human or livestock consumption (EC, 2006). However, in some cases, high levels of toxin and the possible interaction between concomitantly occurring mycotoxins, represent toxicological risks, and food safety is seriously hampered (Balmas *et al.*, 2015).

For *F. culmorum*, chemotypes have been recognized within the type B trichothecene mycotoxins (Pasquali *et al.*, 2016). Distinct chemotypes are recognized according to their production of deoxynivalenol (DON) and related derivatives or nivalenol (NIV) (Scherm *et al.*, 2013). Discrimination of DON and NIV may provide insight into the toxigenic potential of *F. culmorum* strains. To our knowledge, all the *F. culmorum* strains from wheat in Italy belong to 3-ADON (Quarta *et al.*, 2005; Covarelli *et al.*, 2014) apart from two NIV strains found in two Italian regions, Tuscany and Emilia-Romagna (Prodi *et al.*, 2010), and one isolate that was characterized as NIV in Sardinia (Balmas *et al.*, 2015). Search for chemotypes associated with FFR throughout the Middle East demonstrated that 100% of *F. culmorum* strains in Turkey belong to the 3-ADON chemotype (Yörük and Albayrak, 2012), while in Syria 55% of the strains were 3-ADON and 45% were NIV chemotypes (Alkadri *et al.*, 2013). In other surveys (Yörük and Albayrak 2012; Alkadri *et al.*, 2013; Mert-Türk and Gencer, 2013; Motallebi *et al.*, 2015) dominance of the *F. culmorum* 3-ADON chemotype was further highlighted.

The DON chemotype is most widely found (Scherm *et al.*, 2013), whereas NIV producers are less frequent in many European countries (Bakan *et al.*, 2001; Jennings *et al.*, 2004), and in Tunisia (Kammoun *et al.*, 2010; Rebib *et al.*, 2014), Turkey (Yörük and Albayrak, 2012) and the United States of America (Mirocha *et al.*, 1994).

The nucleotide sequence of the translation elongation factor 1-α (EF1-α) gene, encoding a part of a highly conserved ubiquitous protein involved in translation, was first used in fungi in *Fusarium* (O'Donnell *et al.*, 1998). As a single-locus identification tool, EF1-α shows a high level of sequence polymorphism among related species, hence it was considered a useful genetic region for phylogenetic and taxonomic studies, allowing reliable identification as an alternative to rDNA or β-tubulin (O'Donnell, 2000; Roger *et al.*, 1999).

Knowledge of chemotype distribution within *F. culmorum* populations originating from several agro-ecological areas in Tunisia would provide useful information on strain fitness in the field, representing a reliable resource for the development of effective disease control strategies (Strange and Scott, 2005).

The goals of the present study were: 1) to characterize a representative *F. culmorum* population isolated from FRR-affected durum wheat plants grown in different agro-ecological areas of Tunisia; 2) to assess genetic variability and population structure of Tunisian *F. culmorum* strains, based on EF1-α sequence polymorphism and ISSR markers; 3) to assess the virulence of *F. culmorum* strains on wheat plants to verify the hypothesis that a specific haplotype is associated with FRR; and 4) to examine the distribution of genetic chemotypes and gather information about the potential toxigenicity of

the fungal population that might contaminate durum wheat in Tunisia.

MATERIALS AND METHODS

Sampling, fungal isolation and isolate storage

FRR distribution on durum wheat was monitored during the 2015 growing season. A total of 88 fields were investigated in two climatic regions (sub humid and higher semi-arid) in Northern Tunisia (Bortoli *et al.*, 1969). These regions include more than 90% of the Tunisian wheat production areas (Figure 1). Sixty-eight fields were from the sub-humid region (Bizerte, Mateur and Beja), and 20 fields were from the higher semi-arid region (Bousalem). The fields, separated by approx. 10 km, were randomly selected. For each field in which FRR symptoms were observed, 20 plants were randomly collected along diagonal transects in different field zones. Plant samples were transferred in paper bags to the laboratory and stored at 4°C until analysed.

All fungal strains were obtained from the basal stems of diseased durum wheat plants. Fungal isolation was carried out according to Balmas *et al.* (2015), and monospore cultures were prepared as described by Burgess *et al.* (1994). All monospore strains collected were identified based on morphological traits, as described by Burgess *et al.* (1994).

For further analyses, all strains were stored at -80°C in 15% glycerol in the *Fusarium* collection of the Dipartimento di Agraria, University of Sassari, Italy.

Genomic DNA extraction and molecular characterization

Mycelia of fungal strains were each collected with a sterile spatula from PDA plates, after 5 d of incubation at 25°C in darkness. Genomic DNA was extracted from each isolate according to Aljanabi and Martinez (1997), and stored at 4°C. For each isolate, a partial sequence of the translation elongation factor 1α (EF1-α) was amplified (Balmas *et al.*, 2015), in a total of 50 µL reaction mixture containing: 10-25 µg of DNA template, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each of the primers TEF1 ATGGGTAAGGA(A/G)GACAAGAC and TEF2 GGA(G/A)GTACCAGT(G/C)ATCATGTT, 2 units *Taq* polymerase (Invitrogen). The PCR programme included one cycle at 98°C for 2 min, 35 cycles at 98°C for 15 s, 60°C for 15 s and 72°C for 30 s, followed by a final extension at 72°C for 5 min. PCR products were purified (PureLink™ Quick PCR Purification Kit, Invitrogen) following the manufacturer's instructions, and amplicon concentration was estimated with a fluorometer (Qubit™, Invitrogen), for sequencing optimisation. Sequencing was performed with 3500 Genetic Analyser (Life Technologies). For each isolate, both forward and reverse strands were sequenced and aligned using the multiple alignment program ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalo/). To confirm morphological identification, EF1-α partial sequences were compared with consensus sequences available in *Fusarium*-ID and GenBank databases (O'Donnell *et al.* 2012). Based on the polymorphic nucleotide (T–A) located at position 34 after the first exon of the EF1-α partial sequence gene (Balmas *et al.* 2010, 2015), a Single Nucleotide Polymorphism (SNP) was targeted. All the sequences were then deposited in GenBank (Table 1).

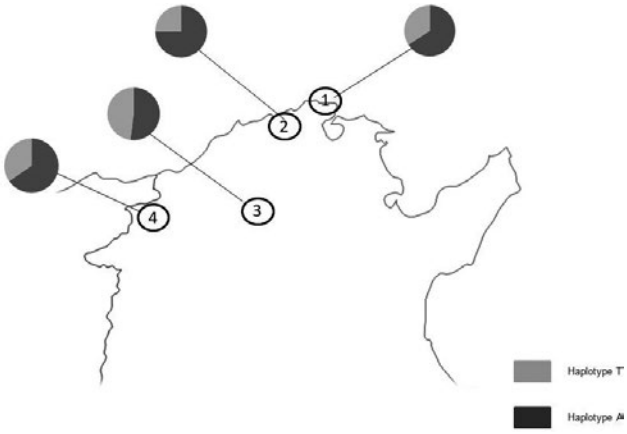

Figure 1. Map indicating sample sites in Tunisia where Fusarium isolates associated with root rot of wheat were obtained. Proportions (%) of haplotypes are indicated. Localities of Bizerte (1), Mateur (2), Beja (3) and Bousalem (4) are also indicated.

To determine the genetic chemotype of each isolate, a *Tri12* multiplex PCR was carried out according to Ward *et al.* (2002) using two different primer sets, 12CON/12NF CATGAGCATGGTGATGTC/TCTCCTCGTTGTATCTGG for NIV discrimination and 12CON/12-3F CATGAGCATGGTGATGTC/CTTTGGCAAGCCCGTGCA for DON discrimination. PCR conditions were performed at 98°C (2 min) for one cycle; 98°C (10 s), 59°C (10 s), and 72°C (20 s) for 30 cycles; and a final cycle of 72°C for 5 min.

Genetic variability among the *F. culmorum* isolates was evaluated by an ISSR-PCR assay, using three different ISSR primers (Table 3). PCR conditions and annealing temperature were modified according to the primer sequence: 48°C for ISSR4 [$(GAGG)_3GG$; Albayrak *et al.* 2016]; and 55°C for both ISSR5 [$(AG)_9G$)] and ISSR6 [$(AC)_8YG$; Mishra *et al.* 2003]. PCR products were visualized on agarose gel (1.5% agarose) after electrophoretic

Table 1. Locations, chemotypes, Single nucleotide polymorphisims (SNP), mean aggressiveness scores and GenBank accession numbers for *Fusarium* isolates associated with Fusarium root rot of wheat in Tunisia.

Isolate	Location	Coordinates	Chemotype[a]	SNP[b]	Aggressiveness[c] Mean ± SD	EF-1α GenBank Accession No.
Fu-BI87	Bizerte	N37 12.423 E940.384	3-ADON	A	90.00 ± 31.6 [abc]	MF510992
Fu-BI84	Bizerte	N37 12.423 E940.384	3-ADON	T	77.50 ± 41.6 [abc]	MF510990
Fu-BI151	Bizerte	N37 13.964 E9 42.388	3-ADON	A	95.00 ± 15.81 [ab]	MF511031
Fu-BI169	Bizerte	N37 13.964 E9 42.388	3-ADON	T	50.00 ± 44.1 [cde]	MF511041
Fu-BI3	Bizerte	N36 36.677 E8 40.158	3-ADON	A	95.00 ± 15.81 [ab]	MF510936
Fu-BI36	Bizerte	N36 36.677 E8 40.158	3-ADON	A	100.0 ± 0.0 [a]	MF510958
Fu-BI135	Bizerte	N36 36.677 E8 40.158	3-ADON	A	77.50 ± 36.2 [abc]	MF511027
Fu-BI33	Bizerte	N37 14.416 E9 43.842	3-ADON	T	100.0 ± 0.0 [a]	MF510955
Fu-BI42	Bizerte	N37 14.416 E9 43.842	3-ADON	A	100.0 ± 0.0 [a]	MF510963
Fu-BI50	Bizerte	N37 14.416 E9 43.842	3-ADON	T	82.50 ± 33.4 [abc]	MF510969
Fu-BI54	Bizerte	N37 14.416 E9 43.842	3-ADON	T	85.00 ± 31.6 [abc]	MF510971
Fu-BI58	Bizerte	N37 14.416 E9 43.842	3-ADON	A	62.50 ± 37.7 [abcd]	MF510975
Fu-BI105	Bizerte	N37 14.416 E9 43.842	3-ADON	T	100.0 ± 0.0 [a]	MF511008
Fu-BI120	Bizerte	N37 14.416 E9 43.842	3-ADON	T	85.00 ± 31.6 [abc]	MF511017
Fu-BI123	Bizerte	N37 14.416 E9 43.842	3-ADON	A	100.0 ± 0.0 [a]	MF511019
Fu-BI13	Bizerte	N37 08.734 E9 46.500	3-ADON	A	55.00 ± 42.2 [bcde]	MF510943
Fu-BI15	Bizerte	N37 08.734 E9 46.500	NIV	A	65.00 ± 39.4 [abcd]	MF510944
Fu-BI4	Bizerte	N37 13.964 E9 42.390	3-ADON	A	92.50 ± 23.72 [ab]	MF510937
Fu-BI118	Bizerte	N37 13.964 E9 42.390	3-ADON	A	90.00 ± 24.15 [abc]	MF511015
Fu-BI67	Bizerte	N37 14.416 E9 43.842	3-ADON	A	87.50 ± 24.30 [abc]	MF510980
Fu-BI150	Bizerte	N37 14.416 E9 43.842	3-ADON	A	90.00 ± 31.6 [abc]	MF511030
Fu-BI170	Bizerte	N37 14.416 E9 43.842	3-ADON	A	100.0 ± 0.0 [a]	MF511042
Fu-BI183	Bizerte	N37 14.416 E9 43.842	3-ADON	A	100.0 ± 0.0 [a]	MF511046
Fu-BI6	Bizerte	N37 06.858 E9 46.944	NIV	T	77.50 ± 41.6 [abc]	MF431609
Fu-BI8	Bizerte	N37 06.858 E9 46.944	NIV	T	72.50 ± 39.9 [abc]	MF510939
Fu-BI28	Bizerte	N37 06.858 E9 46.944	NIV	T	75.00 ± 40.8 [abc]	MF510951
Fu-BI161	Bizerte	N37 07.868 E9 45.396	3-ADON	T	75.00 ± 28.87 [abc]	MF511039
Fu-BI159	Bizerte	N37 07.868 E9 45.396	3-ADON	A	100.0 ± 0.0 [a]	MF511037
Fu-BI152	Bizerte	N37 07.868 E9 45.396	3-ADON	A	90.00 ± 31.6 [abc]	MF511032
Fu-BI149	Bizerte	N37 07.868 E9 45.396	3-ADON	A	100.0 ± 0.0 [a]	MF511029
Fu-BI128	Bizerte	N37 07.868 E9 45.396	3-ADON	A	100.0 ± 0.0 [a]	MF511023
Fu-BI103	Bizerte	N37 07.868 E9 45.396	3-ADON	A	100.0 ± 0.0 [a]	MF511006
Fu-BI35	Bizerte	N37 07.868 E9 45.396	3-ADON	A	100.0 ± 0.0 [a]	MF510957
Fu-BI9	Bizerte	N37 10.539 E9 44.507	3-ADON	A	85.00 ± 33.7 [abc]	MF510940
Fu-BI10	Bizerte	N37 10.539 E9 44.507	3-ADON	T	100.0 ± 0.0 [a]	MF510941
Fu-BI19	Bizerte	N37 10.539 E9 44.507	3-ADON	A	100.0 ± 0.0 [a]	MF510946
Fu-BI22	Bizerte	N37 10.539 E9 44.507	3-ADON	A	100.0 ± 0.0 [a]	MF510948
Fu-BI25	Bizerte	N37 10.539 E9 44.507	3-ADON	A	100.0 ± 0.0 [a]	MF510949
Fu-BI27	Bizerte	N37 10.539 E9 44.507	3-ADON	A	100.0 ± 0.0 [a]	MF510950
Fu-BI30	Bizerte	N37 10.539 E9 44.507	3-ADON	A	87.50 ±27.00 [abc]	MF510952
Fu-BI76	Bizerte	N37 10.539 E9 44.507	3-ADON	T	100.0 ± 0.0 [a]	MF510984
Fu-BI90	Bizerte	N37 10.539 E9 44.507	3-ADON	T	95.00 ± 15.81 [ab]	MF510994
Fu-BI140	Bizerte	N37 10.539 E9 44.507	3-ADON	A	100.0 ± 0.0 [a]	MF511028
Fu-BI156	Bizerte	N37 10.539 E9 44.507	3-ADON	T	100.0 ± 0.0 [a]	MF511036
Fu-BI98	Mateur	N37 08.774 E9 57.402	3-ADON	A	100.0 ± 0.0 [a]	MF511001
Fu-BI117	Mateur	N37 08.774 E9 57.402	3-ADON	A	100.0 ± 0.0 [a]	MF511014
Fu-BI127	Mateur	N37 08.774 E9 57.402	3-ADON	A	100.0 ± 0.0 [a]	MF511022

(Continued)

Table 1. (Continued).

Isolate	Location	Coordinates	Chemotype[a]	SNP[b]	Aggressiveness[c] Mean ± SD	EF-1α GenBank Accession No.
Fu-MA1	Mateur	N37 08.862 E9 33.401	3-ADON	A	100.0 ± 0.0 [a]	MF431610
Fu-MA12	Mateur	N37 08.862 E9 33.401	3-ADON	T	100.0 ± 0.0 [a]	MF510942
Fu-BI21	Mateur	N37 08.862 E9 33.401	3-ADON	A	16.00 ±16.80 [e]	MF510947
Fu-BI97	Mateur	N37 08.862 E9 33.401	3-ADON	A	92.50 ± 23.72 [ab]	MF511000
Fu-BI100	Mateur	N37 08.862 E9 33.401	3-ADON	T	97.50 ± 7.91 [a]	MF511003
Fu-BI112	Mateur	N37 08.862 E9 33.401	3-ADON	A	77.50 ± 36.2 [abc]	MF511013
Fu-MA95	Mateur	N37 08.862 E9 33.400	3-ADON	A	95.00 ±15.81 [ab]	MF510998
Fu-MA101	Mateur	N37 08.862 E9 33.400	3-ADON	A	100.0 ± 0.0 [a]	MF511004
Fu-BI107	Mateur	N37 10.729 E9 45.649	3-ADON	T	100.0 ± 0.0 [a]	MF511010
Fu-BE31	Beja	N36 42.475 E9 10.687	3-ADON	A	95.00 ± 15.81 [ab]	MF510953
Fu-BE32	Beja	N36 42.475 E9 10.687	3-ADON	T	95.00 ± 15.81 [ab]	MF510954
Fu-BE48	Beja	N36 42.475 E9 10.687	3-ADON	A	80.00 ± 42.2 [abc]	MF510967
Fu-BE55	Beja	N36 42.475 E9 10.687	3-ADON	A	100.0 ± 0.0 [a]	MF510972
Fu-BE69	Beja	N36 42.475 E9 10.687	3-ADON	T	82.50 ± 37.4 [abc]	MF510981
Fu-BE78	Beja	N36 42.475 E9 10.687	3-ADON	T	95.00 ± 15.81 [ab]	MF510985
Fu-BE106	Beja	N36 42.475 E9 10.687	3-ADON	A	100.0 ± 0.0 [a]	MF511009
Fu-BE129	Beja	N36 42.475 E9 10.687	3-ADON	A	92.50 ± 16.87 [ab]	MF511024
Fu-BE131	Beja	N36 42.475 E9 10.687	3-ADON	T	67.50 ± 44.2 [abcd]	MF511025
Fu-BE132	Beja	N36 42.475 E9 10.687	3-ADON	T	100.0 ± 0.0 [a]	MF511026
Fu-BE154	Beja	N36 42.475 E9 10.687	3-ADON	T	95.00 ± 15.81 [ab]	MF511034
Fu-BE162	Beja	N36 42.475 E9 10.687	3-ADON	A	100.0 ± 0.0 [a]	MF511040
Fu-BE171	Beja	N36 42.475 E9 10.687	3-ADON	A	100.0 ± 0.0 [a]	MF511043
Fu-BE180	Beja	N36 42.475 E9 10.687	3-ADON	A	100.0 ± 0.0 [a]	MF511044
Fu-BE182	Beja	N36 42.475 E9 10.687	3-ADON	T	100.0 ± 0.0 [a]	MF511045
Fu-BE37	Beja	N36 38.159 E9 06.458	3-ADON	A	9750 ± 7.91 [a]	MF510959
Fu-BE38	Beja	N36 38.159 E9 06.458	3-ADON	A	100.0 ± 0.0 [a]	MF510960
Fu-BE41	Beja	N36 38.159 E9 06.458	3-ADON	A	100.0 ± 0.0 [a]	MF510962
Fu-BE52	Beja	N36 38.159 E9 06.458	3-ADON	A	80.00 ± 36.9 [abc]	MF510970
Fu-BE60	Beja	N36 38.159 E9 06.458	3-ADON	A	92.50 ± 23.72 [ab]	MF510977
Fu-BE63	Beja	N36 38.159 E9 06.458	3-ADON	T	92.50 ± 12.08 [ab]	MF510979
Fu-BE72	Beja	N36 38.159 E9 06.458	3-ADON	A	90.00 ± 31.6 [abc]	MF510982
Fu-BE75	Beja	N36 38.159 E9 06.458	3-ADON	T	97.50 ± 7.91 [a]	MF510983
Fu-BE83	Beja	N36 38.159 E9 06.458	3-ADON	T	100.0 ± 0.0 [a]	MF510989
Fu-BE104	Beja	N36 38.159 E9 06.458	3-ADON	T	92.50 ± 23.72 [ab]	MF511007
Fu-BE126	Beja	N36 38.159 E9 06.458	3-ADON	T	92.50 ± 16.87 [ab]	MF511021
Fu-BO44	Bousalem	N36 34.444 E8 54.849	3-ADON	T	100.0 ± 0.0 [a]	MF510964
Fu-BO46	Bousalem	N36 34.444 E8 54.849	3-ADON	A	90.00 ± 31.6 [abc]	MF510965
Fu-BO47	Bousalem	N36 34.444 E8 54.849	3-ADON	A	100.0 ± 0.0 [a]	MF510966
Fu-BO56	Bousalem	N36 34.444 E8 54.849	3-ADON	A	75.00 ± 31.18 [abc]	MF510973
Fu-BO57	Bousalem	N36 34.444 E8 54.849	3-ADON	A	85.00 ± 31.6 [abc]	MF510974
Fu-BO59	Bousalem	N36 34.444 E8 54.849	3-ADON	A	100.0 ± 0.0 [a]	MF510976
Fu-BO79	Bousalem	N36 34.444 E8 54.849	3-ADON	T	77.50 ± 27.51 [abc]	MF510986
Fu-BO80	Bousalem	N36 34.444 E8 54.849	3-ADON	T	100.0 ± 0.0 [a]	MF510987
Fu-BO82	Bousalem	N36 34.444 E8 54.849	3-ADON	A	100.0 ± 0.0 [a]	MF510988
Fu-BO85	Bousalem	N36 34.444 E8 54.849	3-ADON	A	100.0 ± 0.0 [a]	MF510991
Fu-BO91	Bousalem	N36 34.444 E8 54.849	3-ADON	A	85.00 ± 33.7 [abc]	MF510995
Fu-BO102	Bousalem	N36 34.444 E8 54.849	3-ADON	A	100.0 ± 0.0 [a]	MF511005
Fu-BO110	Bousalem	N36 34.444 E8 54.849	3-ADON	A	100.0 ± 0.0 [a]	MF511011

(Continued)

Table 1. (Continued).

Isolate	Location	Coordinates	Chemotype[a]	SNP[b]	Aggressiveness[c] Mean ± SD	EF-1α GenBank Accession No.
Fu-BO111	Bousalem	N36 34.444 E8 54.849	3-ADON	A	97.50 ± 7.91 [a]	MF511012
Fu-BO119	Bousalem	N36 34.444 E8 54.849	3-ADON	A	100.0 ± 0.0 [a]	MF511016
Fu-BO153	Bousalem	N36 34.444 E8 54.849	3-ADON	T	90.00 ± 31.6 [a]	MF511033
Fu-BO155	Bousalem	N36 34.444 E8 54.849	3-ADON	A	95.00 ± 15.81 [a]	MF511035
Fu-BO160	Bousalem	N36 34.444 E8 54.849	3-ADON	A	92.50 ± 23.72 [ab]	MF511038
Fu-BO2	Bousalem	N36 34.444 E8 54.900	3-ADON	T	90.00 ± 31.6 [abc]	MF510935
Fu-BO7	Bousalem	N36 34.444 E8 54.900	3-ADON	T	100.0 ± 0.0 [a]	MF510938
Fu-BO16	Bousalem	N36 34.444 E8 54.900	3-ADON	A	92.50 ± 23.72 [ab]	MF510945
Fu-BO34	Bousalem	N36 30.263 E8 47.347	3-ADON	T	95.00 ± 15.81 [ab]	MF510956
Fu-BO92	Bousalem	N36 37.668 E8 54.874	3-ADON	T	100.0 ± 0.0 [a]	MF510996

[a] 3-acetyldeoxynivalenol, 3-ADON; nivalenol, NIV.
[b] Single nucleotide polymorphism (SNP) at position 34 after first exon of EF-1α (Balmas *et al.* 2010).
[c] Data followed by the same letters are not significantly different from each other (Tukey test).

Table 2. Statistical significance of impacts of location, chemotype or haplotype on pathogenicity of *Fusarium* isolates associated with root rot of wheat.

Variable	Number	Degrees of freedom	P-value
Location		3	0.32
Bizerte	440		
Mateur	120		
Beja	250		
Bousalem	230		
Chemotype		1	0.00
3-ADON	1000		
NIV	40		
Haplotype		1	0.87
A	660		
T	380		

Table 3. ISSR primers used in this study.

Primer	Sequence	(P/M)[a]	Reference
ISS4	$(GAGG)_3GG$	(4/12)	Albayrak *et al.* (2016)
ISSR5	$(AG)_9G$	(0/5)	Mishra *et al.* (2003)
ISSR6	$(AC)_8YG$	(4/10)	Mishra *et al.* (2003)

[a] *P* number of polymorphic markers with polymorphism level above 2%; M monomorphic markers.

separation in 1 × TAE buffer of 1 h for ISSR5 and ISSR6, or 2 h for ISSR4. Fragment size was estimated by comparison with a 1 Kb Plus DNA Ladder (Invitrogen). Gel images were analysed using a Gel-Doc XR+ System (Bio-Rad). ISSR markers were visually scored as presence (1) or absence (0) of each band.

Pathogenicity experiments

For each *F. culmorum* isolate, agar plugs (8 mm diam.) covered with mycelium were cut from actively growing colony margins of 5-d-old cultures grown on PDA, and were used to inoculate durum wheat seeds. A single durum wheat seed (cv. Saragolla) was deposited on each mycelial plug and was planted in the centre of a plastic pot containing sterilized potting mix composed of equal proportions peat soil, sand and redwood soil. The pots were incubated on a bench in a glasshouse for 20 d, with day and night temperatures of 25-30 °C and 18-25 °C, and with daily irrigation. For each isolate, ten replicates were established. According to, After 3 weeks, disease severity index was assessed using an empirical scale of five disease severity classes of disease severity (Balmas *et al.*, 2006), where 0 = no browning; 1 = 1 to 25%; 2 = 50%; 3 = 75%; and 4 = no plant emergence). For each plant, stem browning were also assessed.

Statistical analyses

Disease severity data were analysed using Minitab version 17.1.0 software. *Post hoc* analyses (Tukey's HSD test of multiple comparisons) were subsequently performed considering 95% confidence level.

A phylogenetic tree was constructed from pairwise distance matrix by UPGMA, applying MEGA version 7.0 software. Bootstraps analysis with 1,000 replicates was carried out to estimate the statistical support for differ-

ent tree branches, and the number on each branch represented the bootstrap value.

Analysis of molecular variance (AMOVA), provided by the Arlequin version 3.5.1.2 software, using 1,023 permutations, was used to calculate the variance within the *F. culmorum* collection, based on ISSR molecular markers.

RESULTS

Distribution of Fusarium culmorum isolates in Tunisia, and their molecular chemotyping

A total of 104 *F. culmorum* isolates were obtained from the basal stems of symptomatic durum wheat plants. The greatest number of isolates (44) was collected from the the sub-humid region of Bizerte.

Among the 104 *F. culmorum* isolates 100 (96%) were of the 3-ADON chemotype, and were obtained from all the Tunisian regions considered. Only four isolates were ascribed to the NIV chemotype, and all NIV chemotype isolates were collected from two fields of the sub-humid region of Bizerte

EF1-α haplotype distribution

The identification of both SNPs (A or T) of the EF1-α partial sequence of the *F. culmorum* isolates confirmed the presence of the two haplotypes within the *F. culmorum* population. Both haplotypes were homogenously and equally distributed in the region of Beja, whereas the A haplotype was dominant in the other three tested regions (Figure 1).

ISSR marker analysis

Based on three ISSR primers (ISSR4, ISSR5, and ISSR6), 27 bands were scored, and ranged from 0.39 to 2.6 Kb. The most informative primer was ISSR4 (12 amplicons). Bootstrapping gave values less than 50%. The cluster analysis with the UPGMA using pairwise genetic distances indicated high similarity between the *F. culmorum* isolates (Figure 2). Results from AMOVA provided an estimated pattern of population differentiation. All ISSR variation was distributed among isolates within populations (98.3% of the total variance). A Small proportion (1.7%) of the variability was explained by differences between populations and was not statistically significant ($P = 0.12$; Table 4). In addition, no statistically significant correlations were detected between cluster-

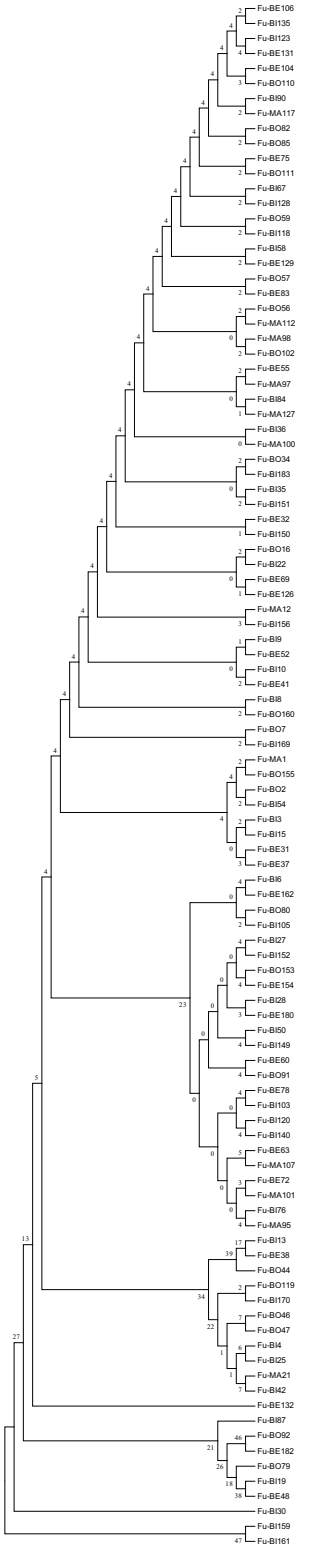

Figure 2 UPGMA analysis of *Fusarium culmorum* populations, based on combined ISSR data analyzed using using pairwise distances matrix. The numbers on the branches represent bootstrap support values.

Table 4 Analysis of molecular variance of ISSR for 104 isolates of *Fusarium culmorum* from four Tunisian populations.

Source of variation	*d.f*[a]	*SSD*[b]	Variance components	Percentage of variation
Among population	*3*	3.142	0.01282 Va	1.71
Within population	99	73.052	0.73790 Vb	98.29
Total	102	76.194	0.75072	
Va and FST : *P* (rand. value > obs. value) = 0.12121 *P* (rand. value = obs. value) = 0.00000 *P*-value = 0.12121±0.01052				

[a] *df* degrees of freedom;
[b] *SSD* sums of squared deviations;
[c] *P* probability of obtaining equal or large value determined by1023 randomizations of the treatments.

ing analyses, virulence, chemotype and haplotype of the isolates.

Aggressiveness of Fusarium culmorum *isolates*

In the pathogenicity test, root symptoms caused by *F. culmorum* isolates were detected in almost all cases (91%), with severity values ranging from 16 to 100%. A highly significant effect of chemotype on isolate aggressiveness was observed, with the 3-ADON isolates being more aggressive than the NIV isolates ($P = 0.007$; Table 1). However, few NIV chemotype strains were analyzed, which did not allow robust statistical analysis. In contrast, no statistically significant effects were detected for geographical origin ($P = 0.324$) or haplotype ($P = 0.877$) on isolate aggressiveness.

DISCUSSION

Fusarium culmorum is reported as the predominant cause of FRR disease in Tunisia, since incidence of the pathogen is promoted by dry springs and irregular rainfall (Gargouri *et al*., 2001). Pedo-climatic conditions in wheat-producing Tunisian regions are very similar to those in Sardinia (Balmas *et al*., 2015), and in Turkey (Tunali *et al*., 2008), where *F. culmorum* is the most common species causing FRR.

The present research aimed to investigate the genetic variability and the structure of *F. culmorum* populations associated with FRR on durum wheat in Tunisia. *F. culmorum* strains were collected from different wheat production areas, showing different climatic conditions. Two distinct chemotypes (3-ADON and NIV) were distinguished in the Tunisian populations of *F. culmorum*, the 3-ADON chemotype was the most common. This confirms previous reports by Rebib *et al*. (2014) on two fields showing FRR symptoms. The NIV chemotype was detected for the first time as associated to FRR in Tunisia, although this chemotype was found only in two fields in the Bizerte region.

Based on chemotype characterization of Italian *Fusarium* species, Covarelli *et al*. (2015) suggested that climatic conditions may strongly affect the occurrence of 3-ADON and 15-ADON, whereas NIV contamination may occur regardless of climatic conditions. Kammoun *et al*. (2010) reported that most isolates (98%) causing Fusarium head blight were 3-ADON producers, while 2% were NIV producers and originated from Bizerte, results which agree with those reported here. However, further investigations are required to better understand if specific agronomic or environmental conditions favour the presence of NIV-chemotype strains (Beyer *et al*., 2014).

While previous reports from Tunisia have focused on FHB chemotyping (Bensassi *et al*., 2009; Kammoun *et al*., 2010), the present study represents the first geographic survey in different areas of Tunisia, and of the chemotypes of *F. culmorum* causing FRR on durum wheat. These results are in agreement with previous studies carried out in northern Mediterranean countries, including Italy, France, Portugal and Yugoslavia (Logrieco *et al*., 2003; Pasquali and Migheli, 2014), as well as Germany and the United Kingdom (Tóth *et al*., 2004; Jennings *et al*., 2004). These reports confirm that the DON chemotype predominates among *F. culmorum* strains recovered from cereal grains.

Genetic chemotyping is an essential tool for characterizing *F. culmorum* populations causing root rot on wheat, but the presence of an amplification product reflects the possibility of a particular toxin being produced, whereas biosynthesis of the toxin remains to be confirmed by chemical analysis.

In our previous study of *F. culmorum* strains collected in the Sardinian region, association of haplotype A (EF1-α polymorphism) with FRR was highlighted (Balmas *et al*., 2015). To verify this association, an *ad hoc* survey on FRR-causing isolates from Tunisia was carried out. In this study, both A and T haplotypes were observed within the Tunisian *F. culmorum* population. No significant association was found between A-haplotype and FRR. It is therefore evident that, at least in Tunisia, the presence of the two haplotypes is not associated with specific fitness or virulence advantage in FRR pathogens on wheat.

ISSR markers were used to assess the genetic variability of *F. culmorum* populations in Tunisia, aiming

to verify the findings of Rebib *et al.* (2014) over a large geographic area. They suggested a high level of similarity among populations comparing two Tunisian fields. The present data confirmed that no clear trends were apparent in the distribution of the genetic variability with regard to the geographic origin within Tunisia. Our observations are in agreement with those reported by Albayrak *et al.* (2016), who showed similarity coefficients of 65.7-94.3% among *F. culmorum* isolates using ISSR4 and 41 other primers. The present report also confirms a previous study on the distribution of RAPD markers, suggesting that the low level of genetic differentiation among Tunisian populations of *F. culmorum* is mostly interpreted as the outcome of asexual reproduction in this pathogen (Gargouri *et al.* 2003). Similarly, Gargouri *et al.* (2003) indicated that no structuring had been observed at small or large geographic scales in this fungal species. Based on this assumption, the lack of a geographic structure in Tunisian populations of *F. culmorum* also suggests that spore dispersal probably occurs over a wide geographic area. These factors probably influence the level of genetic diversity within populations.

Using three markers, two of which (ISSR5 and ISSR6) were the same used in the present work, Mishra *et al.* (2003) found that 81% of the ISSR bands were polymorphic among *F. culmorum* populations: ISSR5 generated 28 ISSR distinct genotypes and ISSR6 produced 22 genotypes among 75 examined isolates. Moreover, the pattern of genetic diversity was largely associated to the geographical origin of the isolates (Mishra *et al.* 2003). In our study, no clear spatial clustering or relationships between variability and geographical regions were observed. Other genotyping assays have failed to show clear correlation between genetic variability and the geographic origin, mostly because the tested populations were from limited agro-ecological areas (Gargouri *et al.* 2003; Mishra *et al.* 2003).

The current survey provides a first insight into the genetic diversity of the *F. culmorum* population causing FRR in the main durum wheat growing regions of Tunisia. Further analyses with greater numbers of *F. culmorum* isolates from throughout Tunisia are warranted. Nonetheless, these preliminary data provide knowledge at the country scale on chemotyping and haplotyping, as an aid to ensure food safety monitoring, and for development of effective disease prevention and control strategies.

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Virulence factors of *Fusarium* spp., causing wheat crown and root rot in Iran

Saeedeh DEHGHANPOUR-FARASHAH, Parissa TAHERI*, Mahrokh FALAHATI-RASTEGAR

Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, P.O.Box: 91775-1163, Mashhad, Iran
*Corresponding author: p-taheri@um.ac.ir

Abstract. Crown and root rot of wheat, caused by *Fusarium* spp., limit crop yields worldwide, from rotting of seeds, seedlings, crowns, roots and basal plant stems. Virulence factors and virulence and aggressiveness of *Fusarium* spp. were investigated for isolates from Iran, obtained from wheat plants with crown and root rot symptoms. Forty isolates of *Fusarium* were used in this research. Among the isolates, nivalenol (NIV) was detected as the dominant trichothecene chemotype produced. Production of trichothecenes and zearalenone (ZEA) in autoclaved rice cultures of *Fusarium* isolates was analyzed using high performance liquid chromatography. The levels of NIV ranged from 258 to 1246 μg kg^{-1}, of deoxynivalenol (DON) from 45 to 1411 μg kg^{-1}, and of ZEA from 53 to 3220 μg kg^{-1}. All *Fusarium* isolates produced cellulase and pectinase enzymes. Positive correlation was observed between activity of cell wall degrading enzymes (CWDEs) produced by the isolates and their pathogenicity on wheat leaf segments. Virulence of trichothecene-producing isolates was greater than that of non-trichothecene-producing isolates. Considerable association was detected between the capability of *Fusarium* spp. isolates to produce virulence factors (such as mycotoxins and CWDEs) and their pathogenicity on wheat.

Keywords. Cell wall degrading enzymes, mycotoxins.

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INTRODUCTION

Crown and root rot of wheat, caused by several *Fsarium* species, occur in most cereal producing regions of the world, including Europe, Australia, North America, South America, West Asia, South Africa and North Africa (Smiley *et al*., 1996; Paulitz *et al*., 2002; Smiley *et al*., 2005). In Iran, several *Fusarium* spp. have been isolated from crown and root rot symptoms in wheat growing regions (Besharati Fard *et al*., 2017). Fusaria produce a diverse array of toxic secondary metabolites (mycotoxins), which are involved in pathogenicity of these fungi to host plants. The most important *Fusarium* mycotoxins are trichothecenes and zearalenone, which can contaminate agricultural products, making them unsuitable for food or feed (Ma *et al*., 2013).

Trichothecenes are sesquiterpenoid molecules of which many variants are known. These have been chategorized as type A, including T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS), monoacetoxyscirpenol (MAS), and type B, including deoxynivalenol (DON, vomitoxin), nivalenol (NIV) and their mono- and di-acetylated derivatives (Yli-Mattila, 2010).

The ability of *Fusarium* spp. to produce particular mycotoxins can be investigated using biochemical and molecular techniques. Molecular techniques are based on detection of different gene clusters involved in production of mycotoxins. Chemotype determination can be performed with primers for several genes involved in trichothecene biosynthesis such as *Tri3*, *Tri5, Tri7*, *Tri12* and *Tri13* (Mahmoud and Shehata, 2017). The type of trichothecene produced by an isolate can be predicted based on genetic markers derived from the *Tri* gene cluster, containing the genes involved in tricothecene biosynthesis. Different mycotoxins have different toxicological properties. NIV is more toxic than DON to humans and domestic animals. Trichothecenes such as DON are potent inhibitors of eukaryotic protein biosynthesis (Van der Lee *et al*., 2015). Trichothecenes also play important roles as virulence factors in fungal pathogenesis.

Based on several previous reports, different types of trichothecene chemotypes are produced by *F. graminearum* and *F. culmorum*, and possibly by other *Fusarium* species (Khaledi et al. 2017, Desjardins et al. 1993; Sarver et al. 2011; Li et al. 2016). DON is one of the factors influencing virulence and aggressiveness of *Fusarium* spp. Some studies have reported that chemotype diversity depends on geographical distribution. Both DON and NIV chemotypes are reported from several countries in Asia, Africa, Europe, South and North America. The DON chemotype is reported as the major trichothecene chemotype present in North America, while the NIV chemotype has not been detected in this region. The NIV chemotype was most frequently isolated from some Asian and European countries (Gilbert *et al*., 2002; Zeller *et al*., 2003; Panthi *et al*., 2014).

Among the *Fusarium* isolates, NIV, 3-ADON and 15-ADON chemotypes were detected from different fields of Mazandaran and Golestan provinces in the northern region of Iran (Haratian *et al*., 2008; Malihipour *et al*., 2012), while 15-ADON was the only chemotype detected among the isolates collected from fields of Ardabil province in the North West of Iran (Malihipour *et al*., 2012; Davari *et al*., 2013). Among *Fusarium* isolates from fields of Golestan province in the North of Iran, the NIV genotype occurred more frequently, followed by 3-acetyl deoxynivalenol (3-ADON) and 15-acetyl deoxynivalenol (15-ADON) genotypes (Khaledi *et al*., 2016).

DON is the most predominant mycotoxin associated with progress of disease in host plants (Mirocha et al. 2003; Taheri 2018). DON is more phytotoxic on cereals than NIV (Desjardins 2006), disrupting normal cell function by inhibiting protein biosynthesis, and is a major virulence factor of *Fusarium* spp. pathogenic on cereals (Khaledi et al. 2017; Yu et al. 2008; Zhang et al. 2010).

Zearalenone (ZEA), a polyketide mycotoxin, has chronic estrogenic effects on mammals, causing reproductive problems in farm-raised pigs, experimental animals, livestock and humans (Gaffoor and Trail, 2006). ZEA can be produced pre- or post-harvest in maize and other cereals. Contaminations to ZEA by *Fusarium* spp. in maize, wheat and barley were reported from North, West and South of Iran (Karami-Osboo and Mirabolfathy, 2008; Ehsani *et al*., 2014). Production of ZEA by *F. acuminatum*, *F. crookwellense*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. sporotrichioides* and *F. semitectum* have been reported (Jiménez *et al*., 1996; Ezekiel *et al*., 2008).

The other mechanism involved in aggressiveness and virulence of *Fusarium* spp. is production of extracellular enzymes, which degrade host plant cell walls. These cell wall degrading enzymes (CWDEs), such as cellulases and pectinases, are crucial in the processes of colonization and disease establishment (Wanyoike *et al*., 2002; Kikot *et al*., 2010). The CWDEs are involved in softening the cell walls, increasing accessibility of cell wall components for degradation by other enzymes, which enables success of further infection steps and spread of fungal mycelia into the inner host plant tissues (Roncero *et al*., 2003; Ortega *et al*., 2013).

The objectives of the present study were: (i) to investigate the capability of *Fusarium* spp. isolates (obtained from wheat plants with crown and root rot symptoms) for producing various virulence factors such as trichothecenes, ZEA and CWDEs such as cellulase and pectinase; and (ii) to examine pathogenicity and aggressiveness of *Fusarium* isolates on wheat, and determine relationships between ability of the isolates to produce virulence factors and their pathogenicity.

MATERIALS AND METHODS

Fungal inoculum preparation

Forty isolates, belonging to eight species of *Fusarium*, were obtained from wheat plants showing symptoms of crown and root rot in Yazd province of Iran. The isolates were deposited in the fungal collection in Ferdowsi University of Mashhad, Iran (Table 1). Fun-

Table 1. Capability of *Fusarium* spp. isolates obtained from wheat plants for production of pectinase and cellulase, based on quantitative (μg mL^{-1}) and qualitative (mm) analyses, together with virulence and aggressiveness traits of *Fusarium* spp. isolates on inoculated wheat leaf segments. Virulence was evaluated on the leaf segments at 7 d post inoculation.

Isolate code	Maximum of enzyme activity (μg mL^{-1})		Zone diameter (mm)		Virulence on leaf segments (LL[a], mm)	Aggressiveness on leaf segments (hpi[b])
	Pectinase±SE	Cellulase±SE	Pectinase on pectin agar media±SE	Cellulase on CMC agar media±SE		
Ab1	4028±89l mn [c]	1129.5±20.5 abc	6.6±0.8 efg	7.3±0.3 fg	11±2.3 def	24
Ab2	3865±1 no	674±28 kl	5±0.1 ij	3.6±0.3 lm	6±0.5 j	96
Ab3	3727±10.5 rst	735.5±23.5 ij	5±1.1 ij	4.6±0.8 jkl	7.6±0.6 gh	96
Ar1	4309±86 gh	1087.5±27.5 abcde	8.3±0.3 bcde	8.3±0.3 cd	12.6±2.4 de	36
Ar2	4264±49 ghijkl	1010±10 bc	7.3±0.8 de	6±0.5 hi	10±0.1 def	48
As1	4221±207.5 hi	954±31 efg	7.6±0.8 cd	7±0.5 gh	9±2 ef	36
As2	5218±28 bc	1139.5±4.5 abc	12.3±1.3 a	10.3±0.3 ab	25±1.7 a	12
As3	4135±42.5 jk	965±15 efg	6.6±0.8 efg	6±1.5 hi	9.6±1.4 ef	72
Ba1	3770±182.5 qr	787.5±7.5 hi	6±0.1 gh	4.3±0.3 jkl	8.3±2 gh	96
Ba2	4008±3 lmn	690.5±3.5 jkl	7±0.5 ef	5±0.5 jk	8.6±0.8 fg	72
Ba3	3982±17 lmn	967.5±7.5 defg	7±0.5 ef	5.6±0.6 ij	9.3±1.2 ef	48
Ba4	4460±182 ghij	1068±61.5 abcde	10±0.5 abcd	10±0.1 abc	17±1.7 bc	24
Ba5	3938±66.5 mn	514.5±31.5 no	4.6±0.3 jk	3±0.5 m	6±0.1 j	120
Ba6	3725±30 rst	845±25 ghi	6.3±0.6 fg	5.6±0.8 ij	9±1.5 ef	72
Ba7	5031±28.5 cd	1082±22 abcde	10.6±0.6 ab	9.6±0.3 abcd	20.6±1.8 ab	24
Ba8	4021±84 lmn	750±23 hi	6±1.5 gh	6±0.1 hi	8.3±1.6 gh	72
De1	5445±130 ab	1145±12 abc	12.3±1.4 a	11.3±0.8 a	25.6±2 a	12
De2	4089±11 lm	956±4 efg	8±0.1 bcde	4.6±0.3 jkl	8.3±1.6 gh	96
Kh1	3733±108.5 rst	543±133 mno	4.6±1.2 jk	4±0.1 kl	6.6±0.3 ij	120
Kh3	3790±60 pq	700±10 jkl	6.3±0.8 fg	5±0.1 jk	8.3±0.8 gh	96
Kh4	4864±112 def	971±36 defg	9±0.5 bc	9±0.5 bcd	15.6±1.6 bcde	12
Kh5	4171±58.5 ij	810±20 hi	5.6±1.2 hi	4±0.5 kl	7±1.1 hij	120
Kh6	4487±160 ghi	1122.5±12.5 abc	9.3±0.3 b	8.6±0.3 bcde	12±2.6 def	24
Meh1	3831±54 op	476±32 no	4.6±0.3 jk	3±0.1 m	5.6±0.3 j	120
Meh2	4392±75.5 ghijk	1085±50 abcde	6±1.5 gh	7.3±0.3 fg	10±0.5 def	48
Meh3	4080.5±30.5 kl	1009±36 bc	7.3±0.3 de	6±1 hi	9.3±2.3 ef	48
Mey1	3872±62.5 no	1167± 7.5 a	10.3±0.6 abc	9.3±0.3 bc	16±3.7 bcd	24
Mey2	3942±47 mn	599±14 lm	6±1 gh	4.6±0.3 jkl	7.6±0.6 gh	96
Mey3	3679±26 st	498±127 no	4.3±0.8 kl	3.3±0.8 lm	6±0.5 j	120
Mey4	5017±7.5 cd	1102.5 ±2.5 abcde	7.3±1.2 de	8±0.1 de	11±2.8 def	24
Ta1	3671±81 st	443.5±94.5 o	4±0.5 l	3±0.5 m	5±0.1 j	120
Ta2	3763±77 qr	1117.5±2.5 abcd	7±0.1 ef	5±1.1 jk	10.6±0.6 def	72
Ta4	3777±99 pq	1045±95 abcde	7.6±0.3 cd	7±0.5 gh	11.3±0.3 def	36
Ta5	5174±39 bcd	1159.5±15.5 ab	10.3±0.8 abc	10±0.5 abc	20.3±1.6 abc	24
Ta6	3546.5±106.5 t	829±6 ghij	5.6±1.4 hi	5±0.5 jk	8±2 gh	72
Ya1	4098.5±91.5 kl	890±20 fgh	6.6±1.3 ef	5.6±0.3 ij	9.3±0.3 ef	72
Ya2	4903±27 cde	1057 ±67.5 abcdef	8.3±0.3 bcde	7.6±0.3 ef	11.6±1.6 def	36
Ya3	4604±209.5 efg	1003±2 cd	8.6±0.3 bcd	9±0.1 bcd	14.3±3.9 bcdef	24
Ya4	5641.5±195.5 a	1151±8 abc	9±0.1 bc	9.3±0.3 bc	14±5.6 cd	24
Ya5	4552±289.5 fgh	1062.5±52.5 abcde	7.6±0.6 cd	8±0.5 de	12±3.5 def	36

[a] LL, Lesion length.

[b] hpi, hours post inoculation

[c] Different letters indicate significant differences ($P = 0.05$), according to Duncan analysis. Each experiment was repeated twice, with similar results.

gal inocula were produced in Mung Bean Broth (MBB) using the method of Zhang *et al.* (2013). Conidium suspensions were diluted in water containing 0.05% (v/v) Tween 20, to final concentration of 1×10^5 conidia mL^{-1}.

Virulence assays

Spring wheat cultivar (cv. Falat), obtained from Agricultural Research Center of Khorassan Razavi province in Iran, was grown in a greenhouse, with 12 h photoperiod, RH of 75%, and a day:night temperature regime of 18°C:12 °C. After 14 d, 7 cm segments from the mid-section of the first leaf of plants were harvested, and placed adaxial surface up on the surfaces of 0.5 % water agar in Petri plates, as described by Browne and Cooke (2004). Leaf segments were each inoculated at the center of the adaxial surface with 5 µL of conidium suspension. Sterile distilled water containing 0.05% (v/v) Tween 20 was applied on the control (non-inoculated) leaf segments. Petri dishes were incubated at 25°C with a 12 h:12 h light:dark cycle. After 7 d, the lesion length at the point of inoculation on each leaf was determined. The experiment was replicated three times for each isolate, and repeated twice.

Assessment of aggressiveness

Aggressiveness, as another quantitative component of pathogenicity, was investigated for each fungal isolate on detached leaves of wheat plants (cv. Falat) in laboratory conditions using the methods described by Malihipour *et al.* (2012) and Pariaud *et al.* (2009). Analysis of aggressiveness was determined based on hours post inoculation (hpi) for disease symptom appearance.

Qualitative analyses of cell wall degrading enzymes

For determining cellulase activity**,** Glucose Yeast Extract Peptone Agar containing 0.5% carboxy-methyl-cellulose in Petri plates was used for qualitative investigation of cellulase activity. After 3 to 5 d of fungal colony growth, plates were flooded with 0.2% aqueous Congo red solution and de-stained with 1M NaCl for 15 min. Appearance of yellow areas around fungal colonies in red medium indicated cellulase activity (Hankin *et al.*, 1971).

Pectinolytic activity was determined by growing the fungi in Petri plates containing Pectin Agar (containing 5 g L^{-1} pectin, 1 g L^{-1} yeast extract, and 15 g L^{-1} agar in distilled water; pH 5.0). After incubation of 3 to 7 d at 28°C, pectin utilization was detected by flooding the culture plates with freshly prepared iodine-potassium iodide solution (1.0 g iodine + 5.0 g potassium iodide in 330 mL distilled water) (Hankin *et al.*, 1971). Clear zones formed around fungal colonies indicated pectinolytic activity.

In each assay, Petri plates each inoculated with a PDA plug without fungus were used as as negative controls.

Quantitative analysis of cell wall degrading enzymes

For cellulase assays, fungal cultures were prepared in 500 mL capacity Erlenmeyer flasks each containing 250 mL of culture medium, as described by Abdel-Razik (1970). After inoculation, incubation was carried out under shaking (150 rpm) at 27°C and darkness for 10 d. Cellulase activity was assessed using the method of Wood and Bhat (1988). Absorbance was measured at 550 nm, and the amount of reducing sugar released was calculated from the standard curve for glucose. One unit of cellulase activity was defined as the amount of enzyme that catalyzed glucose at 1.0 µ mol min^{-1} during the hydrolysis reaction.

For pectinase assays, the fungal cultures were prepared in 500 mL capacity Erlenmeyer flasks each containing 250 mL culture medium, as described by Mac-Millan and Voughin (1964). Pectinase activity was determined based on the amount of reducing sugar (D-galacturonic acid) released into the culture supernatant. The amount of D-galacturonic acid was determined using the dinitrosalicylic acid colorimetric method of Colowich (1995), and absorbance was measured at 540 nm. The unit of enzyme activity was defined as the amount of enzyme that released galacturonic acid at 1 µ mol min^{-1}, according to the standard curve. The standard curve was developed based on the absorbance for different concentrations of D-galacturonic acid.

Detection of the genes responsible for production of NIV, DON and zearalenone

For detection of DON, NIV and zearalenone genes, the *Tri5*, *Tri13* and *PKS4* (polyketide synthase) genes were amplified by PCR, using the primers pairs *Tri5F* (5′-AGCGACTACAGGCTTCCCTC-3′) and *Tri5R* (5′-AAACCATCCAGTTCTCCATCTG-3′) for *Tri5*, *Tri13F* (5′-TACGTGAAACATTGTTGGC-3′) and *Tri13R* (5′-GGTGTCCCAGGATCTGCG-3′) for *Tri13*, and *PKS4F* (5′-CGTCTTCGAGAAGATGACAT-3′) and *PKS4R* (5′-TGTTCTGCAAGCACTCCGA-3′) for *PKS4* (Doohan *et al.*, 1999; Waalwijk *et al.*, 2003; Meng *et al.*,

2010). The PCR cycles consisted of an initial denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation (95°C for 35 s), annealing (60°C for 30 s), extension (72°C for 30 s), and final extension at 72°C for 7 min. The PCR products were detected on 1% agarose gels.

Mycotoxin analyses in laboratory cultures

Mycotoxin production by the *Fusarium* isolates in laboratory cultures was investigated using the method of Alvarez *et al.* (2009). Briefly, 25 g of rice grains (*Oryza sativa* L.) were soaked in 100 mL of sterile distilled water for 6 h in 500 mL capacity flasks. Water was then drained and the rice grain was autoclaved twice. Five mL of inoculum suspension (1×10^5 conidia mL^{-1}) of each isolate was added to each flask and incubated at 26 ± 1°C in darkness for 3 weeks. The rice-fungus mixtures were each ground in a mortar and then dispensed in an Erlenmeyer flask with 75 mL of acetonitrile:methanol:water (80:5:15, v/v/v). HPLC analysess was carried out on a Waters Alliance 2695 separations module coupled to a Waters 474 scanning fluorescence detector (Waters Corporation) that was set at 365 nm excitation and 440 nm emission. To perform chromatographic separations, 500 µL of water:methanol (86:14, v/v) was added to each extract and cleaned with a C18 Spherisorb 5 µm (250 × 4.6 mm; Merck). The mobile phase was water:acetonitrile:methanol (78:12:10 v/v/v) with a flow rate of 2.5 mL min^{-1}. NIV, DON and zearalenone production were measured in µg per kg of sample. Standards of the DON, NIV and ZEA were used to construct a five-point calibration curve of peak areas versus concentrations. The injection volume was 50 µL for both the standard solutions and sample extracts.

Statistical analyses

All experiments were set up in completely randomized designs. The data were analyzed by one-way analysis of variance (ANOVA), and comparison of means was carried out using the Duncan's Multiple Range Test ($P \leq 0.05$. Statistical analyses and correlation tests were performed using software Statistical Package for the Social Sciences (SPSS; version 22).

RESULTS

Virulence and aggressiveness assays

Comparison of the data obtained from inoculation of *Fusarium* spp. isolates on wheat leaf segments revealed that different isolates had different virulence capabilities (Table 1). Significant differences in disease index were recorded among the isolates tested. Leaf assays revealed that the greatest lesion length was produced by *F. solani* isolate De1 and *F. flocciferum* isolate As2. The least disease was observed for the *F. equiseti* isolate Ta1 and *F. oxysporum* isoklate Meh1. Other isolates tested fell between these isolates with various levels of virulence on wheat leaf segments (Table 1, Figure 1). The results of the aggressiveness test on detached leaves showed more rapid development of disease symptoms by *F. solani* isolate De1 and *F. flocciferum* isolate As2, compared to the other isolates tested (Table 1).

Analysis of cell wall degrading enzymes

Qualitative and quantitative analysis of CWDEs showed that all the *Fusarium* isolates were capable of producing pectinase and cellulose enzymes (Table 1). In the quantitative assays, the amounts of CWDE activity among isolates varied from 443.5 to 1167 µg mL^{-1} for cellulase and 3546.5 to 5641.5 µg mL^{-1} for pectinase. Quantitative results of CWDE assays agreed with the qualitative results. The *F. equiseti* isolate Mey1 and *F. pseudograminearum* isolate Ta5 had the greatest *in vitro*

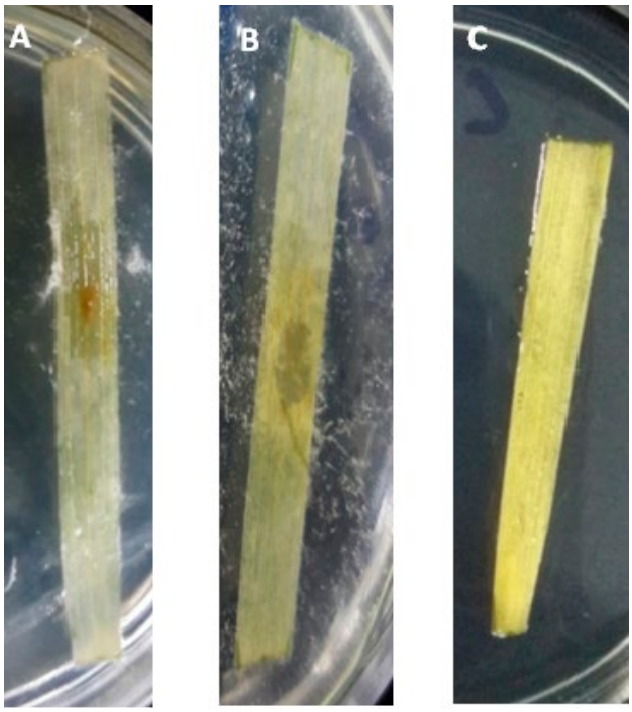

Figure 1. Disease symptoms caused by *Fusarium solani* isolate De1 and *F. flocciferum* isolate As2 on wheat leaves (A and B) and negative control (C).

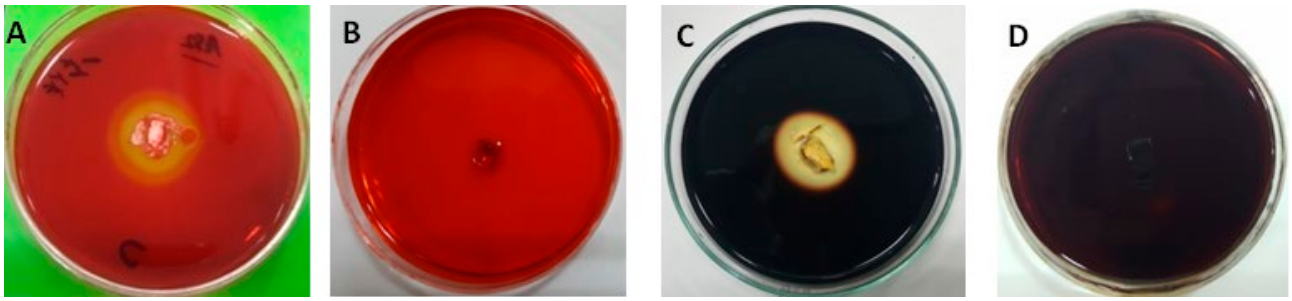

Figure 2. Cellulolytic activity of *Fusarium flocciferum* isolate As2 on GYP medium (A) and its negative control (B). Pectinolytic activity of this isolate on pectin agar medium (C) and its negative control (D).

cellulase activity of all the isolates. The least cellulase activity was measured for *F. equiseti* Ta1 and *F. oxysporum* Meh1. The *Fusarium oxysporum* Ya4 and *F. solani* De1 had the greatest pectinase activities. Least pectinase was measured for *F. flocciferum* Ta6 and *F. equiseti* Ta1. Based on the size of clear culture medium zones for cellulase activity, maximum activity was observed for *F. solani* De1 and *F. flocciferum* As2, while least activity was measured for *F. equiseti* Ta1, *F. oxysporum* Meh1 and *F. equiseti* Ba5.The least pectinase activity was measured for *F. equiseti* Ta1 and *F. pseudograminearum* Mey3, and *F. solani* isolate De1 and *F. flocciferum* isolate As2 gave the greatest pectinase activities (Figure 2).

Detection of trichothecene and zearalenone genotypes by PCR

The *Tri5* gene encodes trichodiene synthase, which catalyses the first step in trichothecene biosynthesis. This gene was detected using the primer set TRI5 (F)/ TRI5 (R), which produced a unique PCR product of 544 bp for the isolates which contained the *Tri5* gene (Figure 3A, Table 2) (Doohan *et al.*, 1999; Covarelli *et al.*, 2015). The results obtained from PCR of the *Tri5* gene showed amplification of this gene in 43% of the isolates, which produced either NIV or DON.

Also, the Tri13F/Tri13R primers for amplification of *Tri13* gene amplified a fragment in the range of 200 to 300 bp from DON producers and 400 to 450 bp from NIV producing isolates of *Fusarium* (Figure 3B, Table 2). Among the isolates producing trichothecenes, results obtained from PCR of the *Tri13* gene showed amplification of this gene for 35% of DON-producing isolates, and 65% of NIV- producing isolates.

The *PKS4* (polyketide synthase) gene of *F. graminearum* has been reported to be essential in production of ZEA (Lysøe *et al.*, 2006). The size of PCR products obtained in detecting this gene was approx. 280 bp (Fig-

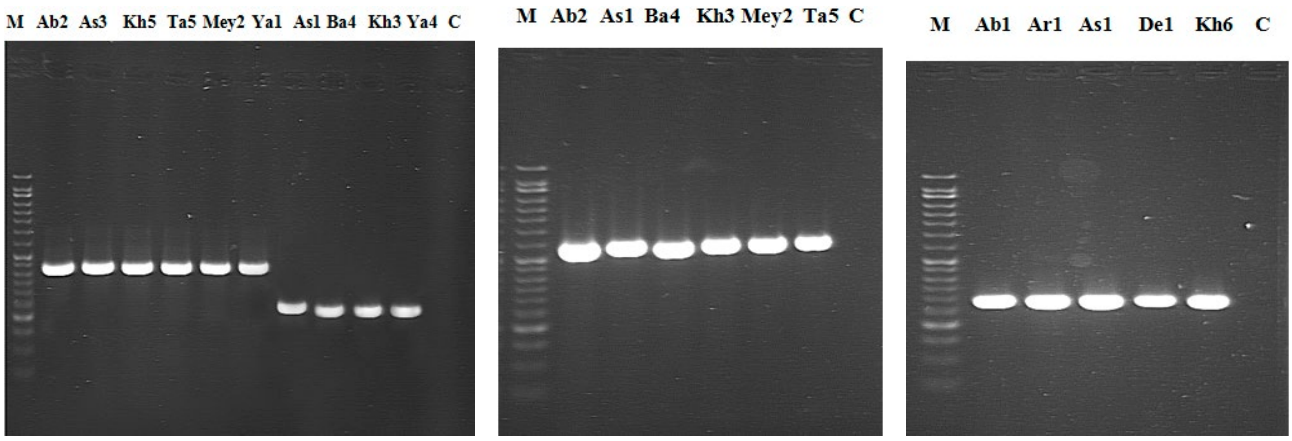

Figure 3. Amplification products using the primer pairs *Tri5F*/*Tri5R* (A), *Tri13F*/*Tri13R* (B), *PKS4F*/*PKS4R* (C), M: marker (1,500 bp). The primer set *Tri5F*/*Tri5R* produced a unique PCR product with the size of 544 bp for the isolates which contain the *Tri5* gene. The *Tri13F*/*Tri13R* primers amplified a fragment in the range of 200 to 300 bp from DON-producing *Fusarium* isolates and 400 to 450 bp from NIV-producing isolates. The *PKS4F*/*PKS4R* primer set produced a fragment of 280 bp for the zearalenone-producing isolates.

Table 2. Origin and species of *Fusarium* isolates obtained from wheat plants in Yazd province of Iran, together with the presence of trichothecene- (NIV and DON) and zearalenone- (ZEA) specific markers detected in the isolates by PCR assays, and their capability for mycotoxin production analyzed by HPLC.

Isolate code	Sample site	Species	Trichotecene genotype	PCR assay results			Toxin production *in vitro* (μg kg−1)		
				Tri5	Tri13	PKS4	NIV	DON	ZEA
Ab1	Abarkuh	*F. acuminatum*	-	-	-	+	-	-	ND
Ab2	Abarkuh	*F. culmorum*	NIV	+	+	+	298	ND	186
Ab3	Abarkuh	*F. culmorum*	DON	+	+	+	ND	1411	252
Ar1	Ardakan	*F. pseudograminearum*	NIV	+	+	+	1007	ND	3075
Ar2	Ardakan	*F. flocciferum*	-	-	-	-	-	-	-
As1	Ashkezar	*F. acuminatum*	DON	+	+	+	ND	379	ND
As2	Ashkezar	*F. flocciferum*	-	-	-	+	-	-	ND
As3	Ashkezar	*F. equiseti*	NIV	+	+	+	850	ND	ND
Ba1	Bafq	*F. solani*	-	-	-	+	-	-	ND
Ba2	Bafq	*F. acuminatum*	-	-	-	+	-	-	ND
Ba3	Bafq	*F. proliferatum*	-	-	-	+	-	-	ND
Ba4	Bafq	*F. culmorum*	DON	+	+	+	ND	1093	114
Ba5	Bafq	*F. equiseti*	-	-	-	-	-	-	-
Ba6	Bafq	*F. proliferatum*	-	-	-	+	-	-	67
Ba7	Bafq	*F. pseudograminearum*	DON	+	+	+	ND	65	3220
Ba8	Bafq	*F. equiseti*	-	-	-	+	-	-	ND
De1	Taft	*F. solani*	-	-	-	+	-	-	ND
De2	Taft	*F. flocciferum*	-	-	-	-	-	-	-
Kh1	Khatam	*F. flocciferum*	-	-	-	-	-	-	-
Kh3	Khatam	*F. equiseti*	DON	+	+	+	ND	103	ND
Kh4	Khatam	*F. culmorum*	NIV	+	+	+	1135	ND	230
Kh5	Khatam	*F. acuminatum*	NIV	+	+	+	350	ND	73
Kh6	Khatam	*F. pseudograminearum*	NIV	+	+	+	1083	ND	1760
Meh1	Mehriz	*F. oxysporum*	-	-	-	+	-	-	53
Meh2	Mehriz	*F. equiseti*	-	-	-	+	-	-	ND
Meh3	Mehriz	*F. solani*	-	-	-	-	-	-	-
Mey1	Meybod	*F. equiseti*	-	-	-	+	-	-	105
Mey2	Meybod	*F. equiseti*	NIV	+	+	+	480	ND	ND
Mey3	Meybod	*F. pseudograminearum*	NIV	+	+	+	258	ND	1385
Mey4	Meybod	*F. oxysporum*	-	-	-	+	-	-	ND
Ta1	Taft	*F. equiseti*	-	-	-	+	-	-	ND
Ta2	Taft	*F. equiseti*	-	-	-	+	-	-	96
Ta4	Taft	*F. pseudograminearum*	NIV	+	+	+	954	ND	1670
Ta5	Taft	*F. pseudograminearum*	NIV	+	-	+	1246	ND	2525
Ta6	Taft	*F. flocciferum*	-	-	-	-	-	-	-
Ya1	Yazd	*F. culmorum*	NIV	+	+	+	743	ND	365
Ya2	Yazd	*F. equiseti*	-	-	-	+	-	-	ND
Ya3	Yazd	*F. equiseti*	-	-	-	+	-	-	78
Ya4	Yazd	*F. oxysporum*	DON	+	+	+	ND	45	ND
Ya5	Yazd	*F. oxysporum*	-	-	-	-	-	-	-

ure 3C, Table 2). Overall, 83% of the *Fusarium* isolates produced this amplicon in the PCR assays.

Mycotoxin analyses using HPLC

Data of detection of trichothecenes produced by *Fusarium* isolates on rice grain showed that among 17 isolates which amplified the *Tri5* gene, 657% produced NIV and 35% produced DON. The levels of NIV ranged from 258 to 1,246 μg kg^{-1}, of DON from 45 to 1,411 μg kg^{-1}, and of ZEA from 53 to 3,220 μg kg^{-1} (Table 2).

DISCUSSION

This study was a detailed investigation of trichothecene genotypes and quantification of NIV, DON and ZEA by HPLC from *Fusarium* species associated to wheat crown and root rot in Iran. Activities of CWDEs and their relationships with virulence factors, aggressiveness and pathogenicity of *Fusarium* spp. isolates to wheat leaf tissues were also evaluated.

Different types of virulence factors can be produced by different *Fusarium* species pathogenic on cereals (Taheri 2018). Mycotoxins, such as trichthecenes and ZEA, and CWDEs, are among the main factors involved in virulence and aggressiveness of *Fusarium* spp. on host plants. We conclude that the two most virulent *Fusarium* isolates in this study (*F. solani* isolate De1 and *F. flocciferum* AS2), which do not produce tricothecenes, may produce other types of virulence factors, such as CWDEs as demonstrated here. Involvement of other virulence factors, such as lipases, xylanases, protein kinases, other proteins and various transcription factors (reviewed by Taheri, 2018), in pathogenicity of these fungi, needs to be investigated in the future studies.

Quantitative and qualitative activities of CWDEs, including cellulase and pectinase which are involved in the infection processes of *Fusarium* spp. on wheat, were investigated here. Aggressiveness of *Fusarium* spp. involves different mechanisms or components, such as production and release of extracellular enzymes which degrade host plant cell walls. The CWDEs are crucial in the processes of pathogen colonization and establishment of disease. Once infection is established, mycotoxins are released and these interfere with the metabolism, physiological processes and structural integrity of host cells (Ortega *et al*., 2013). These enzymes are particularly important for phytopathogenic fungi without specialized penetration structures (Gibson *et al*., 2011). The activities of CWDEs produced by the *Fusarium* isolates, which caused maximum or minimum virulence on wheat leaves, were compared for possible associations between the CWDEs and virulence. The isolates *F. solani* De1 () and *F. flocciferum* As2, which showed the greatest virulence on wheat leaves, had greater enzyme activities at different time points investigated. In contrast, the isolates Ta1 and Meh1 had the least virulence capability, and the lowest levels of CWDE activity. Correlation analysis revealed high levels of direct association between the capability of *Fusarium* spp. in producing CWDEs and their virulence on the wheat leaves (Table 3). Similarly, Khaledi *et al*. (2016) demonstrated the association of aggressiveness and virulence of *Fusarium* spp. isolates causing head blight of wheat with the levels of CWDEactivity.

Table 3. Correlation analyses between activity of cell wall degrading enzymes (in quantitative and qualitative assays) produced by *Fusarium* spp. isolates and their virulence on wheat leaves.

Correlation	Virulence on leaf segments	*P* value (two-tailed)
Pectinase (quantitative)	0.78231	< .0001
Pectinase (qualitative)	0.94502	< .0001
Cellulase (quantitative)	0.71337	< .0001
Cellulase (qualitative)	0.90916	< .0001

In the present study, trichothecene genotype detection revealed 50% amplification of the *Tri5* gene for *F. acuminatum* isolates, 27% amplification for *F. equiseti* isolates, and 25% amplification for those of *F. oxysporum*. *Fusarium pseudograminearum* and *F. culmorum* isolates had 100% amplification of the *Tri5* gene. However, this gene was not detected in isolates of *F. solani*, *F. proliferatum* and *F. flocciferum*. In accordance with our data, Tan and Niessen (2003) showed that *F. solani* was not capable of producing trichothecenes and ZEA, and this species lacked the *Tri5* gene required for biosynthesis of trichothecenes. Khaledi *et al*. (2016) reported that some *F. proliferatum* isolates causing wheat head blight amplified the *Tri5* gene and this species has the ability of trichothecenes biosynthesis, which is in agreement with the findings from the present study.

The *Tri13* gene from the *Fusarium* trichothecene biosynthetic gene cluster is responsible for conversion of DON to NIV (Lee *et al*., 2001). Our results showed that the NIV was produced by 65% of the isolates, whereas 35% of the isolates produced DON. There are few reports on the geographical distribution of trichothecene chemotypes produced by *Fusarium* spp. in different regions of Iran. Our data showed that the distribution of DON and NIV was not equal in different parts of the studied province, and that NIV was the dominant chemo-

type. Other investigations in Iran showed dominance of the NIV chemotype in Mazandaran (Haratian *et al*., 2008) and Golestan provinces (Abedi-Tizaki *et al*., 2013; Khaledi *et al*., 2016). Other studies in different regions of the world such as Africa, Asia and Europe have confirmed the presence of NIV and DON chemotypes, but only the DON type has been detected in North America (Miedaner *et al*., 2000). Both NIV and DON chemotypes have been identified together, in Europe and South America, and the DON chemotype was dominant in these regions. In Asian countries such as Korea and Japan, the NIV chemotype had the greatest distribution (Gale *et al*., 2011; Lee *et al*., 2002).

The results of the present study relating to virulence of *Fusarium* isolates on wheat leaf segments showed that all isolates were pathogenic to wheat (cv. Falat), and differences in virulence were observed. Some reports have showned that trichothecenes are virulence factors in plants, and they may contribute to colonization of wheat crowns by the pathogen (Mudge *et al*., 2006). Maier *et al*., (2006) showed that NIV and DON act as virulence factors on wheat, while only the NIV chemotype is virulent on maize (. In general in the present study, the isolates with NIV chemotype were more aggressive than the other chemotypes of trichothecenes produced by *Fusarium* spp. This is in agreement with the observations of other researchers (Cumagun *et al*., 2004; Khaledi *et al*., 2016).

The polyketide synthase gene *PKS4*, which is involved in ZEA biosynthesis, was used in our study for developing a PCR-based assay to detect ZEA-producing *Fusarium* isolates (Meng *et al*., 2010). The isolates of *F. flocciferum* and *F. solani* did not produce ZEA. The results of our study showed that the levels of ZEA were not correlated with virulence and aggressiveness of *Fusarium* spp. isolates. This is similar to results of Kuhnem *et al*. (2015), who found that the level of ZEA produced by *F. graminearum* was not related to severity of the disease caused by this species on maize.

Finding novel and effective ways to prevent or decrease production of different types of virulence factors by *Fusarium* spp. may be helpful in management of destructive diseases caused by these important and commonly occurring phytopathogenic fungi.

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Research Papers

Soilborne diseases caused by *Fusarium* and *Neocosmospora* spp. on ornamental plants in Italy

Vladimiro GUARNACCIA[1,2], Dalia AIELLO[3,*], Giancarlo POLIZZI[3], Pedro W. CROUS[1,4], Marcelo SANDOVAL-DENIS[1,4]

[1] *Westerdijk Fungal Biodiversity Institute, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands*
[2] *DiSAFA, University of Torino, Largo Paolo Braccini, 2, 10095 Grugliasco, TO, Italy*
[3] *Dipartimento di Agricoltura, Alimentazione e Ambiente, Università degli Studi di Catania, Via S. Sofia 100, 95123 Catania, Italy*
[4] *Faculty of Natural and Agricultural Sciences, Department of Plant Sciences, University of the Free State, P.O. Box 339, Bloemfontein 9300, South Africa*
*Corresponding author: dalia.aiello@unict.it

Abstract. During surveys conducted in 2010–2014, several disease symptoms were observed on eight ornamental plant species in different nurseries located in Sicily (Southern Italy). Two *Neocosmospora* and 31 *Fusarium* isolates were recovered from symptomatic plants. Fungus identification was based on morphological characteristics and phylogenetic analyses of fragments of the intergenic spacer (IGS), internal transcribed spacer (ITS) and large subunit (LSU) regions of the rDNA; partial fragments of the beta-tubulin (*TUB*), RNA polymerase largest subunit (*RPB1*), RNA polymerase second largest subunit (*RPB2*) and translation elongation factor 1-alpha (*EF-1α*) genes. The *Fusarium* species recovered from ornamental plants were *F. agapanthi* (from *Agapanthus africanus*), *F. anthophilum* (*Dasylirion longissimum*), *F. fujikuroi* (*Trachycarpus princeps*), *F. oxysporum* (*Bougainvillea glabra*, *Cordyline australis* 'Purpurea', *Dasylirion longissimum*, *Eremophila laanii* and *Philoteca myoporoides*) and *F. proliferatum* (*T. princeps*), while *N. solani* was isolated from crown and root rot of *Ficus carica*. The pathogenicity of representative isolates collected from each host was tested on seedlings or cuttings grown in a growth chamber. All the *Fusarium* and *Neocosmospora* isolates tested were pathogenic and reproduced symptoms identical to those observed in the field, except for *F. fujikuroi* on *T. princeps* and *F. oxysporum* on *D. longissimum* that were non-pathogenic.

Keywords. Morphology, multigene phylogeny, pathogenicity, root rot, wilt.

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INTRODUCTION

During the last decade, Italy has significantly increased production of ornamental plants in nurseries, and several new species and products have been introduced for cultivation in greenhouses and open fields. Movement

of ornamental plants through the peninsula led to the spread of pathogens to new areas, and introduction of new pathogens from abroad (Gullino and Garibaldi, 2006; Polizzi *et al.,* 2012; Aiello *et al.,* 2017, 2018).

In Sicily (Southern Italy), production of ornamentals has increased in the eastern area, where it replaced lemon orchards due to decline in demand for these fruits. Plant growth in nurseries is compromised by several foliar and root diseases, and among these diseases those caused by species of Nectriaceae are exceptionally common (Polizzi *et al*., 2007; Vitale *et al.,* 2009; Aiello *et al.,* 2014, 2015; Gullino *et al.,* 2015).

Fusarium Link *sensu lato* was recently segregated into several *Fusarium*-like genera (i.e., *Bisifusarium* L. Lombard, Crous & W. Gams [*Fusarium dimerum* species complex (SC)], *Neocosmospora* E.F. Sm. [*Fusarium solani* SC] and *Rectifusarium* L. Lombard, Crous & W. Gams [*Fusarium ventricosum* SC]). These taxa are among the most important human, animal or plant pathogens, affecting an extensive variety of hosts (O'Donnell *et al.,* 2008, 2010; Lombard *et al.,* 2015). *Fusarium* and *Fusarium*-like genera are well-known as responsible for diseases on ornamental plants, including flowering crops, herbaceous ornamentals such as begonia, carnation and chrysanthemum, woody ornamentals such as *Bougainvillea*, *Hebe*, *Hibiscus* and *Pyracantha* spp. (Horst and Nelson, 1997; Sinclair and Lyon, 2005; Polizzi *et al.,* 2010a, 2010b, 2011; Bertoldo *et al.,* 2015; Lupien *et al.,* 2017), and palms such as *Arecastrum*, *Phoenix* and *Washingtonia* spp. (Elliott *et al.,* 2004).

Considering the importance of diseases caused by *Fusarium*-like fungi, the high economic losses caused by these pathogens and the relevance of these crops, surveys were conducted over a 5-year period in ornamental nurseries located in the Catania province, eastern Sicily, Italy. During the surveys conducted from 2010 to 2014, large numbers of palms, perennial herbaceous shrubs, and young cuttings were detected showing symptoms of crown and root rots, damping-off, wilt and dieback. The aims of the present study were to identify the Fusaria obtained from these affected ornamentals, using morphological characteristics and DNA sequence analyses, and to evaluate the pathogenicity of representative isolates on the hosts from which they were isolated.

MATERIALS AND METHODS

Field sampling and pathogen isolations

During 2010–2014, surveys were performed in ornamental plant-producing regions located in eastern Sicily (Table 1). The disease incidence (DI) was recorded for each host, based on the number of symptomatic plants in the total of those present in five investigated nurseries. Additionally, approx. 20 plants per species per nursery showing wilt, crown or root rot or damping-off symptoms, were randomly collected for analysis. Fragments (each 5 × 5 mm) of symptomatic tissues were cut from the margins of lesions, surface-sterilised in a sodium hypochlorite solution (10%) for 20 s, followed by 70% ethanol for 30 s, and rinsed three times in sterilised water. Tissue fragments were dried in sterilised filter paper, placed on 2% potato dextrose agar (PDA) amended with 100 μg mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (PDA-PS), and were incubated at 25°C until characteristic *Fusarium*-like colonies were observed. Pure cultures were obtained by transferring single conidia to fresh PDA, with the aid of a Nikon SMZ1000 dissecting microscope.

Fungal isolates and morphological characterization

The cultural and micromorphological features of all the isolates included in this study were evaluated fol-

Table 1. Hosts, locations, symptoms and incidence (%) of diseases caused by *Fusarium* and *Neocosmospora* in Sicily (Southern Italy).

Hosts	Locations	Geographical coordinates	Collection year	Symptoms	Incidence %[a]
Agapanthus africanus	Carruba, Nursery 1	37.698004, 15.193944	2014	Damping-off	50
Bougainvillea glabra	Carruba, Nursery 1	37.698004, 15.193944	2010	Wilt	30
Cordyline australis 'Purpurea'	Carruba, Nursery 2	37.699090, 15.197300	2014	Wilt	20
Dasylirion longissimum	Riposto, Nursery 3	37.733699, 15.194320	2014	Wilt	10
Eremophila laanii	Carruba, Nursery 1	37.698004, 15.193944	2010	Wilt	50
Ficus carica	Carruba, Nursery 1	37.698004, 15.193944	2013	Crown and Root rot	50
Phyloteca myoporoides	Milazzo, Nursery 4	38.201964, 15.239530	2013	Wilt	30
Trachycarpus princeps	Grotte, Nursery 5	37.681233, 15.180316	2013	Root rot	40

[a] Number of symptomatic plants on the total of those cultivated.

lowing the procedures of Aoki *et al.* (2003), with some modification as described previously (Sandoval-Denis *et al.,* 2018).

DNA extraction, PCR amplification and sequencing

Fungus isolates were grown on PDA for 4-7 d at room temperature, under a natural day/night photoperiod. Total genomic DNA was extracted from fresh mycelium scraped from each colony surface, using the Wizard® Genomic DNA purification Kit (Promega Corporation). Fragments of seven nuclear loci, including the translation elongation factor 1-alpha (*EF-1α*), the intergenic spacer region of the rDNA (*IGS*), the internal transcribed spacer region of the rDNA (*ITS*), the large subunit of the rDNA (*LSU*), the RNA polymerase largest subunit (*RPB1*), RNA polymerase second largest subunit (*RPB2*) and beta-tubulin (*TUB*), were PCR amplified as described previously (O'Donnell *et al.,* 2009; 2010, Sandoval-Denis *et al.,* 2018). The PCR products were sequenced using the following primer pairs: EF-1/EF-2 for *EF-1α* (O'Donnell *et al.,* 2008), iNL11/iCNS1 plus the internal sequencing primer pair NLa/CNSa for IGS (O'Donnell *et al.,* 2009), ITS4/ITS5 for ITS (White *et al.,* 1990), LR0R/LR5 for LSU (Vilgalys and Hester, 1990; Vilgalys and Sun, 1994), Fa/G2R for *RPB1* (O'Donnell *et al.,* 2010), 5f2/7cr and 7cf/11ar for *RPB2* (Liu *et al.,* 1999; Sung *et al.,* 2007), and 2Fd/4Rd for TUB (Woudenberg *et al.,* 2009). Sequences generated in this study were uploaded to the GenBank and the European Nucleotide Archive (ENA) databases.

Phylogenetic analyses and molecular identification

Sequence alignments were performed individually for each locus using MAFFT on the European Bioinformatics Institute (EMBL-EBI) portal (http://www.ebi.ac.uk/Tools/msa/mafft/). BLASTn searches on GenBank, and pairwise sequence alignments on the *Fusarium* MLST database of the Westerdijk Fungal Biodiversity Institute (http://www.westerdijkinstitute.nl/fusarium/), were performed using *EF-1α* and *RPB2* sequences. This was to assess the distribution of the Fusaria isolates among the different *Fusarium* species complexes or *Neocosmospora*. Following this preliminary identification, different loci combinations were selected for each of the *Fusarium* species complexes and *Neocosmospora* isolates, according to the phylogenetic informativeness for each locus as compiled in published literature. These combinations were as follows: *EF-1α*, ITS, *RPB1*, *RPB2* and *TUB* for the *F. fujikuroi* species complex (FFSC) (Edwards *et al.* 2016); *EF-1α* and IGS for the *F. oxysporum* species complex (FOSC), and collapsed to haplotypes according to O'Donnell *et al.* (2009); *EF-1α*, ITS, LSU and *RPB2* for the genus *Neocosmospora* (O'Donnell *et al.,* 2008).

The different gene datasets were analysed independently and combined, using Maximum likelihood (ML) and Bayesian methods (BI) as described previously (Sandoval-Denis *et al.,* 2018).

Pathogenicity tests

Pathogenicity tests were performed on potted healthy seedlings or cuttings of all symptomatic species recovered with a subset of 16 representative isolates (Table 2). Each experiment was conducted twice, obtaining similar results in both tests. For each experiment three replicates per isolate were used with 20 to 50 plants per replicate. All plants were inoculated by placing two colonised 1 cm^2 plugs (PDA from 9-d-old mycelium cultures, grown at 25 ± 1°C in the dark) at the base of each plant stem. Uninoculated plants for all the host species served as controls. After inoculation, plants were covered with a plastic bag for 48 h and maintained at 25 ± 1°C and 95% relative humidity (RH) under a 12 h fluorescent light/dark regime until the symptoms were observed. All plants were irrigated two to three times per week, and were examined each week for disease symptoms. Disease incidence (DI) was determined for each host species. Fungi were re-isolated from symptomatic tissues and identified, to fulfil Koch's postulates.

RESULTS

Field sampling and pathogen isolations

Symptoms referable to *Fusarium* spp. were detected on eight ornamental species in five nurseries investigated in Eastern Sicily, Italy (Figure 1). The diseases were observed on seedlings and unrooted and rooted cuttings (1 to 12-month-old) during propagation stages in the greenhouses. Disease incidence varied from 10 to 50%, according to the host species (Table 1). The symptoms observed on ornamental plants consisted of damping-off, crown and root rot, and wilt (Table 1).

Damping-off consisted of root rot and stem decay at soil level, and occurred on young seedlings. Rotted roots were dark brown or black, and were partially or completely destroyed. Crown rot sometimes occurred in association with root rot. As consequence of crown and root rot, basal leaves turned necrotic while infected

Table 2. Collection details and sequence accession numbers of isolates included in this study, as well as disease incidence from pathogenicity tests conducted with the isolates.

Species	Culture number[a]	Host	Incidence %[b]	GenBank/ENA accession number[c]						
				ITS	*EF-1α*	IGS	LSU	*RPB1*	*RPB2*	*TUB*
Fusarium agapanthi	CPC 27740#	*Agapanthus africanus*	100	LS422776	LS420058		LS422776	LS420106	LS420122	LS420041
	CPC 27741#	*Agapanthus africanus*	100	LS422777	LS420059		LS422777	LS420107	LS420123	LS420042
Fusarium anthophilum	CPC 27742#	*Dasylirion longissimum*	75	LS422778	LS420060		LS422778	LS420108	LS420124	LS420043
	CPC 27743	*Dasylirion longissimum*		LS422779	LS420061		LS422779	LS420109	LS420125	LS420044
	CPC 27744	*Dasylirion longissimum*		LS422780	LS420062		LS422780	LS420110	LS420126	LS420045
Fusarium fujikuroi	CPC 27719#	*Trachycarpus princeps*	0	LS422781	LS420063		LS422781	LS420111	LS420127	LS420046
Fusarium oxysporum	CPC 27729#	*Philoteca myoporoides*	100		LS420064	LS420138				
	CPC 27730#	*Philoteca myoporoides*	100		LS420065	LS420139				
	CPC 27731	*Philoteca myoporoides*			LS420066	LS420140				
	CPC 27732	*Philoteca myoporoides*			LS420067	LS420141				
	CPC 27733	*Philoteca myoporoides*			LS420068	LS420142				
	CPC 27734#	*Eremophila laanii*	100		LS420069	LS420143				
	CPC 27735#	*Eremophila laanii*	100		LS420070	LS420144				
	CPC 27738#	*Bougainvillea glabra*	100		LS420071	LS420145				
	CPC 27739#	*Bougainvillea glabra*	100		LS420072	LS420146				
	CPC 27745#	*Dasylirion longissimum*	0		LS420073	LS420147				
	CPC 27746#	*Cordyline australis* 'Purpurea'	100		LS420074	LS420148				
	CPC 27747#	*Cordyline australis* 'Purpurea'	100		LS420075	LS420149				
	CPC 27748	*Cordyline australis* 'Purpurea'			LS420076	LS420150				
	CPC 27749	*Cordyline australis* 'Purpurea'			LS420077	LS420151				
	CPC 27750	*Cordyline australis* 'Purpurea'			LS420078	LS420152				
	CPC 27751	*Cordyline australis* 'Purpurea'			LS420079	LS420153				
Fusarium proliferatum	CPC 27711#	*Trachycarpus princeps*	100	LS422782	LS420080		LS422782	LS420112	LS420128	LS420047
	CPC 27712	*Trachycarpus princeps*		LS422783	LS420081		LS422783	LS420113	LS420129	LS420048
	CPC 27713	*Trachycarpus princeps*		LS422784	LS420082		LS422784	LS420114	LS420130	LS420049
	CPC 27714	*Trachycarpus princeps*		LS422785	LS420083		LS422785	LS420115	LS420131	LS420050
	CPC 27715	*Trachycarpus princeps*		LS422786	LS420084		LS422786	LS420116	LS420132	LS420051
	CPC 27716	*Trachycarpus princeps*		LS422787	LS420085		LS422787	LS420117	LS420133	LS420052
	CPC 27717	*Trachycarpus princeps*		LS422788	LS420086		LS422788	LS420118	LS420134	LS420053
	CPC 27718	*Trachycarpus princeps*		LS422789	LS420087		LS422789	LS420119	LS420135	LS420054
	CPC 27720	*Trachycarpus princeps*		LS422791	LS420089		LS422791	LS420121	LS420137	LS420056
Neocosmospora solani	CPC 27736#	*Ficus carica*	100	LT991945	LT991907		LT991952		LT991914	
(= *Fusarium solani*)	CPC 27737#	*Ficus carica*	100	LT991946	LT991908		LT991953		LT991915	

[a] CPC: Culture collection of P.W. Crous, housed at Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; Strains used in the pathogenicity tests are indicated with #.
[b] Percentage calculated from the number of symptomatic plants relative to the total number inoculated.
[c] ENA: European Nucleotide Archive; *EF-1α*: Translation elongation factor 1-alpha; IGS: Intergenic spacer region of the rDNA; ITS: Internal transcribed spacer regions of the rDNA and 5.8S region; LSU: Partial large subunit of the rDNA; *RPB1*: RNA polymerase largest subunit; *RPB2*: RNA polymerase second largest subunit; *TUB*: Beta-tubulin.

Figure 1. Natural and artificial symptoms referable to *Fusarium* and *Neocosmospora* spp. **a, b**. Wilt of *Bougainvillea glabra* cuttings (a) and *Cordyline australis* seedlings (b). **c**. Root rot with subsequent leaf chlorosis of *Trachycarpus princeps*. **d**. Damping-off of *Agapanthus africanus*. **e**. Wilt of *Phyloteca myoporoides.* **F to i**. Vascular discolouration and wilt after *Fusarium oxysporum* inoculation of *Bougainvillea glabra* (f and g), *Eremophila laanii* (h) and *Phyloteca myoporoides* (i). **j**. Crown and root rot caused by *Neocosmospora solani* (= *Fusarium solani*) on *Ficus carica* (left) compared with control plants (right).

Table 3. Characteristics of the data partitions used for phylogenetic analyses in this study.

Species[a]	Data partition[b]	ML evolutionary model[c]	Number of characters[d]				
			Total	Conserved	Variable	Informative	BI unique sites
F. fujikuroi SC	ITS	SYM+I	459	420	39	32	50
	RPB1	SYM+I+G	1279	1038	241	148	195
	RPB2	GTR+I+G	1570	1251	319	216	332
	EF-1α	SYM+G	455	317	133	76	148
	TUB	SYM+I+G	507	389	117	65	140
F. oxysporum SC	*EF-1α*	GTR+I+G	591	448	143	101	67
	IGS	GTR+I+G	2190	1420	744	554	218
Neocosmospora	ITS	GTR+I+G	496	404	90	65	117
	LSU	GTR+I+G	481	450	31	14	25
	RPB2	GTR+I+G	1603	1235	365	243	275
	EF-1α	GTR+G	324	222	97	48	107

[a] SC: species complex.
[b] *EF-1α*: Translation elongation factor 1-alpha. IGS: Intergenic spacer region of the rDNA. ITS: Internal transcribed spacer regions of the rDNA and 5.8S region. *RPB1*: RNA polymerase largest subunit. *RPB2*: RNA polymerase second largest subunit. *TUB*: Beta-tubulin.
[c] G: Gamma distributed rate variation among sites. GTR: Generalised time-reversible. I: Proportion of invariable sites. ML: Maximum-likelihood; SYM: Symmetrical model.
[d] BI: Bayesian inference.

plants sometimes wilted and died. Wilted plants had conspicuous vascular brown discolourations from the crown to the canopy.

A total of 33 monosporic *Fusarium*-like isolates were collected (Table 2). Among these, two isolates were obtained from damping-off, 12 from root rot, and 19 were from wilted plants.

Phylogenetic analyses and species identification

Pairwise sequence alignments on the *Fusarium* MLST database and GenBank BLASTn searches demonstrated that 16 isolates belonged to the FOSC and 15 isolates to the FFSC, while two isolates were assigned to the genus *Neocosmospora* (*F. solani* species complex) (Table 2).

Subsequent more inclusive multilocus phylogenetic analyses identified a total of five *Fusarium* spp. and one *Neocosmospora* sp. The alignment characteristics and statistics are summarized in Table 3. The phylogenetic analyses of the 15 FFSC isolates from ornamentals revealed a total of four species (*F. agapanthi* O'Donnell, T. Aoki, J. Edwards & Summerell, *F. anthophilum* (A. Braun) Wollenw., *F. fujikuroi* Nirenberg and *F. proliferatum*) from different hosts (Figure 2). Isolates belonging to FOSC were studied based on a two-gene analysis using *EF-1α* and IGS sequences and incorporated in the original alignments previously published by O'Donnell *et al*. (2009) including representatives of 257 known FOSC haplotypes. The FOSC isolates from ornamentals belonged to 15 different haplotypes: isolates CPC 27748 and 22749, from *Cordyline australis* 'Purpurea' showed identical DNA sequences, and corresponded to haplotype 122 of FOSC; isolate CPC 27733 from *Philoteca myoporoides* belonged to haplotype 188, while each of the remaining isolates corresponded to a previously undescribed haplotype (Figure 3). The phylogeny of the genus *Neocosmospora* was based on *EF-1α*, ITS, LSU and *RPB2* sequences, and showed that two isolates from *Ficus carica* (CPC 27736 and 27737) belonged to *N. solani* (Martius) L. Lombard & Crous (= *F. solani*) (Figure 4).

Pathogenicity tests

Fourteen *Fusarium* and two *Neocosmospora* isolates tested were pathogenic to the different inoculated original hosts, and produced symptoms similar to those observed on diseased plants in nurseries (Figure 1). Two isolates were non-pathogenic. Damping-off occurred on *Agapanthus africanus*, crown and root rot on *F. carica* and root rot with subsequent leaf chlorosis appeared on *Trachycarpus princeps*. The remaining host plants showed vascular discolouration and wilted. The DI (%) caused by *Fusarium* and *Neocosmospora* species on different hosts ranged from 75 to 100%, after 15 d to 3 months (Table 2).

All *F. agapanthi*, *F. proliferatum*, and *N. solani* isolates were pathogenic, and caused 100% DI on *A. africanus*, *T. princeps* and *F. carica,* whereas *F. anthophilum*

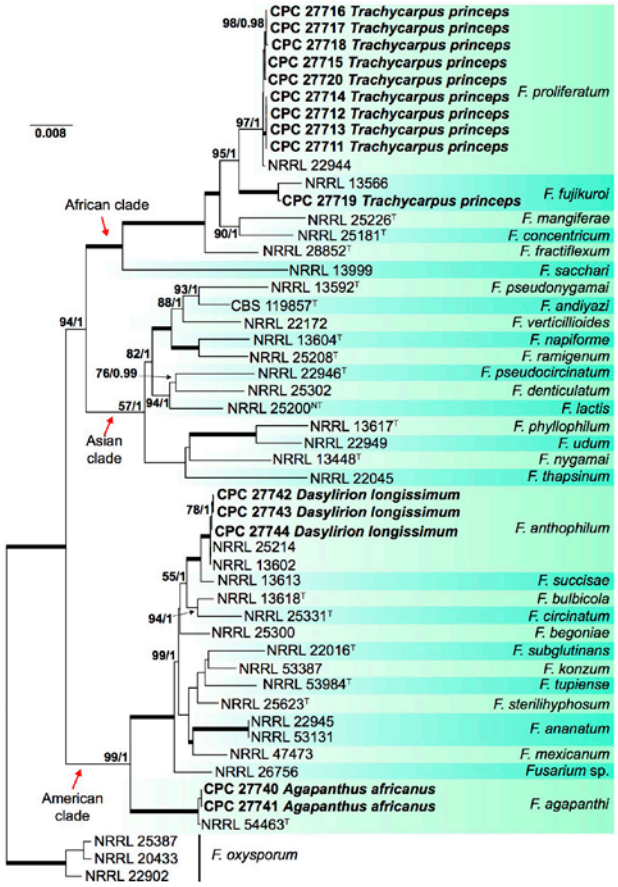

Figure 2. Maximum-likelihood (ML) phylogram of the *Fusarium fujikuroi* species complex obtained from combined ITS, *RPB1*, *RPB2*, *EF-1α* and *TUB* sequences. Branch lengths are proportional to distance. Numbers on the nodes are ML bootstrap values greater than 55%; and Bayesian posterior probability values greater than 0.95. Full supported branches and isolates obtained from ornamentals plants are indicated in bold. Ex-type strains are indicated with T and ex-neotype strains are indicated with NT.

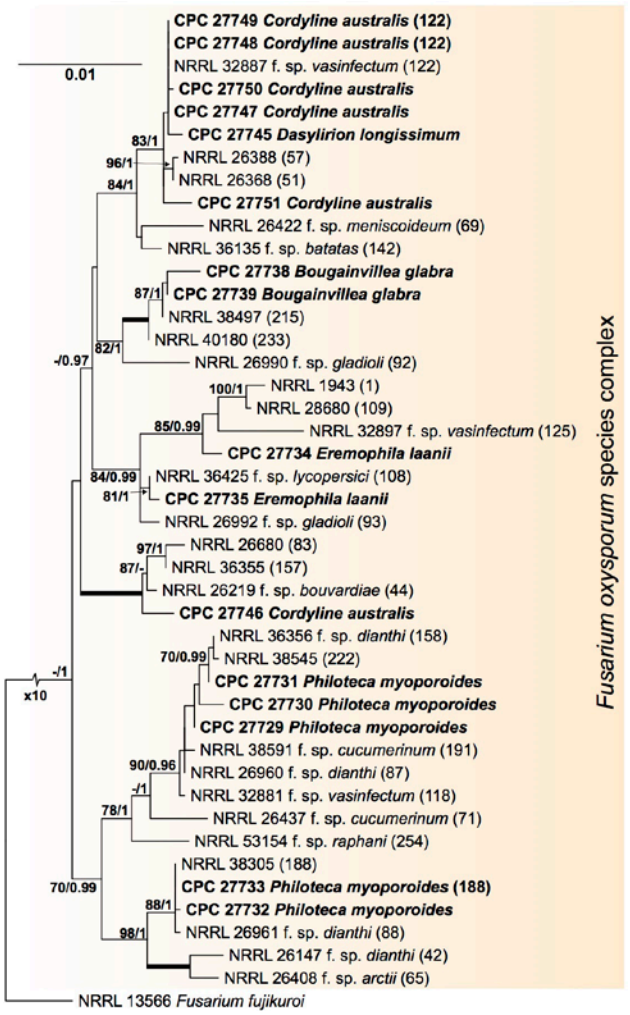

Figure 3. Maximum-likelihood (ML) phylogram of the *Fusarium oxysporum* species complex obtained from combined *EF-1α* and IGS sequences of isolates obtained in this study and representatives of the most closely related haplotypes. Branch lengths are proportional to distance. Numbers on the nodes are ML bootstrap values greater than 55%; and Bayesian posterior probability values greater than 0.95. Full supported branches and isolates obtained from ornamentals plants are indicated in bold. Numbers between parentheses indicate the corresponding haplotype.

caused disease with lower DI on *Dasylirion longissimum* (75%). *Fusarium oxysporum* isolates gave high DI (100%) on *Bougainvillea glabra*, *C. australis* 'Purpurea', *Eremophila laanii* and *P. myoporoides*, but was non-pathogenic on *D. longissimum*. Similarly, *F. fujikuroi* caused no symptoms on the original host *T. princeps*. The pathogens were re-isolated from the artificially inoculated plants, and were identified as previously described, fulfilling Koch's postulates. No symptoms were observed on control (uninoculated) plants.

DISCUSSION

The most important plant pathogenic *Fusarium* species is the soil-borne *F. oxysporum* Schltdl. (Gordon and Martyn, 1997; Gullino *et al.*, 2012), currently encompassing nearly 150 *formae speciales* (ff. spp.) and races. The broad host plant range of this fungus includes valuable ornamental plants such as *Chrysanthemum*, *Dianthus*, *Gerbera*, *Gladiolus*, and *Lilium* spp. (Engelhard and Woltz, 1971; Linderman, 1981; Farr and Rossman, 2018), on which it causes symptoms ranging from vascular wilt to crown and root rot (Engelhard and Woltz, 1971; Linderman, 1981).

Fusarium proliferatum (Matsush.) Nirenberg ex Gerlach & Nirenberg is another important species, which has

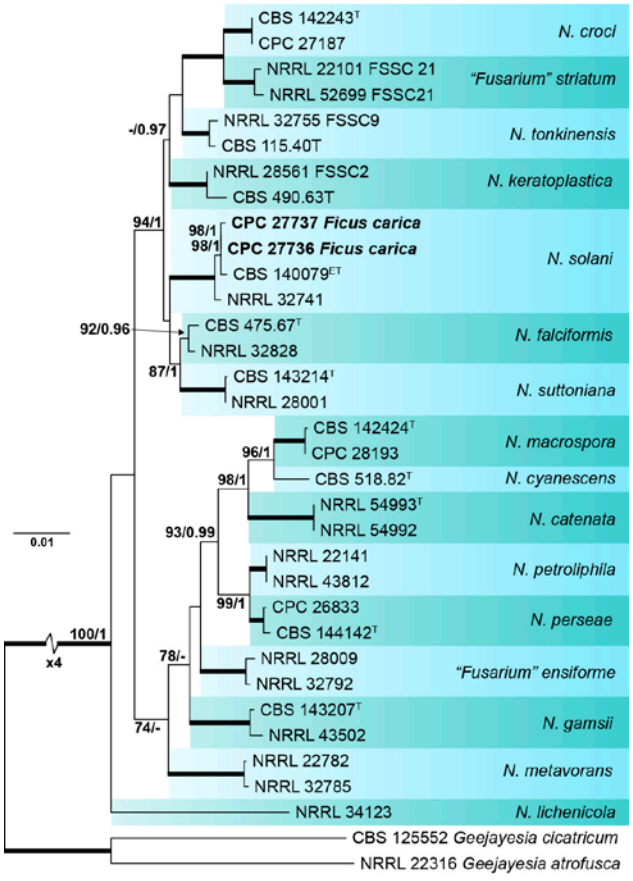

Figure 4. Maximum-likelihood (ML) phylogram of *Neocosmospora* (= *Fusarium solani* species complex) obtained from combined *EF-1α*, ITS, LSU and *RPB2* sequences. Branch lengths are proportional to distance. Numbers on the nodes are ML bootstrap values greater than 55%; and Bayesian posterior probability values greater than 0.95. Full supported branches and isolates obtained from ornamentals plants are indicated in bold. Ex-type and ex-epitype strains are indicated with ^{T} and ^{ET} respectively.

been described as the causal agent of blight, dieback and wilt of several palms belonging to the genera *Chamaerops*, *Phoenix*, *Ravenea*, *Trachycarpus* and *Washingtonia* (Polizzi and Vitale, 2003; Armengol *et al.,* 2005).

In the present study, two *Neocosmospora* and 31 *Fusarium* isolates were recovered from eight ornamental species in Sicily over a 5-year period. Disease symptoms were observed in five ornamental nurseries, and included damping-off, crown and/or root rot, and wilt. The isolates obtained from symptomatic tissues were identified based on single and multilocus phylogenetic analyses of seven loci (*EF-1α*, IGS, ITS, LSU, *RPB1, RPB2,* and *TUB*), as well as morphological characters. Our study revealed considerable diversity in the composition of *Fusarium*-like fungal populations recovered from nurseries.

As confirmed in the pathogenicity tests, all the *F. oxysporum* isolates caused symptoms except on *D. longissimum*. However, this host showed disease symptoms when inoculated with *F. anthophilum*. The inoculated isolate of *F. fujikuroi* produced no symptoms on *T. princeps*, while the remaining *Fusarium* species investigated, *F. agapanthi, F. proliferatum* and *N. solani*, were pathogenic to the respective tested hosts from which they were isolated.

Fusarium and *Neocosmospora* species are widespread in nurseries in Italy (Polizzi *et al.,* 2003; 2010a; 2010b; 2011; Bertoldo *et al.,* 2015), where they represent a limiting factor for production of ornamental plants cultivated in Sicily. These pathogenic species have very broad host ranges worldwide (Farr and Rossman, 2018). However, there are no known reports of diseases caused by *F. anthophilum* on *D. longissimum*. Moreover, *F. agapanthi* was originally described as pathogenic on *Agapanthus praecox* in Australia and Italy (Edwards *et al.,* 2016). However, this pathogen was isolated in the present study, causing serious seedling damping-off of *A. africanus*, suggesting that it may also be more prevalent on other species of *Agapanthus*. Previous studies have reported *F. proliferatum* associated with palms belonging to the genera *Chamaerops*, *Phoenix*, *Trachycarpus* and *Washingtonia* (Polizzi and Vitale, 2003; Armengol *et al.,* 2005). Our study presents a new report for *F. proliferatum* as a pathogen of *T. princeps*.

The FOSC includes soil-borne pathogens responsible for vascular wilts, stem cankers, rots, and damping-off of a wide range of agronomical and horticulturally important crops (Baayen *et al.,* 2000; Michielse and Rep, 2009; O'Donnell, 2009). Members of this complex collectively represent the most commonly found and economically important species complex within *Fusarium*. *Fusarium oxysporum* was the predominant species found in all the nurseries sampled, and unlike other species, it was recovered from multiple hosts. Recently, Polizzi *et al.* (2010a; 2010b; 2011) identified *F. oxysporum* associated with wilt diseases of *B. glabra*, *E. laanii* and *P. myoporoides*. However, no reports were previously known of diseases caused by *F. oxysporum* on *C. australis*, as reported here.

The present study is also the first report of *N. solani* causing crown and root rot of *F. carica* cuttings. This plant species is often cultivated for fruit production, and thousands of cuttings cultivated for ornamental purposes were investigated because serious losses were observed from of crown and root rot, leading to plant death. *Neocosmospora* is a species-rich genus containing at least 60 phylogenetically distinct species (O'Donnell, 2000; Zhang *et al.,* 2006; O'Donnell *et al.,* 2008; Nalim *et al.,*

2011). These fungi generally cause crown and/or root rot of infected host plants, while symptoms on aboveground plant portions may manifest as cankers, wilting, stunting and chlorosis, or as lesions on stems and/or leaves (Coleman, 2016; Guarnaccia *et al*., 2018).

The high disease incidence observed in the investigated ornamental nurseries probably depends on the prevailing climatic conditions, farming practices and environmental conditions such as temperature, humidity, irrigation systems or the use of non-disinfected plant growth substrates. Potted plant production could promote infections, since plants are frequently stressed due to being containerised during the production processes. Moreover, several wounds can occur during transplanting. Thus, prevention is a major strategy to control *Fusarium* diseases, and an accurate diagnosis of *Fusaria* species occurring in a particular area is significant for the selection of effective disease management strategies.

This study provides the first overview of *Fusarium* and *Neocosmospora* diversity associated with diseased ornamental plants in Southern Italy, and includes information on the pathogenicity of these fungi. It also provides the first reports of several new pathogen/host combinations, such as *N. solani* associated with crown and root rot of *F. carica*, and *F. agapanthi*, *F. anthophilum*, *F. oxysporum* and *F. proliferatum* as pathogens, respectively, of *A. africanus*, *D. longissimum*, *C. australis* and *T. princeps*.

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Research Papers

Distribution of large-spored *Alternaria* species associated with early blight of potato and tomato in Algeria

Djida AYAD[1,2,*], Djihad ARIBI[1], Bruno HAMON[2], Abdelaziz KEDAD[1], Philippe SIMONEAU[2], Zouaoui BOUZNAD[1]

[1] *ENSA-El Harrach, Laboratoire de Phytopathologie et Biologie Moléculaire. Algiers, Algeria*
[2] *UMR 1345 IRHS, Université d'Angers-INRA-Agrocampus Ouest, SFR QUASAV, 42 Rue G. Morel, F-49071 Beaucouzé, France*
*Corresponding author: medaydji@gmail.com

Abstract. Potato and tomato are important crops in Algerian agriculture, and both are threatened by abiotic and biotic stresses, and early blight is a major disease affecting both crops. Surveys carried out from 2012 to 2015 in 12 major growing regions for these crops yielded a total of 247 *Alternaria* isolates having morphological and cultural characteristics of sections *Alternaria* and *Porri*. Since early blight symptoms and morphological characteristics of the isolates did not allow sharp distinction between the different large-spored species of *Alternaria*, the isolates in section *Porri*, often considered primary causes of the diseases, were selected for molecular characterization by diagnostic PCR using specific primers. This allowed species identification of 147 *Alternaria* isolates as *A. solani*, *A. protenta, A. grandis* or *A. linariae*. These species were present on potato and tomato crops at varying frequencies, depending on the hosts and on bioclimatic locations. Pathogenicity tests for the four species, on detached leaflets and whole seedlings, showed that all were pathogenic to potato and tomato, with varying virulence. These results suggest that parasitic specialization of these *Alternaria* species on solanaceous plants should be reconsidered.

Keywords. Solanaceous crops, parasitic specialization, prevalence.

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INTRODUCTION

Potato (*Solanum tuberosum* L.) and tomato (*S. lycopersicum* L.) are important crops grown in Algeria. They are distributed differently in the various bioclimatic zones of the country, and are grown with different cultural practices. Potato crops occupy significant area in several regions of Algeria, from north (Ain Defla) to south (El Oued), and from east (Skikda, Guelma) to west (Mostaganem, Mascara). Tomato, however, is mainly cultivated in the northern part of the country, less extensively and mostly in plastic houses, as in the Biskra region. Both crops are often cultivated side by side or follow one another in rotations for several years, in many coastal regions of the

country. Intensification of these two crops without compliance to good agricultural practices led to the development of important diseases caused by fungi and oomycetes, including early blight and late blight.

The first reference mentioning *A. solani* Sorauer (= *Macrosporium solani* Ellis & G. Martin) as a pathogen causing potato leaf blight was that of Galloway (1891) in Australia. Chester (1892) then reported this pathogen in the United States of America, other solanaceous cultivated plants (Van der Waals *et al.*, 2001; Odilbekov, 2015). A few years later, *A. solani* and *A. alternata* (Fr.) Keissl. were identified on potato leaves but *A. alternata* was considered first as saprophytic (Jones and Grout, 1897). It was not until 1984 that *A. alternata* was recognized as pathogenic on several solanaceous plants (Droby *et al.* 1984). The name "early blight" for the disease caused by *A. solani* was coined by Jones (1893) to distinguish this from late blight. The symptoms of the early blight appear on the leaves as dark, elongated or circular lesions each with concentric rings surrounded by a yellow halo.

On the basis of cultural techniques and morphological characteristics of the conidia (size and beak length), Simmons (2000, 2007) described several new *Alternaria* species on *Solanaceae*, that may be responsible for early blight on potato and tomato. Thus, new species were described, which are morphologically very similar to *A. solani*. These include *A. grandis* E.G. Simmons on *S. tuberosum*, *A. tomatophila* E.G. Simmons on *S. lycopersicum*, *A. cretica* E.G. Simmons & Vakal. On *S. lycopersicum* var. *esculentum*, and *A. subcylindrica* E.G. Simmons & R.G. Roberts on *S. lycopersicum* var. *cerasiforme*.

More recently, molecular and genomics techniques applied to *Alternaria* allowed redefinition of various species with large conidia commonly isolated from *Solanaceae*, and considered as the primary causes of early blight. Woudenberg *et al.* (2014), by synonymizing *A. linariae* (Neerg.) E.G. Simmons with *A. cretica* E.G. Simmons & Vakal., *A. cucumericola* E.G. Simmons & C.F. Hill, *A. subcylindrica*, *A. tabasco* E.G. Simmons & R.G. Roberts, and *A. tomatophila*, have expanded the host range of *A. linariae* to *Solanaceae*, *Cucurbitaceae*, and *Scrophulariaceae*. *Alternaria linariae*, a common pathogen of tomato, was also reported on potato during surveys carried out in Algeria (Ayad *et al.*, 2018). *Alternaria Grandis*, which was confused for a long time with *A. solani*, has been regularly reported in many countries on potato (Lourenço *et al.,* 2009; Cardoso, 2014; Bessadat *et al.*, 2016; Landschoot *et al.*, 2017). *Alternaria grandis* was also recently isolated from tomato (Bessadat *et al.*, 2017) in the northwest of Algeria. *Alternaria protenta*, which is closely related to *A. solani* and whose host range was extended to *Asteraceae*, *Euphorbiaceae*, *Gramineae* and *Solanaceae* by Woudenberg *et al.* (2014), was recently detected on potato in Belgium (Landschoot *et al.*, 2017) and in Algeria (Ayad *et al.*, 2017).

The aims of the present study were, firstly, to identify at the species level the large-spored *Alternaria* isolates obtained from surveys carried out during three cropping years on different potato and tomato fields across the main bioclimatic areas of Algeria. Since early blight symptoms and the morphological characteristics of the isolates do not allow distinction between the different large-spored *Alternaria* spp., specific identification of the isolates was based on PCR techniques. The second objective was to assess the aggressiveness of isolates on their respective potato and tomato hosts, and to clarify the parasitic specialization of replace by large-spored *Alternaria* species by artificial cross inoculations *in vitro* and under conditions similar to those occurring in the field.

MATERIALS AND METHODS

Surveys and sampling

Surveys were carried in 60 locations across 12 regions in Algeria, with different cropping systems (fields and greenhouses). Three to four plots were sampled in each region during three successive cropping years: 2012–2013, 2013–2014 and 2014–2015. The majority of the localities were selected from the main potato- and tomato-producing regions represented by different bioclimatic areas of Algeria: Mediterranean climate (Algiers, Tipaza, Guelma and Skikda), semi-arid climate (Mostaganem, Chlef, Mascara, Ain Defla and Bouira), and arid climate (Laghouat, Biskra and El Oued) (Figure 1). Sampling was carried out from potato and tomato plant organs showing the typical symptoms of early blight: 164 isolates were collected from potato leaves essentially and 83 from leaves, stems and fruits of tomato.

Isolation, purification and conservation of isolates

Isolations were performed according to the method of Van der Waals *et al.* (2004). Small tissue pieces (3 to 4 mm^2) were cut from individual lesion edges, disinfected in 1% active NaOCl for 3 min, washed twice in sterile distilled water, and then dried with absorbent sterile paper. Four pieces of tissues were plated on individual Petri plates containing Potato Dextrose Agar (PDA), and these were incubated at 22°C under continuous light for 1 to 2 weeks. The purification of the *Alternaria* spp. isolates as single conidium cultures was car-

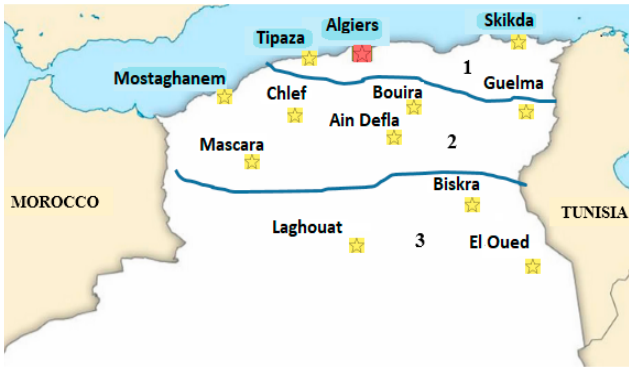

Figure 1. Geographic localities in the different bioclimatic areas: 1 = Mediterranean climate, 2 = semi-arid climate, 3 = arid climate

ried out between the 3rd and the 5th day of incubation. Plates containing the single conidium cultures were then placed under near-UV (12h dark and 12h light) to induce sporulation and allow further morphological characterization.

Single conidium cultures for short-term use were grown in Petri dishes containing a PDA for 12 d then stored at 4°C. For medium term use, 12-d-old PDA cultures in test tubes were stored at 4°C. For long term use, mycelium/agar plugs (8 mm diam.) were stored at 80°C in cryotubes containing sterile glycerol (30% w/w).

Identification of the large-spored Alternaria species using molecular markers

On the basis of the cultural and the morphological characteristics, 155 isolates with large conidia (out of a total 247 isolates) were selected for molecular characterization by PCR using specific primers, for analysis of genomic DNA by PCR/RFLP, using double enzymatic digestion of a portion of the calmodulin gene and sequencing the calmodulin and the RPB2 genes. Isolates were grown on PDA medium at room temperature for 10 d. Total genomic DNA from fungal samples was extracted according to the method described by Goodwin and Lee (1993). The DNA extracts of the 155 isolates were amplified using two sets of specific primer pairs to differentiate between *A. linariae* and *A. solani* and related species (*A. grandis* and *A. protenta*) (Gannibal *et al.*, 2014). The primer pair OasF7 and OasR6 amplified a 164 bp fragment from the Alt a1 gene of *A. solani*, *A. grandis* and *A. protenta*, and the primer pair OatF4 and OatR2 amplified a 438 pb fragment from the calmodulin encoding gene of *A. linareae*. The PCR conditions were as described by Gannibal *et al.* (2014). To differentiate between *A. grandis* and *A. solani sensu lato* (i.e. including *A. protenta*), a portion of the calmodulin gene was amplified from the isolates that each gave a positive signal with the primer pair OasF7 – OasR6 using the primer pair CALDF1 / CALDR1 (Lawrence *et al.*, 2013). The resulting PCR products were double digested with the restriction enzymes *Rsa*I and *Hae*II. The digestion products were then separated by electrophoresis in 2% agarose gels and visualized under UV light after staining with ethidium bromide. Predicted restriction patterns mainly differed by the size of the larger fragment, i.e. 420 bp for *A. solani sensu lato* and 292 bp for *A. grandis* (Figure 2). Identification of the *Alternaria* species was validated by sequencing the fragment of the calmodulin locus amplified as described above from 42 isolates. To confirm the presence of *A. protenta*, a portion of the RPB2 locus was amplified from the isolates identified as *A. solani* based on their calmodulin sequence, using the primer pair RPB2-5F2 (Sung *et al.*, 2007) and RPB2-7cR (Liu *et al.*, 1999), and the resulting PCR product was sequenced. Sanger sequencing was performed by GATC Biotech. Sequence analyses were carried out using the Phylogeny.fr web service (Dereeper *et al.*, 2008). Multiple sequence alignments were generated with MUSCLE and curated using the Gblocks algorithm. Maximum likelihood (ML) analyses were performed with PhyML. The robustness of the ML topologies was evaluated using the Shimodaira-Hasegawa (SH)-like test for branches.

Pathogenicity tests of large-spored Alternaria species on their respective hosts

To confirm pathogenicity of isolates, inoculations onto detached leaflets and whole plants were performed on susceptible varieties of tomato (cv. Marmande) and

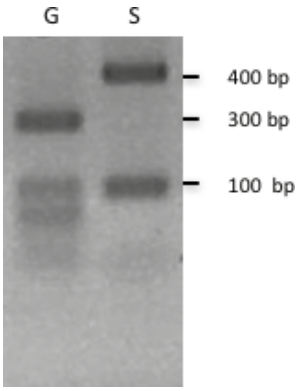

Figure 2. Typical restriction patterns obtained by double digestion of PCR product corresponding to a portion of the calmodulin gene from *A. solani* and *A. grandis*. Size of the larger fragment, 420 bp for *A. solani sensu lato* (S) and 292 bp for *A. grandis* (G)

potato (cv. Spunta). These isolates were selected for their ability to produce conidia. Conidium suspensions were prepared from 15-d-old cultures. The final concentration of the suspensions was adjusted to 10^4 conidia mL^{-1}. The inoculations were performed by depositing drops (20 µL each) on detached leaflets or by spraying suspensions onto whole plants. Disease severity was evaluated at 15 d –post-inoculation, using a visual rating scale from 1 to 9 expressing the extension of necrosis CIP (International Potato Center) (2008).

RESULTS

Characterization of the large-spored Alternaria species according to early blight symptoms on potato and tomato

Isolation from plant samples showing typical early blight symptoms yielded 247 isolates (164 from potato and 83 from tomato). On the basis of the cultural and morphological characteristics of the colonies and conidia, the isolates were divided in three groups. The first (Group A: 92 isolates) included all the isolates with abundant conidium production and small catenulate conidia and identified as species belonging to section *Alternaria*. The second (Group B: 92 isolates) contained all isolates producing large solitary beaked conidia, related to species belonging to the section *Porri*. The third (Group C: 63 isolates) consisted of large-spored isolates that only formed sterile mycelium after culture purification. In relation to the host plants, 89% of the isolates belonging to the section *Porri* originated from potato, and 73% of the non-sporulating isolates originated from tomato. No correlation was found between the presence of yellow halos on symptomatic leaves and the production of a diffusible pigment on growth medium by the isolates from the diseased samples. Similarly, the various forms of symptoms generated colonies producing either large or small conidia. Thus, there was no relationships between necrosis size (small, medium or large necrotic lesions), the types of the colonies in culture medium (sporulating or non-sporulating) and the conidium types (large solitary or small catenulate conidia).

Molecular characterization and identification of large-spored Alternaria species

Given the very similar morphological characteristics of the conidia, and the difficulty to distinguish the different *Alternaria* species within the section *Porri*, molecular markers and DNA-sequencing were used for their identification. The small-spored isolates (Group A) were not included in this examination. The three molecular approaches allowed clear species identification for 147 of 155 isolates from Groups B and C, while eight isolates could not be identified due to problems in DNA extraction and amplification at the selected loci (Table 1). Preliminary tests were carried out for the identification of the 147 isolates using two pairs of specific primers that differentiate *A. solani*, *A. protenta* and *A. grandis* (OasF7/OasR6) from *A. linariae* (OATF4/OATR2). All the tested isolates generated positive signals with one of the two primer pairs, confirming the presence of these species on potato and tomato crops in Algeria. In this analysis, 37 isolates including seven from potato and 30 from tomato, were identified as *A. linariae* by amplification of the 438 pb from the gene encoding for calmodulin (Table 1). A PCR product corresponding to a fragment of the Alt a1 gene (164 bp) was amplified from the remaining 110 isolates. These isolates, of which 86 were isolated from potato and 24 from tomato, could be assigned to either *A. solani* or to phylogenetically related-species, i.e. *A. grandis* and *A. protenta*. Further identification was conducted in order to differentiate between *A. solani* and *A. grandis* isolates using PCR/RFLP, by amplifying the calmodulin locus followed by restriction enzyme digestion (*Hae*II and *Rsa*I). Based on the characteristic electrophoretic profiles of the restricted PCR products, *A. solani* and *A. protenta* were separated from *A. grandis*. Thus, of the 110 isolates that gave positive signals with the OasF7/OasR6 primer pair, 93 were *A. solani*, 12 were *A. grandis* and five showed abnormal enzymatic restriction digestion patterns that may be due to partial digestion with at least one of the restriction enzymes. These five isolates, as well as 37 other representative isolates obtained from the two host plants collected in nine of the twelve surveyed Algerian regions and representing the three identified species (*A. linariae*, *A. grandis* and *A. solani*), were selected for species confirmation by DNA sequencing which was firstly performed at the calmodulin locus. Phylogenetic analyses of sequences derived from the calmodulin locus of the 42 isolates (Figure 3) separated the isolates into two clades: the first (A) contained the isolates previously identified by PCR with specific primers as *A. linariae*, and the second (B) included two sub clades (B1 and B2) regrouping isolates previously identified as *A. grandis* (sub clade B1) and *A. solani* (sub clade B2). DNA sequencing at the RPB2 locus then carried out on the isolates of the sub clade B2 gave the phylogenetic tree shown in Figure 4, in which among the 17 isolates of *A. solani*, three were identified as *A. protenta*, two of which were isolated from the potato and one from tomato.

Table 1. Identification of large-spored of *Alternaria* spp isolates.

Isolate	Species[a]	Host	Geographic origin	PCR OATF4/ OATR2	PCR OAsF7/OAsR6	PCR RFLP Profile[b]	CAL	Rpb2
DA001	***A. linariae***	Potato	Tipaza	+	-	ND	MH243795	_
DA002	A. linariae	Potato	Tipaza	+	-	ND	MH243769	_
DA003	***A. linariae***	Potato	Tipaza	+	-	ND	MH243793	_
DA005	*A. solani s.l*	Potato	Alger	-	+	S	_	_
DA006	***A. linariae***	Potato	Alger	+	-	ND	MH243789	_
DA007	***A. linariae***	Potato	Alger	+	-	ND	MH243794	_
DA008	***A. solani***	Potato	Alger	-	+	S	MH243805	MH243818
DA009	***A. grandis***	Potato	Alger	-	+	?	MH243790	_
DA010	*A. solani s.l*	Potato	Alger	-	+	S	_	_
DA011	***A. solani***	Potato	Alger	-	+	S	MH243806	MH243820
DA012	*A. solani s.l*	Potato	Alger	-	+	S	_	_
DA013	*A. solani s.l*	Potato	Alger	-	+	S	_	_
DA014	*A. solani s.l*	Potato	Alger	-	+	S	MH243796	_
DA015	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA016	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA017	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA018	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA019	***A. solani***	Potato	Bouira	-	+	S	MH243808	MH243822
DA020	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA021	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA022	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA025	***A. solani***	Potato	Bouira	-	+	S	MH243786	MH243823
DA026	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA028	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA029	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA030	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA031	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA032	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA033	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA034	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA035	*A. solani s.l*	Potato	Mascara	-	+	S	_	_
DA036	*A. solani s.l*	Potato	Mascara	-	+	S	_	_
DA037	*A. linariae*	Tomato	Mostaganem	+	-	ND	_	_
DA038	***A. grandis***	Potato	Alger	-	+	G	MH243770	_
DA039	*A. linariae*	Potato	Alger	+	-	ND	_	_
DA040	***A. linariae***	Tomato	Mascara	+	-	ND	MH243792	_
DA041	*A. linariae*	Tomato	Mostaganem	+	-	ND	_	_
DA042	*A. linariae*	Tomato	Mostaganem	+	-	ND	_	_
DA043	*A. solani s.l*	Potato	Mostaganem	-	+	S	_	_
DA045	*A. solani s.l*	Potato	Mascara	-	+	S	_	_
DA046	*A. solani s.l*	Potato	Mostaganem	-	+	S	_	_
DA047	***A. grandis***	Potato	Mostaganem	-	+	G	MH243771	_
DA048	*A. solani s.l*	Potato	Mostaganem	-	+	S	_	_
DA049	*A. solani s.l*	Potato	Chlef	-	+	S	_	_
DA050	***A. solani***	Potato	Chlef	-	+	S	MH243772	MH243809
DA051	***A. grandis***	Potato	Chlef	-	+	G	_	_
DA052	***A. grandis***	Potato	Chlef	-	+	G	MH243773	_

(Continued)

Table 1. (Continued).

Isolate	Species[a]	Host	Geographic origin	PCR OATF4/ OATR2	PCR OAsF7/OAsR6	PCR RFLP Profile[b]	CAL	Rpb2
DA053	*A. solani s.l*	Potato	Chlef	-	+	S	_	_
DA054	***A. grandis***	Potato	Mostaganem	-	+	G	MH243774	_
DA055	***A. grandis***	Potato	Mostaganem	-	+	G	_	_
DA056	*A. solani s.l*	Potato	Mostaganem	-	+	S	_	_
DA057	***A. solani***	Potato	Mascara	-	+	?	MH243791	MH243819
DA058	*A. solani s.l*	Potato	Mascara	-	+	S	_	_
DA059	*A. solani s.l*	Potato	Mostaganem	-	+	S	_	_
DA060	***A. grandis***	Potato	Mostaganem	-	+	G	MH243775	_
DA061	***A. grandis***	Potato	Mostaganem	-	+	G	_	_
DA062	***A. protenta***	Tomato	Mostaganem	-	+	S	MH243797	MH243810
DA063	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA064	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA065	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA066	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA067	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA068	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA069	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA070	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA071	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA072	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA073	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA074	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA075	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA076	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA077	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA078	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA079	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA080	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA081	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA082	***A. protenta***	Potato	El Oued	-	+	?	KX870505	KX870507
DA083	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA084	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA085	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA086	***A. protenta***	Potato	El Oued	-	+	S	KX870506	KX870508
DA087	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA088	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA089	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA090	***A. solani***	Potato	El Oued	-	+	S	MH243776	MH243811
DA091	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA092	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA093	*A. solani s.l*	Potato	Alger	-	+	S	_	_
DA094	***A. solani***	Potato	Alger	-	+	S	MH243798	MH243812
DA096	*A. solani s.l*	Potato	Alger	-	+	S	_	_
DA097	*A. solani s.l*	Potato	Alger	-	+	S	_	_
DA098	*A. solani s.l*	Potato	Alger	-	+	S	_	_
DA099	***A. grandis***	Tomato	Alger	-	+	G	MH243777	_
DA100	***A. linariae***	Tomato	Alger	+	-	ND	MH243807	_

(Continued)

Table 1. (Continued).

Isolate	Species[a]	Host	Geographic origin	PCR OATF4/ OATR2	PCR OAsF7/OAsR6	PCR RFLP Profile[b]	CAL	Rpb2
DA101	***A. linariae***	Tomato	Alger	+	-	ND	MH243778	_
DA102	*A. linariae*	Tomato	Alger	+	-	ND	_	_
DA103	*A. linariae*	Tomato	Alger	+	-	ND	_	_
DA104	*A. linariae*	Tomato	Alger	+	-	ND	_	_
DA107	*A. linariae*	Tomato	Alger	+	-	ND	_	_
DA108	*A. solani s.l*	Potato	Guelma	-	+	S	_	_
DA109	***A. linariae***	Tomato	Tipaza	+	-	ND	MH243779	_
DA110	*A. linariae*	Tomato	Tipaza	+	-	ND	_	_
DA111	***A.linariae***	Tomato	Tipaza	+	-	ND	MH243780	_
DA112	*A. linariae*	Potato	Skikda	+	-	ND	_	_
DA113	***A. solani***	Tomato	Guelma	-	+	S	MH243799	MH243821
DA114	***A. solani***	Potato	Guelma	-	+	?	MH243788	MH243813
DA115	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA116	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA117	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA118	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA119	***A. linariae***	Tomato	Biskra	+	-	ND	MH243800	_
DA120	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA121	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA122	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA123	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA124	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA125	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA126	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA127	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA128	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA129	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA130	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA131	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA132	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA133	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA134	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA135	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA136	***A. solani***	Tomato	Biskra	-	+	S	MH243801	MH243814
DA137	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA138	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA139	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA140	***A. solani***	Tomato	Biskra	-	+	S	MH243802	MH243815
DA141	***A. solani***	Tomato	Biskra	-	+	S	MH243803	MH243816
DA142	***A. solani***	Tomato	Biskra	-	+	S	MH243804	MH243817
DA143	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA144	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA145	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA146	*A. solani s.l*	Potato	Tipaza	-	+	S	_	_
DA147	***A. linariae***	Tomato	Tipaza	+	-	ND	MH243781	_
DA148	*A. linariae*	Tomato	Mostaganem	+	-	ND	_	_
DA149	*A .linariae*	Tomato	Mostaganem	+	-	ND	_	_

(Continued)

Table 1. (Continued).

Isolate	Species[a]	Host	Geographic origin	PCR OATF4/ OATR2	PCR OAsF7/OAsR6	PCR RFLP Profile[b]	CAL	Rpb2
DA150	*A. linariae*	Tomato	Mostaganem	+	-	ND	_	_
DA152	***A. grandis***	Tomato	Alger	-	+	G	MH243782	_
DA153	***A. linariae***	Tomato	Alger	+	-	ND	MH243783	_
DA154	***A .grandis***	Tomato	Alger	-	+	?	MH243787	_
DA155	***A. grandis***	Tomato	Alger	-	+	G	MH243784	_
DA156	***A. grandis***	Tomato	Alger	-	+	G	MH243785	_

[a] Species names in bold characters were supported by sequence data; *A. solani s.l* stands for *A. solani sensu lato* (i.e. including *A. protenta*). [b] PCR-RFLP profiles: S = typical of *S. solani*; G = typical of *A. grandis*; ND = not determined; ? = atypical restriction profile

Figure 3. Phylogenetic tree reconstructed by the maximum likelihood method from the alignment of calmodulin sequences of 42 isolates belonging to the *Alternaria* section *Porri*. Bootstrap support values greater than 80 % are indicated by arrows. The calmodulin sequences of the following strains were included as references: *A. linariae* CBS 109156 (GenBank number JQ646257), *A. protenta* CBS 116696 (GenBank number JQ646236), *A. solani* CBS 109157 (GenBank number KJ397981), *A.grandis* CBS 109158 (GenBank number JQ646249). The calmodulin sequence from *A. alternata* EGS 34016 (Genbank number JQ646208) was used to root the tree.

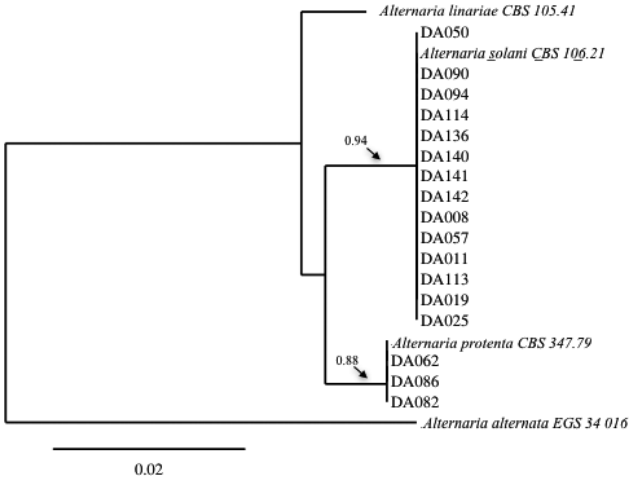

Figure 4. Phylogenetic tree reconstructed by the maximum likelihood method from the alignment of RPB2 sequences of 17 isolates belonging to cluster B2 in the calmodulin phylogeny. Bootstrap support values greater than 80 % are indicated by arrows. The RPB2 sequences of the following strains were included as references: *A. linariae* CBS 105.41 (GenBank number KJ718353), *A. solani* CBS 106.21 (GenBank number KJ718410) *A. protenta* CBS 347.79 (GenBank number KJ718392). The RPB2 sequence from *A. alternata* EGS 34016 (Genbank number JQ646490) was used to root the tree.

Distribution and prevalence of the large-spored Alternaria species in the bioclimatic regions of Algeria

The surveys carried out through the different Algerian potato and tomato growing regions revealed the presence of *A. grandis*, *A. linariae* and the species complex *A. solani*-*A. protenta* at variable levels. The frequencies of these species varied according to the host plants (potato or tomato) and to the geographical locations (Table 2). *Alternaria solani sensu lato* (i.e. including *A. protenta*) was the most common species in Algeria, representing 65% of the large-spored isolates, followed by *A. linariae* (25%) and *A. grandis* with low isolation frequency (10%). Isolation frequencies of these species varied with the sampled crop, although they were all found on both potato and tomato. *Alternaria solani sensu lato* (80%) and *A. grandis* (64%) were more prevalent on potato than on tomato. Conversely, *A. linariae*

Table 2. Frequencies of the four large-spored *Alternaria* species according to the host plants (potato and tomato) and to the geographical locations

Origin	*A. solani-A protenta*	*A. grandis*	*A. linariae*
Host plant origin			
Potato	80.2[a]–82.8[b]	64.3–9.6	18.9–7.5
Tomato	19.8–35.2	35.7–9.2	81.1–55.6
Geographic origin (bioclimatic area)			
Mediterranean	16.7–39.0	50.0–17.0	48.6–44.0
Semi-arid	34.3–70.2	50.0–14.9	18.9–14.9
Arid	49.0–79.7	0–0	32.4–20.3

[a] Percentage of the total number of isolates of the same species according to the host plant or the geographic origin
[b] Percentage of the total number of isolates from the same host or from the same geographic origin

Figure 5. Symptoms on detached leaflets (a, d) and whole tomato (c, e) or potato (b, f) plants inoculated by *A. linariae* isolate DA002 (a, b and c) or by *A. grandis* isolate DA009 (d, e and f)

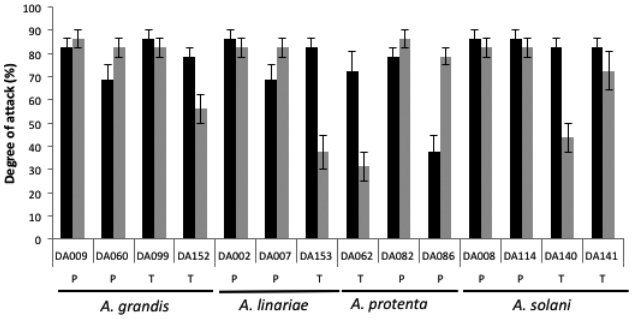

Figure 6. Disease severity evaluated using a visual rating scale (0-100%) at 15 days post-inoculation of tomato (black bars) and potato (grey bars) leaves with isolates representing the four large-spored *Alternaria* species. Letters (P or T) under the isolates identities refer to the host of origin (Potato or Tomato).

was more frequently isolated from tomato than from potato. Locations of sample collection could be divided into three temperature and precipitation zones (Mediterranean, semi-arid or arid). In Mediterranean and arid zones, isolates were almost equally distributed between potato and tomato. *Alternaria linariae* was over-represented in the Mediterranean zone (44% of the collected isolates) compared to the arid zone where it represented only 20% of the collected isolates. The reverse situation was observed for *A. solani sensu lato*. *Alternaria grandis* was not isolated from samples collected from the arid zone, although it represented 15% of isolates from the semi-arid zone and 17% of those from the Mediterranean zone. Only small-spored *Alternaria* isolates were obtained from samples in two locations, i.e. Ain Defla and Laghouat.

Pathogenicity and parasitic specialization of Alternaria species on potato and tomato

Fourteen isolates representing the four large-spored *Alternaria* species were used in the pathogenicity tests. These were: *A. solani* (isolates DA008, DA114, DA140 and DA141), *A. grandis* (isolates DA009, DA060, DA099 and DA152), *A. protenta* (DA062, DA082 and DA086) and *A. linariae* (DA002, DA007 and DA153). The symptoms (necroses surrounded by yellow halos) obtained on detached leaflets in Petri dish assays were confirmed by high degrees of aggressiveness obtained on whole seedling plants of potato and tomato (Figure 5). All the tested isolates produced symptoms on both plant species irrespective of their host plant of origin (Figure 6). The degrees of aggressiveness for each host/pathogen combination varied for the isolates, however. On average, *A. solani* isolates were more aggressive on both tomato and potato, while *A. protenta* isolates were less aggressive with a marked preference for their original host plants.

DISCUSSION

Early blight has long been attributed to large-spored *Alternaria* species, i.e. *A. solani* and *A. grandis* on potato and *A. solani* and *A. linariae* on tomato. Small-spored species related to *A. alternata* are considered to be responsible for the brown spot disease (Nolte, 2008, Tymon *et al.*, 2016). The surveys carried out in Alge-

ria between 2012 and 2015 have shown the presence of several *Alternaria* species producing large conidia in samples from both hosts with typical early blight symptoms, as well as in samples with symptoms that resemble brown spot. On the other hand, many Algerian isolates of the section *Alternaria* collected by Bessadat *et al.* (2017) have caused typical early blight symptoms after artificial inoculations on tomato, and were therefore considered pathogenic but with low aggressiveness. This complicated the attribution of a given symptom to a given *Alternaria* species. Moreover, morphological similarities between species within the *Alternaria* section *Porri* precludes reliable identification at the species level based only on these criteria. In the present work, molecular tools were used to confirm the identity of large-spored isolates originating from tomato and potato leaves. The diagnostic species-specific primers developed by Gannibal *et al.* (2014) were successfully used to quickly characterized 147 isolates from a collection the 155 large-spored isolates, and to distinguish *A. linariae* from isolates that could correspond to either *A. solani*, *A. grandis* or *A. protenta*. No amplification signal was obtained for eight isolates either due to poor quality of the extracted DNA or because these isolates corresponded to another species within section *Porri*. Although never previously observed in Algeria, it has recently been reported that *A. macrospora* may be responsible for leaf spot of tomato in China (Zhang *et al.*, 2017). To discriminate between *A. grandis*, on one hand, and *A. solani* or *A. protenta*, on the other, we took advantage of sequence polymorphism within the calmodulin gene to develop a PCR-RFLP assay. Reliability of these diagnostic methods was checked by sequencing the calmodulin locus, and taken together they allowed us to accurately type more than 90% of the large-spored isolates without the need for nucleotide sequencing. Applying these tools to our isolates, we observed that *A. solani* and *A. linariae* predominate on their respective potato or tomato hosts in most of the surveyed areas. In agreement with recent observations (Bessadat *et al.*, 2017, Ayad *et al.*, 2018), the presence of *A. linariae* and *A. grandis* in Algeria, traditionally considered to be restricted, respectively, to either tomato or potato, was also confirmed on these two plant hosts. *Alternaria linariae* was found on potato and *A. grandis* on tomato in the coastal regions of Algiers and Tipaza, where these two crops often exist side by side or follow each other in the same plot during the seasons. *Alternaria grandis* has not been isolated from the Saharan regions that are characterized by warm and arid periods during each year. Analyses of nucleotidic polymorphism at the RPB2 locus has shown that *A. protenta* was recorded for the first time on tomato in the coastal region of Mostaganem (northwest of Algeria), where the climate is very different from that of the South. This species has already been reported on potato in Belgium (Landschoot *et al.,* 2017), but also in a particular region of the south of Algeria (El Oued), where the temperatures are high and the potato crops are grown under overhead irrigation (Ayad *et al.*, 2017).

Cross inoculations carried out with isolates representing the four identified large-spored *Alternaria* species (*A. solani*, *A. grandis*, *A. linariae* and *A. protenta*) on potato and tomato, showed that aggressiveness toward the two host plants varied according to the isolates but not with respect to particular fungal species. Similar experiments carried out on detached leaves with *A. linariae* and *A. grandis* (Rodrigues *et al.*, 2010; Cardoso, 2014; Gannibal *et al.*, 2014) have previously shown that these two species were able to cause disease on both plants with more severe symptoms on their respective traditional hosts. This suggests that the parasitic specialization of large-spored *Alternaria* species on solanaceous crops should be reconsidered.

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Research Papers

Endophytic fungal communities of ancient wheat varieties

Giulia CASINI[1,2], Thaer YASEEN[3], Ahmed ABDELFATTAH[4,5*], Franco SANTORO[6], Leonardo VARVARO[1], Sandro DRAGO[2], Leonardo SCHENA[4]

[1] *Department of Agriculture and Forestry Science, University of Tuscia, Via S. Camillo de Lellis s.n.c., 01100 Viterbo (VT), Italy*
[2] *Enbiotech S.r.l., via Quarto dei mille, 6, 90144 Palermo (PA), Italy*
[3] *Regional Office for the Near East and North Africa Region (RNE), Food and Agriculture Organization of the United Nations (FAO), Cairo, Egypt*
[4] *Dipartimento di Agraria, Università Mediterranea di Reggio Calabria, Località Feo di Vito, 89122 Reggio Calabria (RC), Italy*
[5] *Department of Ecology, Environment and Plant Sciences, Stockholm University, SE-106 91, Stockholm, Sweden*
[6] *CIHEAM – Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (BA), Italy*
* Corresponding author: ahmed.abdelfattah@su.se

Abstract. The fungal community composition and structure of two ancient tetraploid wheat varieties, native to the Sicilian territory of Italy, Perciasacchi (winter wheat) and Tumminia (spring wheat) were investigated using High Throughput Sequencing (HTS). This showed a predominance of Ascomycetes and Basidiomycetes including *Alternaria*, *Fusarium*, *Mycosphaerella, Filobasidium, Cystofilobasidium, Cryptococcus*, *Leucosporidium*, *Dioszegia, Puccinia*, *Sporobolomyces, Aureobasidium, Cladosporium, Holtermanniella* and *Gibberella*. Principal Coordinates Analysis (PCoA) and Linear discriminant analysis Effect Size (LEfSe) showed that *Aureobasidium, Leucosporidium* and *Puccinia* differentiated between the two wheat varieties. In addition, the microbial association analysis suggested that some endophytic taxa play important roles within the wheat fungal community. Genera such as *Cryptococcus* and *Cystofilobasidium* were shown to have consistent antagonistic activity against *Gibberella* spp., while, *Acremonium* and a group of unidentified ascomycetes had mutual exclusion relationships with *Puccinia*. Since both *Gibberella* and *Puccinia* contain several economically important pathogens of wheat, the detected fungal interactions may indicate microbial-mediated resistance in these wheat varieties.

Keywords. TS, fungal diversity, microbial ecology, microbiome, domestication.

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INTRODUCTION

Plant-associated microorganisms, collectively referred to as the plant microbiota, are known to influence plant physiological development, and response to environmental changes by conferring stress tolerance, disease resistance, and affecting interactions with competitors and predators

(Lugtenberg and Kamilova 2009; Berendsen *et al.,* 2012). Endophytes are microorganisms that can be isolated from surface-disinfested plant tissues or extracted from within plants, and that do not harm plant growth (Hallmann *et al.,* 1997). Endophytes have gained importance in basic and translational science due to their potential roles as biocontrol agents (Dorworth and Callan 1996; Massart *et al.,* 2015; Abdelfattah *et al.,* 2018).

Wheat (*Triticum* sp.) is a global staple food crop adapted to a wide variety of environmental conditions, including marginal areas (Salmon and Clark, 1913). In Italy, production of durum wheat (*Triticum durum*) accounts for more than 50% of the total European production (European Commission, Eurostat and DG Agriculture and Rural Development). Production of this crop is mainly concentrated in the hot and dry southern regions of Italy, including Sicily (https://gain.fas.usda.gov, http://dati.istat.it). Recently, local ancient wheat varieties have acquired increased importance due to their peculiar organoleptic properties, which has fueled expanding craft milling and bakery enterprises (Shewry and Hey, 2015; Jankielsohn and Miles, 2017). For example, in Sicily, the importance of local ancient tetraploid wheat landraces is increasing. This is due to the quality of their flour, their low nutritional requirements, and their ability to grow in dry environmental conditions typical of the Sicilian region. This has sparked scientific curiosity towards the molecular determinants of such adaptation capabilities.

The possibility of finding microbial strains capable of improving wheat productivity and tolerance to biotic and abiotic factors has motivated scientific studies in the last decade (Coombs and Franco, 2003; Larran *et al.,* 2007; Velazquez-Sepulveda *et al.,* 2012; Hubbard *et al.,* 2014). Advances in sequencing and computational applications, such as metabarcoding (Taberlet *et al.,* 2012), have enabled detailed characterization of microbial diversity (Turner *et al.,* 2013; Ofek-Lalzar *et al.,* 2014). Most studies have focused on the bacteria while little is known about fungi. It is becoming increasingly clear that fungal microbiota play critical roles in plant growth, development and stress tolerance (Shendure and Ji, 2008; Nicolaisen *et al.,* 2014; Abdelfattah *et al.,* 2018).

It remains unclear whether locally-adapted wheat varieties are capable of recruiting distinct microbiota, and, if so, to what extent this differential recruitment is influenced by environment and the host genotype. As a first step towards deciphering the contribution of wheat microbiota to crop adaptation and yield, we here report molecular characterization of the composition of the fungal endophytic communities of two locally adapted Sicilian tetraploid wheat varieties, the winter type Perciasacchi and the spring type Tumminia, grown in two agricultural fields.

MATERIALS AND METHODS

Experimental design

This study was carried out in two fields (Field 1 and Field 2), of almost 1 ha each, located in the Madonie area of the Sicilian inland. Field 1 (37.7813410 N, 14.2852990 E), was located at 850 m above sea level and was previously used for legume culture. Field 2 (37.740245 N, 14.239368 E) was located at 800 m above sea level and was uncultivated for almost 5 years. One half of both fields was sown with Perciasacchi in the second week of November 2015, while the other half was sown with Tumminia in the first week of February 2016. The fields and seed were not subjected to any treatments before or after sowing. Based on the field topography, 23 sampling plots were selected in Field 1 and 27 were selected in Field 2.

Plant sampling, surface sterilization and sample preparation

During the heading phase of both varieties, a total of 50 samples were collected from the selected sampling plots. Each sample consisted of ten plants including their roots. Samples were transported to the laboratory and stored at 4°C before processing. The aerial parts of each plant were cut using sterile scissors and a stem portion, of approx. 15 cm above the crown, was kept for sap extraction. Stem and root samples were surface-sterilized using 5% sodium hypochlorite (NaClO), and then rinsed in sterile water. Sap extraction was carried out with the use of a new method, the CIHEAM-IAMB patented 'Method for the extraction of sap from plant material and apparatus for carrying out the method' (https://patents.google.com/patent/WO2017017555A1/en). This method of extracting plant sap from vessels and xylem using the pressure of a syringe has the advantage of reducing plant components in the final extract that can inhibit enzyme activity, adversely affecting the results of diagnosis. Sap extract was obtained by inserting 1 mL of phosphate-buffered saline (PBS) solution into one terminal of the plant stem using a syringe. PBS with the sap extracts was collected from the other terminal of the plant stem in a sterile 1.5 mL capacity tube, which was later used for plating and DNA extraction. Twenty-eight samples were randomly chosen, seven from each half of each field (14 from each wheat variety), for high throughput sequencing (HTS).

Culture conditions for endophytic fungi and DNA extraction

Endophytic fungi were cultured by aseptically transferring 100 µL of each wheat sap extract to 9 cm diam. Petri dishes containing semi-selective Nutrient Yeast Dextrose Agar (NYDA; containing 10 g L^{-1} glucose, 5 g L^{-1} yeast extract, 8 g L^{-1} nutrient broth, 18 g L^{-1} agar, 250 mg L^{-1} streptomycin sulphate and 250 mg L^{-1} ampicillin) (Janisiewicz and Roitman, 1988). The inoculated dishes were incubated at 26°C for 48-72 h. After incubation, a small portion of the growing mycelium was transferred to new Potato Dextrose Agar (PDA). A small portion of the subsequently grown mycelium was transferred from each plate into a unique sterile 1.5 mL capacity tube, and stored at -20°C. DNA extraction was carried out from 400 µL of wheat sap extract and the mixed fungal mycelium, using the NucleoSpin® Plant II extraction kit (Macherey-Nagel) following the manufacturer's instructions and doubling the pre-lysis and lysis incubation periods.

Metabarcoding analyses

Samples for metabarcoding were grouped into the following four categories including seven biological replicates: P1 (Perciasacchi grown in Field 1), T1 (Tumminia grown in Field 1), P2 (Perciasacchi grown in Field 2) and T2 (Tumminia grown in Field 2).

Amplifications of the fungal ribosomal Internal Transcribed Spacer 2 (ITS2) region were performed using the forward primer ITS86F (Turenne *et al.,* 1999) and mix reverse primers ITS4-Mix 1, ITS4-Mix 2, ITS4-Mix 3, and ITS4-Mix 4 (Tedersoo *et al.,* 2014; 2015). Amplifications of the ITS2 region were performed using KAPA HiFi Hot Start ReadyMix kit (KAPA Biosystems), under a temperature profile of 95°C for 3 min, 35 cycles at 98°C for 20 sec, 56°C for 15 sec, 72°C for 30 sec, followed by an elongation step of 72°C for 1 min. PCR products were visualized on 2% agarose gel in order to verify the successful amplification and the absence of contamination. PCR purification was performed using Agencourt AMPure XP beads kit and following the user manual instructions (Beckman Coulter). Amplicon indexing was carried out using Nextera XT v2 Index Kit (Illumina) and followed by a second PCR purification as previously described. Amplification products were quantified by fluorimetry using Qubit (Invitrogen) and pooled in equimolar concentrations before sequencing reactions in MiSeq (Illumina) according to the manufacturer guidelines (support.illumina.com). Datasets generated during this study were deposited and are available at the National Center for Biotechnology Information (NCBI), Sequence Read Archive (SRA), under the accession number PRJNA449228 (www.ncbi.nlm.nih.gov/bioproject/ PRJNA449228).

Sequencing data analyses

Raw reads were trimmed using Trimmomatic v. 0.32 (Bolger *et al.,* 2014), using a sliding window of six bases, Q-score average of ≥ 20, and a minimum sequence length of 150 bp. The paired-end reads were then assembled using PANDAseq Assembler (Masella *et al.,* 2012), setting a minimum sequence length value of 150 bp and a minimum overlapping value of 20 bp. Reads were then checked and filtered for chimeric sequences using VSEARCH v.1.11.1 (Rognes *et al.,* 2016) and the UNITE dynamic database released on November 20, 2016 (https://unite.ut.ee). The same database was also used for creating Operational Taxonomic Units (OTUs), with a similarity threshold of 99% and for taxonomy assignments with BLAST method (Altschul *et al.,* 1990) as implemented in QIIME v. 1.9.1 (Caporaso *et al.,* 2010). These taxonomic units were collapsed to describe the fungal community at the genus, family, order, class, and phylum levels.

Downstream analyses

The downstream analyses were conducted using QIIME 1.9.1 pipeline (Caporaso *et al.,* 2010) as described by (Abdelfattah *et al.,* 2017). The OTU table was normalized by rarefaction to an even depth of 57,623 for cultured mycelium and 42,448 for sap extract samples. This was to reduce sample heterogeneity as well as to keep samples with acceptable numbers of sequences to be used in statistical and taxonomic analyses. Fungal richness and abundance were calculated through alpha-diversity analyses determined by Shannon's Diversity, Simpson, Chao1, and Observed OTUs indices. The diversity results were then compared using a nonparametric two-sample t-test, and the *P*-values were calculated through 999 Monte Carlo permutations. Beta diversity analysis was performed using Bray Curtis dissimilarity metrics and the results were used to conduct Principal Coordinates Analysis (PCoA). PCoA graphs were also implemented with the taxonomic information and plotted on a 3D graph using EMPeror (Vázquez-Baeza *et al.,* 2013). Beta diversity results were used to compare groups of samples (fields, wheat varieties and sample categories) using Permanova analyses. Differentially abundant taxa between the two fields, the wheat varieties

and the four sample categories were detected using the Linear discriminant analysis Effect Size (LEfSe) (Segata *et al.,* 2011), setting *P*-value thresholds for the factorial Kruskal-Wallis test and the pairwise Wilcoxon test of 0.05, and the logarithmic Effect Size (LDA) cut-off >2.

Fungal association network

Inferred fungal associations (co-occurrence and mutual exclusion) within each wheat variety were computed using the CoNet (v1.1.1. beta) plugin within Cytoscape (v3.6.1). The associations of OTUs present in at least 20 samples were identified using an ensemble of correlation metrics (Spearman and Pearson coefficients) and distance metrics (Bray–Curtis and Kullback–Leibler dissimilarity measures). For each association metric and each edge, 100 renormalized permutation and bootstrap scores were generated following the ReBoot procedure (Faust *et al.,* 2012). The measure-specific *P*-values from multiple association metrics were merged using the Simes method (Sarkar and Chang, 1997), and false-discovery rate corrections were performed using Benjamini–Hochberg multiple testing correction (Benjamini and Hochberg, 1995). Only 1,000 top- and 1,000 bottom-ranking edges from each association measure were kept in the network analysis, and only edges supported by at least two of the four association metrics were retained in the final network inference of associations among taxa.

Table 1. Sequencing results obtained from analyses of DNA extracted from cultured mycelium isolated from four wheat variety and field categories: P1 = Perciasacchi from Field 1; T1 = Tumminia from Field 1; P2 = Perciasacchi from Field 2; and T2 = Tumminia from Field 2.

Category	No. of reads	Total OTUs	Rarefied OTUs[a]
P1	83,331	927	927
T1	127,221	960	960
P2	57,623	615	615
T2	84,962	753	753

[a]Rarefaction depth 57,623

Table 2. Sequencing and alpha diversity results from analyses of DNA extracted from wheat sap extracted from four wheat variety and field categories: **P1** = Perciasacchi from Field 1; **T1** = Tumminia from Field 1; **P2** = Perciasacchi from Field 2; and **T2** = Tumminia from Field 2.

Category	Sequencing results			Alpha diversity metrics			
	No. of reads	Total OTUs	Rarefied OTUs[a]	Simpson	Shannon	Chao1	Observed Species
P1	459,150	5,454	3,181	0.95	5.62	1249.40	651.52
T1	299,769	2,646	1,805	0.96	5.67	1252.82	555.92
P2	566,026	4,047	2,815	0.92	4.61	1072.07	507.20
T2	949,383	6,612	3,799	0.93	5.15	908.93	498.35

[a] Rarefaction depth 42,448

RESULTS

Sequencing data processing

HTS generated a total of 1,509,410 unpaired reads from fungal mycelium and 4,492,327 unpaired reads from wheat sap extract. After trimming, pairing, quality and chimera filtering, 687,093 high-quality fungal sequences were retained from mycelium and 2,330,956 from sap extract. The resulting sequences were then assigned to 2,943 OTUs for mycelium samples and 6,885 OTUs for sap samples. A summary of the sequencing results is shown in Table 1 and Table 2.

Cultured endophytic fungi from wheat

The sequencing of the ITS2 region of the endophytic fungi isolated in culture plates provided the taxonomic composition of the cultivable endophytic fungi (Figure 1). A total of 12 species from ten genera were identified. The number of genera per sample varied from four to nine. Ascomycota was the representative phylum of all the cultured genera, where *Mycosphaerella*, *Acremonium*, *Cladosporium*, *Aureobasidium,* and *Fusarium* were shared between all the assessed samples. Other genera were detected only in some sample categories: *Fusarium* and *Alternaria* from P1, T1 and T2, *Microdochium* from P1 and T1, and *Monographella* from T1 and T2).

Endophytic fungal community composition

The taxonomic assignment of the OTUs obtained from the HTS of wheat sap samples elucidated the composition of the endophytic fungal community among the four sample categories (P1, P2, T1 and T2). Overall, 28 OTUs were identified, belonging to 26 fungal genera (Figure 2). However, the relative abundance (RA) of the detected fungal taxa showed some differences between the sample categories, at the phylum and class levels (Figure 3). For example, members of Ascomycota were predominant in P1 (60% RA) and T2 (68% RA). In contrast, Basidiomycota were the most abundant in P2 (39%

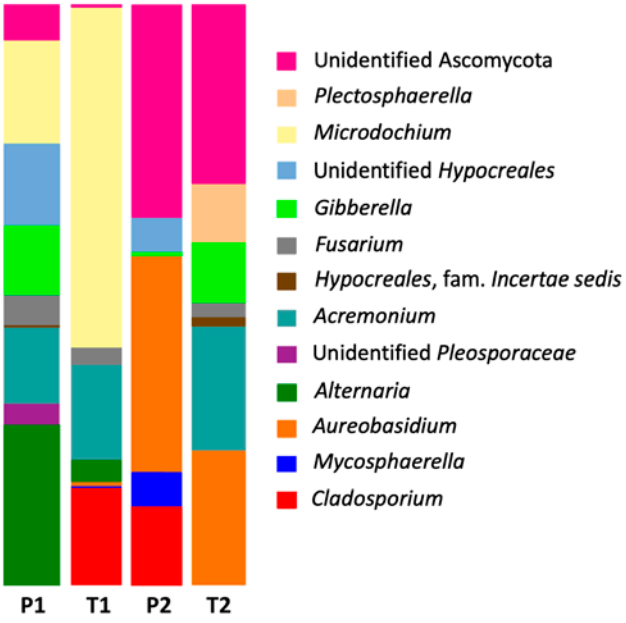

Figure 1. Taxonomic composition of cultured endophytic fungi. Genera distributions of endophytic fungi grown in cultures and analyzed using HTS, among four wheat variety and field categories: P1 = Perciasacchi from Field 1; T1 = Tumminia from Field 1; P2 = Perciasacchi from Field 2; and T2 = Tumminia from /Field 2.

RA) and T1 (59% RA). Field 2 gave the greatest number of unidentified fungal sequences, in particular in the T2 category. Dothideomycetes and Tremellomycetes were the main representative fungal classes in all the samples, with an average RA of 30%, followed by Sordariomycetes (7.5%), Agaricomycetes (2.5%), Microbotryomycetes (4.4%), Pucciniomycetes (3.4%), Leotiomycetes (1.0%), Ustilaginomycotina *incertae-sedis* (0.4%), and Taphrinomycetes (0.2%). At the genus level, *Alternaria*, *Mycosphaerella*, *Cladosporium*, *Filobasidium*, *Holtermanniella*, *Cystofilobasidium* and *Cryptococcus* were detected in all the sample categories.

Richness and diversity of endophytic fungi

The number of detected fungal OTUs per sample varied from 2,646 to 6,612. Alpha diversity indices showed that samples collected from Field 1 had greater fungal diversity compared to those from Field 2 (Table 2). The two-sample t-test based on Shannon index revealed a significant difference between the two fields ($P = 0.02$). However, there was no significant differences, either between the same variety grown in different fields or between the two varieties grown within the same field.

The PCoA plots, showing beta-diversity results, showed that samples collected from the two fields tended to segregate separately. In addition, the two wheat varieties clustered into different groups within each field (Figure 4). In agreement with PCoA and alpha diversity results, statistical comparisons using non-parametric Permanova tests showed significant variation of the fungal communities between the two fields ($P = 0.001$). In addition, the fungal communities were significantly different between the two wheat varieties ($P = 0.044$). A significant difference was also detected where the same varieties were grown in different fields ($P = 0.002$ for Field 1 and 0.005 for Field 2).

Some of the detected taxa had significantly different RAs in the investigated fields and varieties. In particular, *Mycosphaerella*, *Dioszegia*, *Filobasidium*, *Protomyces* and *Alternaria* varied significantly between the two fields (Figure 5), while *Aureobasidium*, *Leucosporidium* and *Puccinia* varied between the two varieties (Figure 6).

Fungal interactions

The co-occurrence and mutual exclusion of specific OTUs were analyzed for each wheat variety. The resulting networks, after statistical calculations and removal of unstable edges/links, were characterized, for Perciasacchi by 132 nodes (OTUs) linked with 420 edges with a clustering coefficient of 0.606, and for Tumminia, by 134 nodes (OTUs) linked with 583 edges and a clustering coefficient of 0.731 (Figure 7). Overall, the interactions between fungal phylotypes were characterized by a greater number of co-occurrences (401 in Perciasacchi and 510 in Tumminia) compared to mutual exclusions (19 in Perciasacchi and 73 in Tumminia). In both varieties, *Cryptococcus, Cystofilobasidium* and *Holtermanniella* were the dominant genera, with consistent co-occurrence interactions between each other as well as within each genus. *Cryptococcus* and *Cystofilobasidium* had mutual exclusion relationships with *Gibberella* species. On the other hand, *Gibberella*, *Acremonium* and a group of unidentified ascomycetes had a mutual exclusion interaction with *Puccinia*. *Cryptococcus* excluded *Sporobolomyces* only in Perciasacchi.

DISCUSSION

This study has demonstrated the limits of culture-dependent methods for characterization of the endophytic fungal communities. Metabarcoding analyses from crude sap extract allowed the detection of 26 genera belonging to Ascomycota and Basidiomycota, while

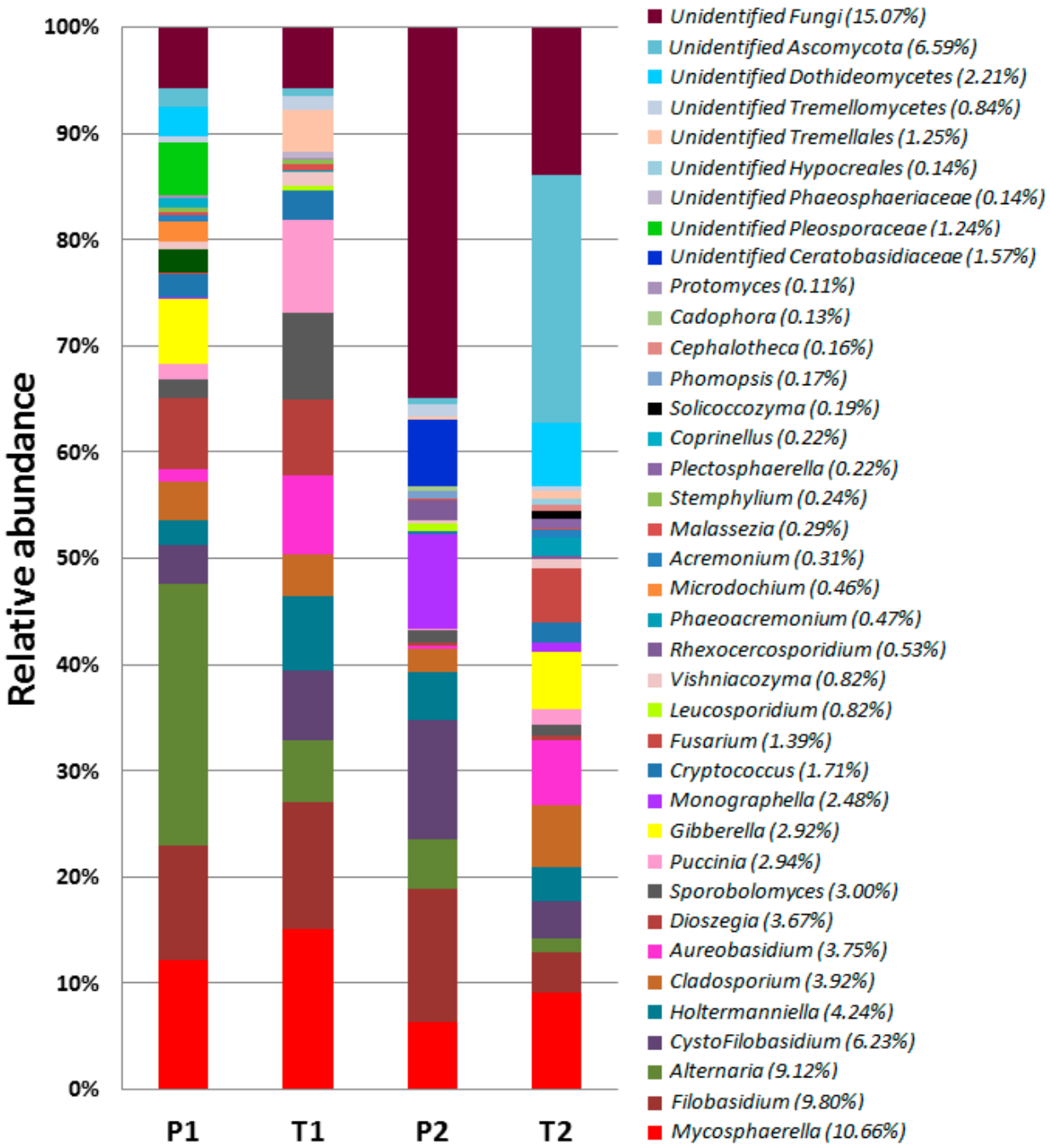

Figure 2. Endophytic fungal richness in two ancient wheat varieties. Distributions and relative abundance of the most abundant genera detected among four sample categories, from analyses of wheat sap samples. Average relative abundance for each genus is reported in parentheses. P1 = Perciasacchi from Field 1; T1 = Tumminia from Field 1; P2 = Perciasacchi fromField 2; and T2 = Tumminia from Field 2. Mean values of relative abundance are indicated in the parenthesis.

fungal isolations allowed the detection of only ten genera belonging to Ascomycota. Since isolations were carried out using one culture medium under one condition, a broader range of culturing conditions may be required to capture greater proportion of wheat endophytes. Nevertheless, nine out of 12 fungal species grown in plate cultures were also detected in the wheat sap extracts, therefore a good correspondence was obtained between the fungal taxa identified by the two methods. The variability in detection of the two methods could be due to

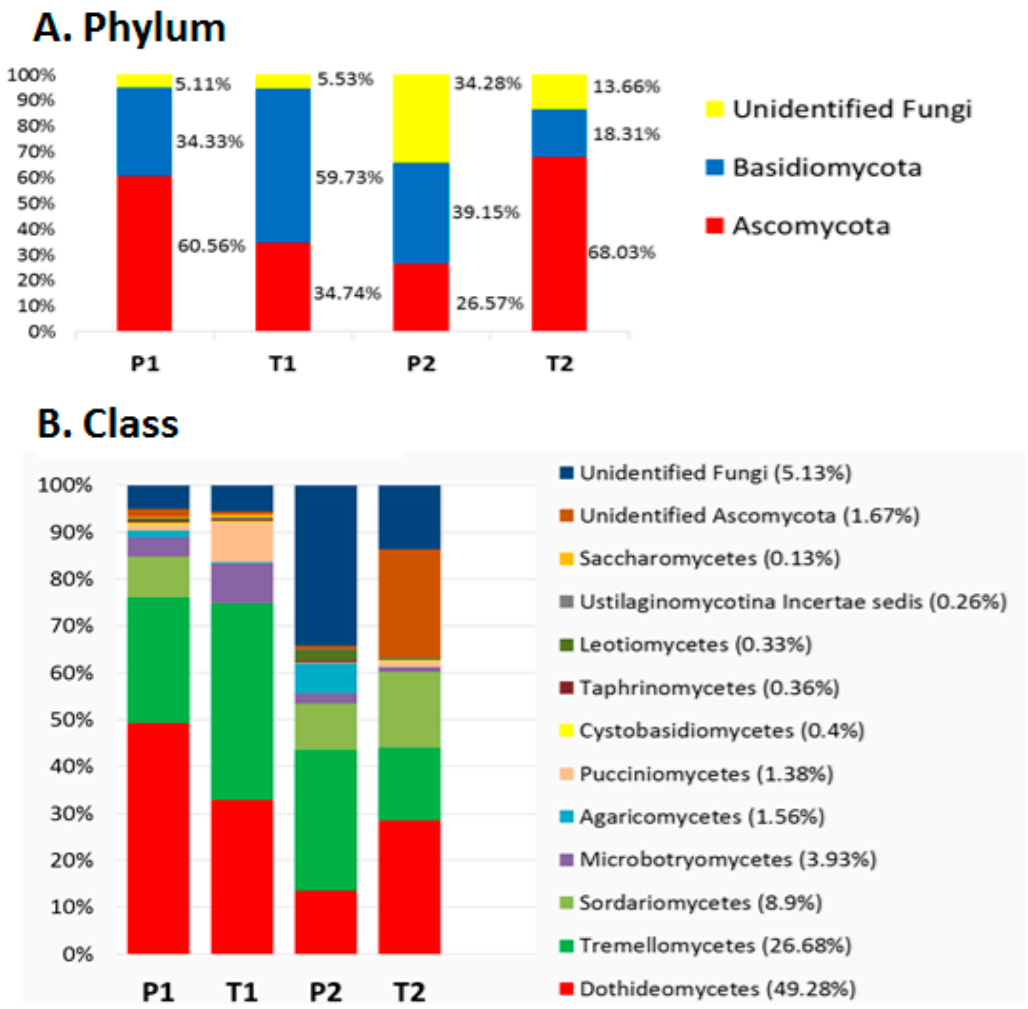

Figure 3. Structure and richness of the wheat endophytic fungal communities. Richness of fungal phyla (A) and classes (B) resulting from downstream analyses of the HTS performed on DNA extracted from wheat sap. Average relative abundance for each class is reported in parentheses. Sample categories are: P1 = Perciasacchi from Field 1; T1 = Tumminia from Field 1; P2 = Perciasacchi from Field 2; and T2 = Tumminia from Field 2.

bias of the culturing technique, which may have favored some taxa over others. Alternatively, the choice of primers for HTS analyses may have impact on the numbers of detected taxa (Tedersoo *et al.,* 2015). Moreover, it is important to highlight that the sap extract obtained with the patented method was successful in fungal isolation and molecular analyses.

The variations observed in the endophytic fungal communities between each wheat variety grown in the two fields could be explained by the different locations and agronomic conditions at the two field sites. Field 1 had been to crop rotation with legumes, while Field 2 was not cultivated for five previous years. Previous studies have reported that geographical location as well as crop management practices can affect the composition of soil microbial communities and, as consequence, the composition of endophytic microbial communities (Göre and Bucak, 2007; Sapkota *et al.,* 2017; Soman *et al.,* 2017).

The fungal communities were different between the two wheat varieties. This is not surprising since host genotype is considered to be a major factor determining the composition of endophyte communities (Sapkota *et al.,* 2015). These results highlight the importance of investigating microbial diversity of ancient crop varieties as possible sources of beneficial microorganisms.

Alternaria, *Cladosporium, Sporobolomyces, Dioszegia* and *Cryptococcus* are reported to be ubiquitous, and they have been detected in the present study as well as in the phyllospheres and grain of several commercial wheat

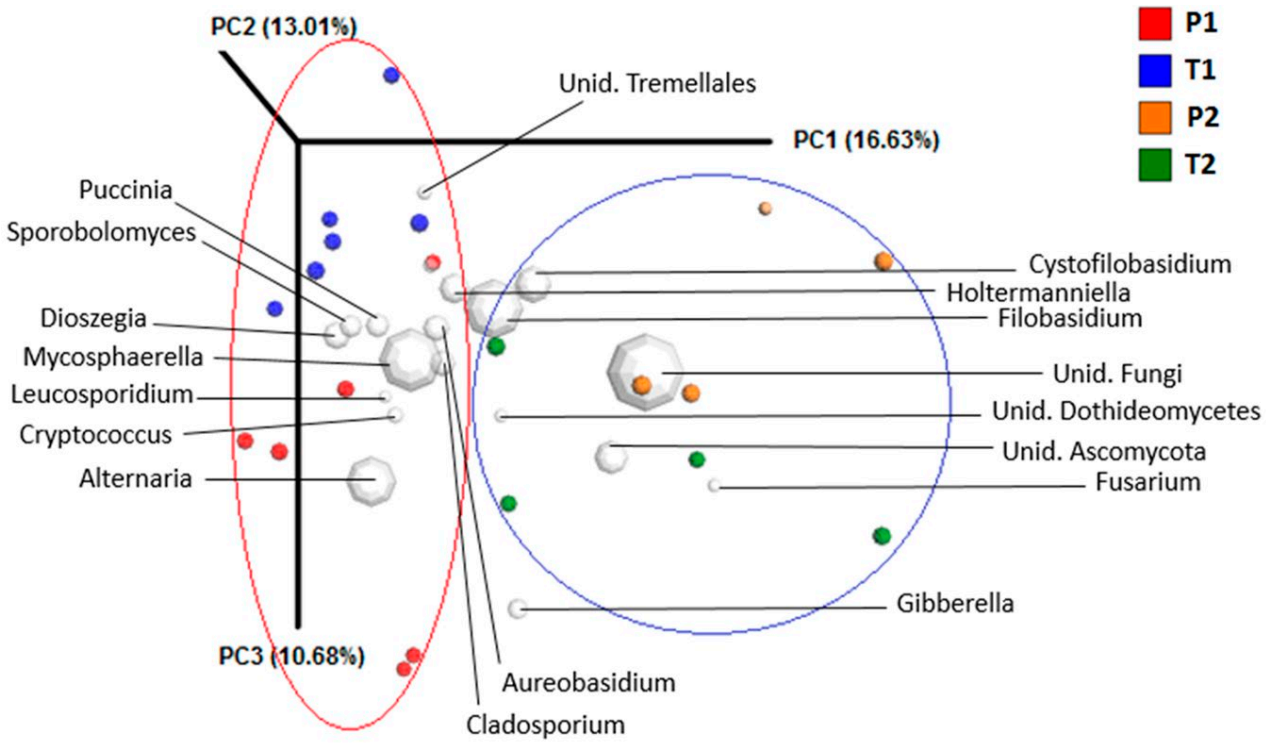

Figure 4. Principal Coordinates Analysis (PCoA). Integration of taxonomy in the PCoA graph showing the distribution of the 18 most abundant endophytic fungal genera among the four sample categories (white symbols). The taxonomic plot weight and the distance from the sample plots are proportional to the relative abundance of each genus. Red circle: cluster of Field 1 samples; blue circle: cluster of Field 2 samples. The different coloured symbols indicate the sample categories: P1 = Perciasacchi from Field 1; T1 = Tumminia from Field 1; P2 = Perciasacchi from Field 2; and T2 = Tumminia from Field 2.

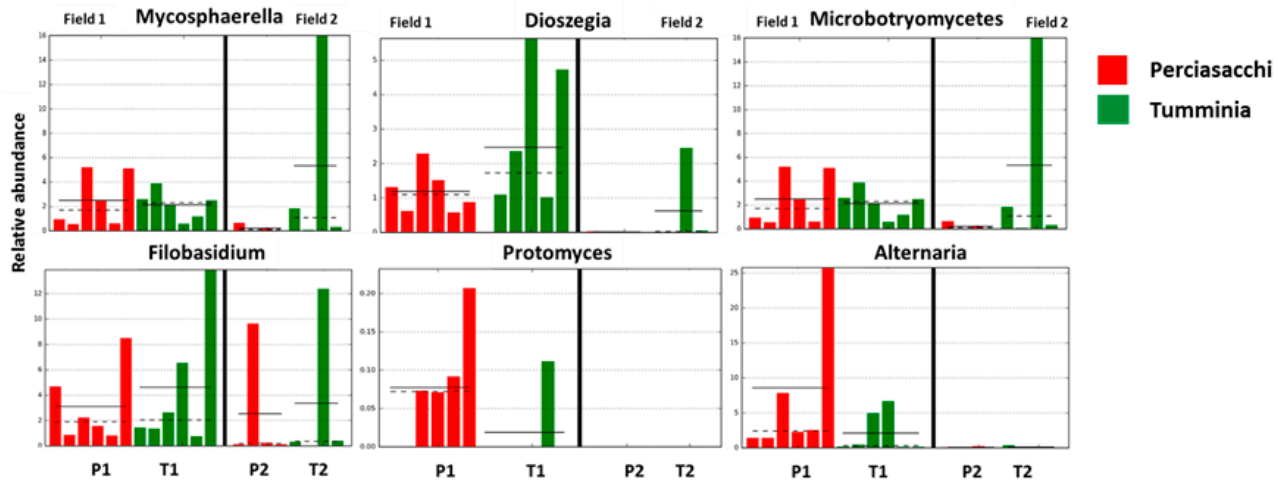

Figure 5. Differentially abundant genera between two ancient wheat varieties and two fields. Histograms indicate significant differences calculated by LEfSe analyses (Pa< 0.05 and LDA cut-off >2). P1 = Perciasacchi from Field 1; T1 = Tumminia from Field 1; P2 = Perciasacchi from Field 2; and T2 = Tumminia from Field 2. Category means (straight line) and medians (dotted line) are indicated.

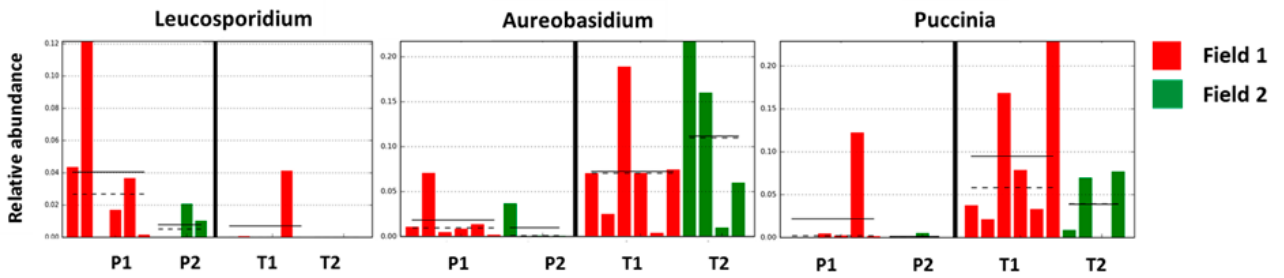

Figure 6. Discriminant fungal genera between two ancient wheat varieties. Differential genera between variety Perciasacchi (red) and Tumminia (green), resulting from LEfSe analysis ($P < 0.05$, LDA cut-off > 2). Histograms represent differential taxa distributions between the two wheat varieties and among the four sample categories: P1 = Perciasacchi from Field 1; T1 = Tumminia from Field 1; P2 = Perciasacchi from Field 2; and T2 = Tumminia from Field 2). Category means (straight line) and medians (dotted line) are indicated.

varieties (Nicolaisen *et al.,* 2014; Sapkota *et al.,* 2015). On the other hand, genera such as *Filobasidium*, *Holtermanniella* and *Cystofilobasidium* were detected in Perciasacchi and Tumminia, but have not been detected in the phyllospheres of modern cultivars. Conversely, *Pyrenophora*, *Epicoccum*, *Phoma* and *Sphaeosphaeria* belonging to core OTUs in the phyllospheres of modern wheat cultivars (Nicolaisen *et al.,* 2014) were not found in the present study. Differences between ancient and modern cultivars may be the consequence of different breeding histories as well as cultivar selection and/or growing conditions. Further research comparing varieties with contrasting pedigrees is necessary to firmly define taxa shared, or differentiating between, wheat genotypes, as previously reported for other crop species (Liu *et al.,* 2018).

Despite the presence of important fungal pathogens, no disease symptoms were observed in the collected samples from both fields. The presence of non-pathogenic fungi and plant resistant genes may account for this observation. However, an important contribution from beneficial fungi for maintaining plant health is likely. The analysis of the association networks suggested that key endophytes played roles within the wheat fungal communities detected. For instance, Basidiomycete endophytes such as *Cryptococcus* and *Cystofilobasidium* were the predominant genera and may have had consistent antagonistic activity against *Gibberella* species. Similarly, *Acremonium* and a group of unidentified ascomycetes mutually excluded *Puccinia*. Since both *Gibberella* and *Puccinia* contain several pathogens that cause economically important diseases in wheat, the detected interactions may indicate microbial-mediated resistance in wheat varieties. Furthermore, *Holtermanniella* and *Cystofilobasidium* were detected in the two ancient wheat varieties, and have not been reported to colonize modern wheat cultivars. This indicates possible negative impacts of breeding and domestication on native beneficial microbiomes.

In conclusion, the results from this study have expanded knowledge of the endophytic fungal communities associated with ancient wheat varieties, and have allowed formulation of hypotheses on the roles of the fungi in host plants. Further studies, including comparison between ancient and modern wheat microbiota, are required to clarify the roles of specific fungi, and to facilitate their exploitation as alternative means for improving plant health and resilience.

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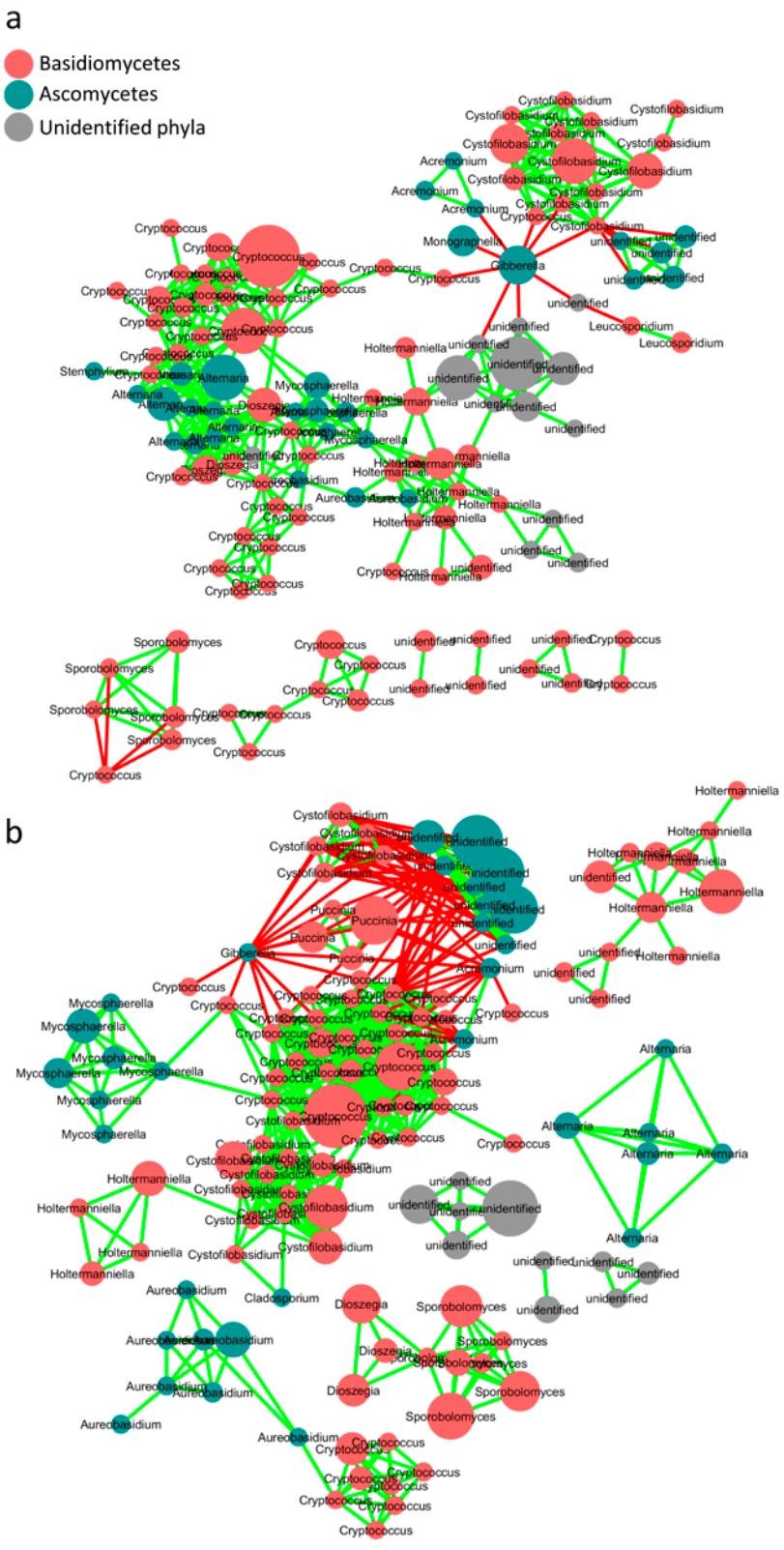

Figure 7. Microbial association network showing the interactions (co-occurrence and mutual exclusion) represented by green links for wheat variety Perciasacchi (a) and red links for Tumminia (b) The size of the nodes indicates OTU abundance. Node colours differentiate fungal phyla. Interactions were calculated by CoNet and visualised in Cytoscape 3.6.

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Research Papers

A fast and reliable method for *Diplodia seriata* inoculation of trunks and assessment of fungicide efficacy on potted apple plants under greenhouse conditions

Elena ARRIGONI[1,2], Claudia Maria OLIVEIRA LONGA[1], Dario ANGELI[1], Matteo SOINI[1], Ilaria PERTOT[1,3,#], Michele PERAZZOLLI[1,#,*]

[1] *Department of Sustainable Agro-ecosystems and Bioresources, Research and Innovation Centre, Fondazione Edmund Mach, Via E. Mach 1, 38010 San Michele all'Adige, Italy*
[2] *Department of Agricultural and Environmental Sciences, University of Udine, via delle Scienze 206, 33100 Udine, Italy*
[3] *Center Agriculture Food Environment (C3A), University of Trento, via E. Mach 1, 38010 San Michele all'Adige, Italy*
[#] These authors jointly supervised this work
*Corresponding author: michele.perazzolli@fmach.it

Abstract. *Diplodia seriata* is a polyphagous and widespread pathogen that infects trunks, shoots, fruit and leaves of apple plants. Fungicides applied in integrated pest management programmes can act against *D. seriata*. However, introduction of scab-resistant apple cultivars and the consequent reduction in fungicide applications under low input disease management may increase the incidence of disease caused by *D. seriata*. Despite potential outbreaks of trunk canker, no fast and reproducible protocols for artificial inoculation of *D. seriata* and fungicide efficacy tests are available for apple plants. In this study, protocols for mycelium plug- and conidium suspension-inoculation of apple stems and shoots were optimised; canker disease assessments were carried out on potted apple plants under greenhouse conditions and coupled with *D. seriata* DNA quantification with quantitative PCR (qPCR). Efficacy tests of commercial fungicides showed that captan, dithianon and fluazinam inhibited *D. seriata* mycelium growth and conidium viability *in vitro*, while penconazole and ziram did not. However, dithianon spray applications did not reduce trunk canker severity and amount of *D.* seriata DNA in artificially inoculated plants under greenhouse conditions. This optimised protocol for fast and precise assessment of fungicide efficacy is suitable for further investigating the effects of other fungicides against *D. seriata*.

Keywords. *Botryosphaeria obtusa*, *Malus domestica*, apple stem canker, *Diplodia* infection protocol, qPCR quantification.

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INTRODUCTION

Diplodia seriata (synonym *Botryosphaeria obtusa*) is a plant pathogen in the Botryosphaeriaceae (Ascomycota) that has been isolated from several

different hosts worldwide (Phillips *et al.*, 2007; Úrbez-Torres and Gubler, 2009; Mondello *et al.*, 2017; Pouzoulet *et al.*, 2017; Spagnolo *et al.*, 2017). In particular, *D. seriata* causes trunk canker and shoot dieback, fruit black rot and leaf frog-eye spot on apple plants (Phillips *et al.*, 2007). Trunk canker is characterised by reddish brown lesions that each turn smoky and develop series of alternate rings, rapidly becoming elliptical in shape (Naqvi, 2007). The bark surface becomes rough and cracked with possible callus depositions around the wounds and reddish brown stains on the underlying wood tissues (Naqvi, 2007). *Diplodia seriata* fruit infection (black rot) initially appears as small raised purplish lesions on young fruits and develops into large, brown, firm lesions on ripening apples that may later rot the entire fruit (Venkatasubbaiah *et al.*, 1991). Leaf infections (frog-eye leaf spot) initially appear as reddish-brown flecks that develop into circular brown lesions, often each surrounded by a purple halo, followed by leaf chlorosis and abscission (Venkatasubbaiah *et al.*, 1991). In addition, *D. seriata* is a polyphagous pathogen and causes pear (Choudhury *et al.*, 2014), grapevine (Úrbez-Torres *et al.*, 2008), olive (Kaliterna *et al.*, 2012) and mulberry trunk canker (Arzanlou and Dokhanchi, 2013), olive (Moral *et al.*, 2008) and loquat rot (Palou *et al.*, 2013), and canker of ornamental plants (e.g. *Cotoneaster salicifolius*) (Bobev *et al.*, 2008) and forest plants (e.g. *Castanea sativa*) (Dar and Rai, 2017). Trunk cankers and infected fruit left in orchards are the main sources of pathogen inoculum (Beer *et al.*, 2015), as well as infected tissues of other plant species in close proximity (Cloete *et al.*, 2011). Thus, the removal of diseased shoots and fruit (Brown-Rytlewski and McManus, 2000; Beer *et al.*, 2015) and the use of resistant cultivars (Biggs and Miller, 2004) are recommended to limit the spread of *D. seriata*.

Conidia of *D. seriata* spread from inoculum sources to healthy trunks, shoots, fruit or leaves during rain and penetrate these through natural openings, such as wounds, lenticels and stomata (Naqvi, 2007). Trunk and shoot infections can occur mainly through pruning wounds in winter or summer, when trees are more vulnerable to canker development due to possible drought stress (Brown-Rytlewski and McManus, 2000). However, *Diplodia* spp. have consistently been found at low levels in asymptomatic apple and pear bark, acting as potential sources of new infection (Arrigoni *et al.*, 2018). Likewise, fungi in the Botryosphaeriaceae have been found as endophytes and latent pathogens in woody plants (Slippers and Wingfield, 2007), and endophytic Botryosphaeriaceae can rapidly cause disease when their hosts are under stress (Slippers and Wingfield, 2007).

Diplodia seriata black rot causes significant losses in organic orchards (up to 10% losses estimated in northern Germany), but damage is less frequent when integrated pest management (IPM) is applied (Beer *et al.*, 2015). Thus, it has been hypothesised that fungicides applied to control apple scab, flyspeck or sooty blotch can also act against *D. seriata* (Brown-Rytlewski and McManus, 2000; Beer *et al.*, 2015). Fungicides (benomyl, kresoxim-methyl and trifloxystrobin) directly applied as topical wound treatments reduced the incidence of *D. seriata* trunk canker in apple trees (Brown-Rytlewski and McManus, 2000), and fungicide sprays against apple scab can control sooty blotch and flyspeck (Weber *et al.*, 2016). This is most probably related to side effects of fungicides applied against the main pathogen (i.e. *Venturia inaequalis*), which can reduce the inoculum of some secondary pathogens. However, the introduction of scab-resistant apple cultivars can reduce fungicide applications compared with susceptible cultivars (Simon *et al.*, 2011; Didelot *et al.*, 2016), and the consequent reduction in fungicide applications under low-input disease managements of scab-resistant apple cultivars may cause outbreaks of several secondary emerging pathogens, *D. seriata* included (Ellis *et al.*, 1998). An increasing risk of canker outbreaks was also hypothesised due to climate change, which exposes plants to the risk of abiotic stresses (Slippers and Wingfield, 2007), indicating that specific fungicide applications may be required to control canker agents in the future (Ellis *et al.*, 1998).

Although the risk of emerging secondary pathogens is real, no fast and reproducible protocols are available to assess the efficacy of conventional spray applications against *D. seriata* canker under greenhouse conditions. Most of the studies on *D. seriata* infection and disease management have focused on apple black rot (Biggs and Miller, 2004; Beer *et al.*, 2015) and grapevine trunk disease (Úrbez-Torres and Gubler, 2009; Pitt *et al.*, 2012; Mondello *et al.*, 2017). Trunk canker diseases are rarely studied due to the long incubation times and difficulties in reproducing infection under controlled conditions. The aim of the present study was to optimise a fast and reliable protocol to inoculate and assess *D. seriata* on potted apple plants and to evaluate fungicide efficacy against trunk canker, using conventional spray applications under greenhouse conditions.

MATERIALS AND METHODS

Fungal isolates

Two strains of *D. seriata* (S and VT) were isolated as single conidium cultures from trunks of *Malus domesti-*

ca plants with canker symptoms in the 'Scurelle' (latitude, N46.0647636; longitude, E11.510519599999952; altitude, 482 m) and 'Vigo di Ton' orchards (latitude, N46.2655852; longitude, E11.085680799999977; altitude, 375 m) in the Trento province (northern Italy). The isolates were stored in glycerol at -80°C in the fungal collection of the Fondazione Edmund Mach, and are freely available upon request. The strains were identified by morphology and molecular methods. For molecular characterization, *D. seriata* was grown on potato dextrose agar (PDA, Oxoid) for 14 d at 27°C, and DNA was extracted form 0.2 g of mycelium using the FastDNA spin kit for soil (MP Bimedicals) according to the manufacturer's instructions. DNA was quantified using the Quant-IT PicoGreen dsDNA assay kit (Thermo Fisher Scientific) with a Synergy2 microplate reader (BioTek), and the internal transcribed spacer (ITS) region was amplified using the forward primer ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990; Halwachs *et al.*, 2017). PCR amplification was carried out using the FastStart HighFidelity PCR system (Roche) with 1 µL of extracted DNA (10 ng), 0.25 mM deoxynucleoside triphosphates, 1% (w:v) bovine serum albumin, 4% (v:v) dimethyl sulphoxide, 0.3 µM of each primer and 2.5 U of FastStart High-Fidelity DNA polymerase (Roche), in 50 µL of reaction, and the following programme: denaturation at 95°C for 5 min, 32 cycles of amplification at 95°C for 30 s, annealing at 60°C for 1 min, extension at 72°C and final extension at 72°C for 10 min. The PCR product was purified with the Illustra ExoProStar reaction (GE Healthcare Life Sciences), and single strand sequences were obtained with an ABI PRISM 3730xl DNA analyser (Applied Biosystems, Thermo Fisher Scientific), using the forward primer ITS5 according to the manufacturer's instructions (Sequencing Service of the Fondazione Edmund Mach). Sequences were aligned against the National Center for Biotechnology Information database (NCBI; http://www.ncbi.nlm.nih.gov) to confirm the identity of *D. seriata*, and they were deposited at the Genbank database of NCBI (https://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers MH174673 (*D. seriata* S) and MH174674 (*D. seriata* VT).

Growth conditions for Diplodia seriata strains S and VT

Mycelium growth of *D. seriata* S and *D. seriata* VT was assessed on four growth media: i) 15 g L^{-1} technical agar (Sigma-Aldrich) in distilled water (water agar, WA), ii) 39 g L^{-1} PDA (Oxoid), iii) 2.4 g L^{-1} potato dextrose broth (Fluka, Sigma-Aldrich) supplemented with 15 g L^{-1} technical agar (PDB+A, Oxoid), and iv) 2.4 g L^{-1} potato dextrose broth (Fluka, Sigma-Aldrich) supplemented with 15 g L^{-1} technical agar (Oxoid) and 10 g L^{-1} of carboxymethyl-cellulose (PDB+A+C, Sigma-Aldrich). Plates were incubated at 27°C for 28 d under two photoperiod conditions: complete darkness or 16:8 h light:dark photoperiod. Three double-sterilised pine needles (5 cm long) were added to each WA plate, in order to stimulate the production of pycnidia, as previously reported for Botryosphaeriaceae species (Slippers and Wingfield, 2007; Amponsah *et al.*, 2008). A mycelium plug (5 mm in diam.) of a 7-d-old culture of *D. seriata* S or *D. seriata* VT grown on PDA was placed in each Petri dish (90 mm diam.). The resulting colony diameters were measured after every 3 d of incubation at 27°C, and the daily colony growth rates were then calculated by dividing the maximum colony diameter (that was measured before complete plate coverage) by the number of days of incubation. Pycnidium production on PDA, PDB+A and PDB+A+C was assessed visually as percentage of dish surface covered by *D. seriata* pycnidia (pycnidia density) after 28 d incubation at 27°C. Pycnidium production on WA supplemented with pine needles was expressed as the number of pycnidia produced per cm of pine needle length by counting under a stereoscope (Nikon model SMZ800) after 28 d incubation at 27°C. Four replicates (plates) were assessed for each fungus strain, growth medium, and photoperiod condition, and the experiment was carried out twice.

Diplodia seriata inoculation of apple plants

Two protocols for inoculation (mycelium plugs and conidium suspensions) and two plant tissues (trunks and shoots) were compared. To prepare the mycelium plugs, *D. seriata* S was grown on PDA for 28 d at 27°C in complete darkness, and plugs (5 mm diam.) were obtained with a flame-sterilised cork borer under sterile conditions. Conidium suspensions were prepared by growing *D. seriata* S on WA plates, each supplemented with three double-sterilised pine needles, for 28 d at 27°C with a 16:8 h light:dark photoperiod. Thirty sporulated pine needles were then transferred to 30 mL of distilled sterile water, ten sterile stainless steel spheres were added to each vial which was then shaken by vortexing at the maximum speed for 2 min, in order to break the pycnidia and allow the release of conidia. The conidium suspension was vacuum-filtered using a membrane filter (160 µm pore size, NY6H, Millipore) to remove intact pycnidia and pine needle fragments. The conidium concentration was adjusted to 1×10^5 conidia mL^{-1} using a Thoma cell counting chamber under a light micro-

scope (Nikon model Eclipse 80i), and the suspensions were applied to apple plants with a compressed air hand sprayer.

Three-year-old apple plants of the apple scab-resistant cv. Fujion were planted in 6 L capacity pots and grown under greenhouse conditions for 3 months, at 25±1°C with a 16:8 h light:dark photoperiod and relative humidity (RH) of 60 ± 10%, until each plant reached the petal fall phenological stage (Chapman and Catlin, 1976). Longitudinal wounds (20 mm long, 8 mm wide and 2 mm deep) were then made on 3-y-old trunks and 1-y-old shoots of the plants, using a flame-sterilised scalpel without totally removing the outer bark. Each wound on the *D. seriata*-inoculated plants was inoculated with a mycelium plug (5 mm diam.) or sprayed with 300 µL of conidium suspension (1×10^5 conidia mL^{-1}). On each plant, eight wounds were made alternately on opposite sides on the trunk, with more than 30 cm between wounds longitudinally along each trunk and to avoid canker lesions overlapping. For each plant, four wounds were inoculated with mycelium plugs and four were sprayed with the conidium suspension. Likewise, eight wounds were made on four separate shoots, four of them inoculated with the mycelium plug and four sprayed with the conidium suspension. As controls, a second group of plants was prepared, and each wound was mock inoculated with a sterile PDA plug (5 mm diam.) or with 300 µL of sterile distilled water. Five replicates (plants) were prepared as *D. seriata* S-inoculated and as control plants.

Each wound was covered with the outer bark and wrapped with laboratory plastic film (Parafilm, Bemis) for 7 d, to maintain high humidity and reduce the risk of contamination, as reported for *D. seriata* inoculations on the grapevine (Spagnolo *et al.*, 2017). Plants were incubated at 95±5% RH and 25±1°C for 48 h, and then maintained under greenhouse conditions in a randomised complete block design.

Sixty days after inoculation, trunks and shoots were cut into sections (15 cm long; sufficient to include whole cankers) containing the inoculation site, and surface-sterilised by placing in 1% v:v sodium hypochlorite solution for 45 sec, then washed twice in sterile distilled water for 60 sec. Trunk sections were peeled to remove 2-3 mm of superficial tissue layers and a calliper was used to measure the length of each canker. Disease severity and incidence were assessed for each wound of mycelium plug- or conidium suspension-inoculated trunks and shoots. For each replicate (plant), disease severity was assessed as the mean of canker length (mm) of four inoculated wounds. Disease incidence was expressed as the percentage (%) of infected wounds. In order to remove the effects of the wound scar, the canker lengths of mock-inoculated plants were subtracted from the disease severity scores of mycelium plug and conidium suspension *D. seriata*-inoculated plants.

In vitro fungicide efficacy tests against Diplodia seriata

Five commercial fungicides commonly used in apple protection programmes (Table 1) (Longo *et al.*, 2017) were tested against *D. seriata* mycelium growth and conidium viability *in vitro*. To assess effectiveness against mycelium growth, a small mycelium portion (2 × 1 mm) was collected with a flame-sterilised bacteriological needle from a 28-d-old *D. seriata* S culture on PDA maintained in darkness, and was transferred to a 2 mL sterile plastic tube containing 1 mL of sterile distilled water (control) or fungicide solution at the concentration recommended on the product label (Table 1). To assess activity against conidium viability, a conidium suspension was prepared as for apple plant inoculations (see above), and 500 µL of suspension (1×10^5 conidia mL^{-1}) was added to 500 µL of sterile distilled water (control) or a 2-fold concentrated fungicide solution in 2 mL collection tubes, to give the recommended concentration for each fungicide.

Samples were incubated at room temperature for 5 h under orbital shaking at 80 rpm and centrifuged at 8000*g* for 3 min. The supernatants were discarded and the pellets were washed three times by suspend-

Table 1. Fungicides tested against *Diplodia seriata*.

Active ingredient	Main apple pathogen target	Commercial name	Manufacturer	Dosage (g L^{-1})
Captan	*Venturia inaequalis*	Merpan 80 WDG	Adama	1.5
Dithianon	*V. inaequalis*	Delan 70 WG	Basf Crop Protection	0.4
Fluazinam	*V. inaequalis*	Banjo	Adama	0.7
Ziram	*V. inaequalis*	Diziram 76 WG	FMC Foret S.A.	2.0
Penconazole	*Podosphaera leucotricha*, *V. inaequalis*	Topas 10 EC	Syngenta	0.3

ing in 1 mL of sterile distilled water and centrifuging at 8000*g* for 3 min, to remove fungicide residues. Each mycelium portion was plated on PDA and diameters of resulting colonies were measured after 3 d incubation at 27°C. Pelleted conidia were resuspended in 200 µL of sterile distilled water, 100 µL of each suspension was plated on PDA and conidium viability was assessed as colony forming units per unit of suspension volume (CFU mL^{-1}) after 2 d incubation at 27°C. Six replicates (tubes) were prepared for each fungicide and inoculation procedure, and the experiment was carried out twice.

Fungicide treatments of apple plants inoculated with Diplodia seriata

Three-year-old apple plants (cv. Fujion) grown under greenhouse conditions were inoculated on the trunks with the *D. seriata* S mycelium plugs (5 mm diam.), and conidium suspensions (300 µL of 1×10^5 conidia mL^{-1}), as described above. Eight wounds were made on each plant on diametrically opposite sides of the trunk: four wounds were inoculated with mycelium plugs and four wounds were sprayed with the conidium suspension. Plants were treated with dithianon 7 and 1 d before inoculation, and 1 d and 7 d after inoculation, at the manufacturer's recommended dose rate (equivalent to 0.1 kg ha^{-1} for young plants; Table 1), using a compressed air hand sprayer. As experimental controls, plants were sprayed with water 1 d before *D. seriata* S inoculation. Plastic film was removed from each wound before fungicide or water treatments, and a new plastic film cover was then applied once the plant tissues had completely dried. A group of mock-inoculated plants with sterile PDA plugs (5 mm diam.) or with 300 µL of sterile distilled water was prepared to assess the effects of wounding, as described above. Plastic films were removed 7 d after inoculation, and the plants were kept for 60 d under greenhouse conditions. Trunks were then cut into sections (each 15 cm long), surface-sterilised and peeled to assess disease severity and incidence lesions. Four wood pieces (each 20 mm long, 5 mm wide and 1 mm thick) were collected with a flame-sterilised scalpel from each trunk section. Two wood samples were obtained from each plant, one from each mycelium plug-inoculated trunk (16 wood pieces) and one from each conidium suspension-inoculated trunk (16 wood pieces). These wood pieces were stored at -20°C for DNA extraction and *D. seriata* S quantification. Five replicates (plants) were analysed for each treatment in a randomised complete block design and the experiment was carried out twice.

DNA extraction and Diplodia seriata quantification with real-time quantitative PCR

Each wood sample was ground in a sterile stainless steel jar with 2.5 mL of a cold (4 °C) sterile isotonic solution (0.85% NaCl), using a mixer-mill disruptor (MM 400, Retsch) at 25 Hz for 45 s, as described by Arrigoni *et al.* (2018). DNA was extracted from 500 µL of each ground wood sample using the FastDNA spin kit for soil (MP Bimedicals) according to the manufacturer's instructions, and was quantified using the Quant-IT PicoGreen dsDNA assay kit (Thermo Fisher Scientific) with a Synergy2 microplate reader (BioTek). *Diplodia seriata* was quantified with real-time quantitative PCR (qPCR) using the primer pair DseCQF (5'-CTCTGCAATCGCTGACCCTTG-3') and DseCQR (5'-ACGTGTTTGTCTAACTAGTAGAGAGTACC-3'), that previously showed high sensitivity and positive correlation between the qPCR-quantified *D. seriata* DNA amount and *D. seriata* incidence (Pouzoulet *et al.*, 2017). qPCR assays were carried out using the KAPA SYBR FAST qPCR Master Mix at the concentration recommended in the manufacturer's instructions (1×; Kapa Biosystems), with 0.5 µM of each primer and DNA template ranging in concentration from 0.5 to 4 ng μL^{-1} in 25 µL of reaction volume. Amplification was performed using a Roche Light Cycler 480 (Roche Diagnostics GmbH) with the following programme: 95°C for 3 min; 40 cycles of 95°C for 3 sec, 62°C for 40 sec and an additional melting analysis from 60°C to 95°C, according to Pouzoulet *et al.* (2017). Negative and standard controls containing, respectively, nuclease-free water and 0.01 ng of pure *D. seriata* DNA, were included in every run. A standard curve for absolute quantification of *D. seriata* in apple wood samples was obtained in triplicate by mixing serial dilutions of *D. seriata* S DNA (1 ng, 0.1 ng, 0.01 ng, 1 pg, 0.1 pg) with 10 ng of apple DNA extracted from axenic apple plants grown *in vitro*. Wood samples of five replicates (plants) were analysed for each treatment and inoculation procedure, and the experiment was carried out twice.

Statistical analyses

Data were analysed with Statistica 13.1 software (Dell). Normal distribution (Kolmogorov-Smirnov test, $P > 0.05$) and variance homogeneity of the data (Levene's or Chochran's tests, $P > 0.05$) were checked, and non-parametric tests were used when these parametric assumptions were not respected. Each experimental repetition was analysed individually, and the Kruskal–Wallis test was used to demonstrate equivalent results in the two repetitions of each experiment ($P > 0.05$, non-significant

differences between the two experimental repetitions). For each experiment, data from the two experimental repetitions were pooled and the Kruskal–Wallis test ($P \leq 0.05$) was used to detect significant differences among treatments. Interactions among factors affecting the *D. seriata* growth, pycnidium production, severity and incidence of disease were assessed according to the Kruskal–Wallis test ($P \leq 0.05$), merging data for growth media, fungal strains, inoculated plant organ (trunks or shoots) or inoculation procedures (mycelium plug or conidium suspension).

RESULTS

Diplodia seriata propagation and inoculation under greenhouse conditions

The greatest growth rate was found for *D. seriata* S and *D. seriata* VT on PDA (Figure 1), and no differences were detected between the two photoperiod conditions after merging data for growth media and fungal strains (Kruskal-Wallist test, $P = 0.264$). Pycnidium production, expressed as pycnidium density and numbers of pycnidia, was comparable among growth media and fungal strains (Figure 2), while production was greater with a 16:8 h light:dark photoperiod compared with complete darkness, after merging data for growth media and fungal strains (Kruskal-Wallis test, respectively, $P = 3.9 \times 10^{-8}$ and 9.4×10^{-4}).

Severity of disease was greater from mycelium plug inoculations than from conidium suspension inoculations (Figure 3), while no differences were found between the two inoculation procedures on shoots (Figure 4a). Statistically significant effects of the inoculation procedure (Kruskal–Wallis test, $P = 0.002$), but not plant organ (Kruskal–Wallis test, $P = 0.449$), were detected for disease severity after merging data for trunks and shoots and for conidium suspension and mycelium plug inoculations. Likewise, the inoculation procedure (Kruskal–Wallis test, $P = 0.002$), but not plant organ (Kruskal–Wallis test, $P = 0.307$), affected disease incidence. The mycelium plug inoculations on shoots resulted in greater disease incidence compared to conidium suspension inoculation on trunks (Figure 4b).

Effects of commercial fungicides against Diplodia seriata

Incubation of *D. seriata* S with dithianon, captan or fluazinam reduced mycelium growth (Figure 5a) and conidium viability *in vitro* (Figure 5b). However, penconazole or ziram did not affect *D. seriata* S mycelium growth or conidium viability.

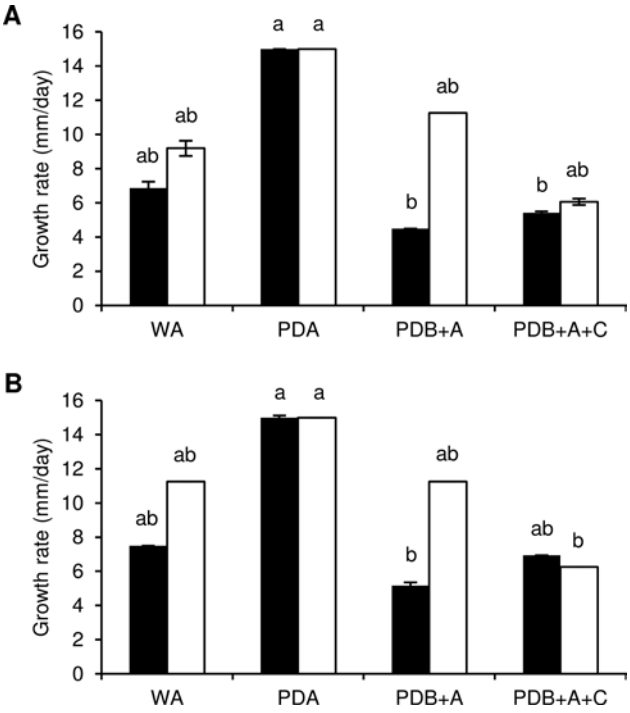

Figure 1. Mean mycelium growth rates of the *Diplodia seriata* strain S (black) and *D. seriata* strain VT (white), grown a) in complete darkness and b) with a 16:8 hour light:dark photoperiod on water agar (WA), potato dextrose agar (PDA), potato dextrose broth supplemented with agar (PDB+A) and PDB+A supplemented with cellulose (PDB+A+C) for 28 d at 27°C. The Kruskal-Wallis test showed no significant differences between the two experimental repetitions ($P > 0.05$, n = 4 replicates per experiment) and data from the two experiments were pooled. Means and standard errors of eight replicates (dishes) from the two experiments are shown for each fungal strain and growth medium. Different letters indicate significant differences (Kruskal-Wallis test; $P \leq 0.05$).

Dithianon was applied before or after *D. seriata* S inoculation of greenhouse-grown plants, and this fungicide did not reduce disease severity (Figure 6a), disease incidence (Figure S1) or amounts of *D. seriata* DNA (Figure 6b) after mycelium plug or conidium suspension inoculation of trunks. Disease severity, disease incidence and amounts of *D. seriata* DNA were affected by the inoculation procedure (Kruskal–Wallis tests, respectively, $P = 4.0 \times 10^{-7}$, $P = 7.1 \times 10^{-7}$ and $P = 3.5 \times 10^{-5}$) and they were greater in the mycelium plug-inoculated plants than in in those inoculated with conidium suspensions, after merging of data for water- and dithianon-treated plants.

DISCUSSION

Diplodia spp. have been found in asymptomatic apple bark (Arrigoni *et al.*, 2018) and Botryosphaeriace-

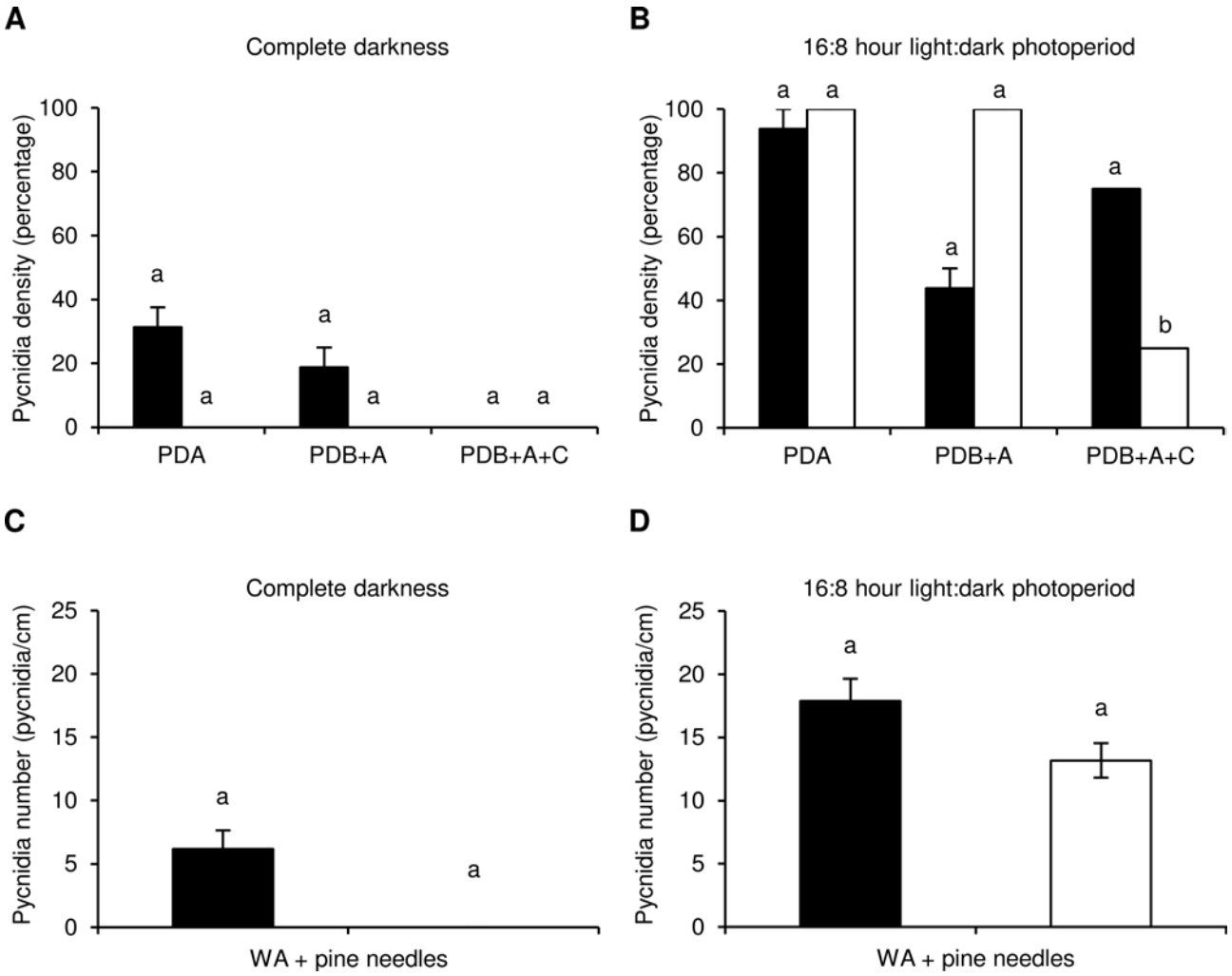

Figure 2. Pycnidium production from *Diplodia seriata* S (black) and *D. seriata* VT (white) grown on potato dextrose agar (PDA), potato dextrose broth supplemented with agar (PDB+A), PDB+A supplemented with cellulose (PDB+A+C) and water agar supplemented with pine needles (WA + pine needles). Pycnidium production was assessed, a, b) as the percentage of Petri plate surface covered by *D. seriata* pycnidia, or c, d) as the numbers of pycnidia per centimetre of pine needles 28 d after growth in complete darkness or with a 16:8 hour light:dark photoperiod at 27°C. The Kruskal-Wallis test showed no significant differences between two experimental repetitions ($P > 0.05$, n = four replicates per experiment) and data from the two experiments were pooled. Means and standard errors of eight replicates (dishes) from the two experiments are shown for each fungal strain and growth medium. Different letters indicate significant differences (Kruskal-Wallis test; $P \leq 0.05$).

ae genera can persist as latent pathogens of trees (Slippers and Wingfield, 2007). This suggests these fungi could be linked to possible plant infections and disease. In apple orchards, *Diplodia* spp. inoculum is probably controlled by fungicide applications in plant protection programmes (Ellis *et al.*, 1998). Therefore, the reduction in chemical fungicides under low-input disease management of apple scab-resistant cultivars (Simon *et al.*, 2011; Didelot *et al.*, 2016) may lead to canker outbreaks (Ellis *et al.*, 1998). In order to investigate fungicide efficacy against apple trunk canker, fast and reproducible methods were developed for *D. seriata* inoculation, disease assessment and *in vitro* fungicide efficacy testing. *Diplodia seriata* S mycelia and pycnidia were obtained on PDA in complete darkness and WA supplemented with pine needles with a 16:8 hour light:dark photoperiod. This agrees with previous studies on Botryosphaeriaceae species (Kim *et al.*, 2005; Slippers and Wingfield, 2007; Amponsah *et al.*, 2008; Phillips *et al.*, 2013; Dheepa *et al.*, 2018). Pycnidium production of *D. seriata* was greater with a 16:8 hour light:dark photoperiod than in complete darkness, confirming the role of photoperiod in

Figure 3. Inoculation procedure of a) 3-y-old apple trunk and b) cut to obtain a longitudinal wound. Each wound was c) inoculated with a mycelium plug or sprayed with conidium suspension (example as in the panel b), and d) covered with laboratory plastic film. Disease severity was assessed on e) mycelium plug-inoculated and f) conidium suspension-inoculated trunks 60 d after incubation under greenhouse conditions. Length (cm) is specified by the ruler on the right.

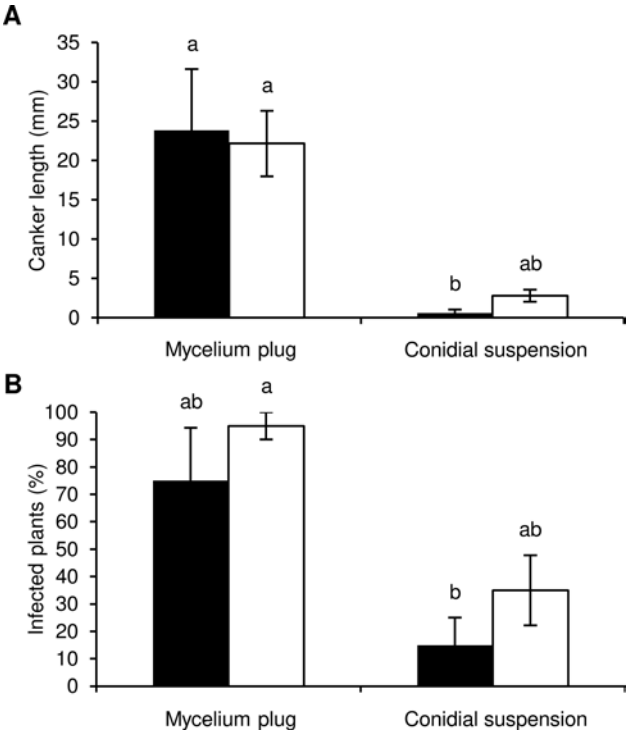

Figure 4. a) Disease severity and b) incidence caused by *Diplodia seriata* S, assessed, respectively, as canker lengths and as percentage of infected plants 60 d after incubation under greenhouse conditions. Three-y-old trunks (black) and 1-y-old shoots (white) of apple plants were inoculated with a *Diplodia seriata* S mycelium plugs or conidium suspensions. Means and standard errors are shown for five replicates (plants) for each plant organ and inoculation procedure. Different letters indicate significant differences (Kruskal-Wallis test; $P \leq 0.05$).

spore production for Botryosphaeriaceae species (Kim *et al.*, 2005; Dheepa *et al.*, 2018), particularly with pine needles (Slippers and Wingfield, 2007; Amponsah *et al.*, 2008; Phillips *et al.*, 2013).

In a previous study, a method for the inoculation of apple plants with mycelium plugs of *Botryosphaeria dothidea* and *B. obtusa* was developed under field conditions, resulting in high variability between experiments (Brown-Rytlewski and McManus, 2000). The lack of reproducibility was attributed to the fluctuating environmental conditions in the field (Brown-Rytlewski and McManus, 2000), so to overcome this variability we developed a method for artificial inoculation of *D. seriata* and fungicide efficacy testing on apple trunks under greenhouse conditions. Mycelium plug inoculations produced severe canker symptoms on the trunks and shoots of potted apple plants, and disease severity was greater in mycelium plug-inoculated plants compared with conidium suspension-inoculated plants, in agreement with Botryosphaeria dieback (e.g. *D. mutila* and *Neofusicoccum* spp.) in grapevine (Amponsah *et al.*, 2011). Severity of disease caused by *D. seriata* on apple trunks and shoots was comparable, indicating that tissue age did not affect disease development under controlled conditions, as occurred for grapevine shoots and trunks inoculated with *Neofusicoccum luteum* (Amponsah *et al.*, 2012a). In the *in vitro* fungicide efficacy tests, three commercial fungicides (fluazinam, dithianon and captan) reduced *D. seriata* viability and growth, while penconazole and ziram did not. Dithianon was reported to have little effect against *D. mutila* on grapevine plants (Amponsah *et al.*, 2012b) and demethylation-inhibiting fungicides showed different efficacy levels against *D. seriata* and *D. mutila in vitro* (Torres *et al.*, 2013), indicating their species-specific susceptibility to fungicides. Penconazole has previously been reported as an inhibitor of *in vitro D. seriata* mycelium growth (Pitt *et al.*, 2012; Mondello *et al.*, 2017), and this fungicide showed lower efficacy than fluazinam (Pitt *et al.*, 2012). Differences in penconazole efficacy could be related to the specific *D. seriata* strains used in this study, and/or to the efficacy of the test protocols used. In particular, mycelia

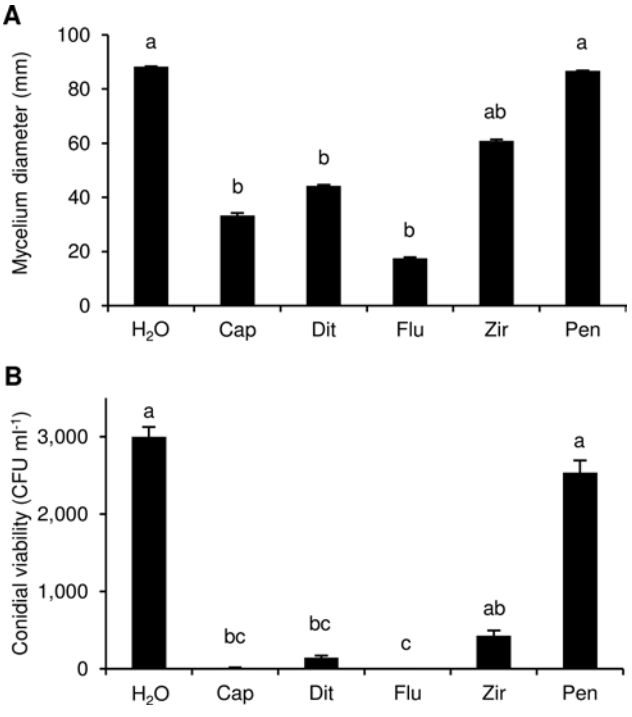

Figure 5. *Diplodia seriata* S a) mycelium growth and b) conidium viability after incubation in water (H_2O) or in presence of the fungicides: captan (Cap), dithianon (Dit), fluazinam (Flu), ziram (Zir) or penconazole (Pen). Mycelia and conidia were incubated for 5 h with the different treatments and then plated onto potato dextrose agar. Mycelium growth and conidium viability were assessed, respectively, as the colony diameter and colony forming units per unit of volume (CFU mL^{-1}) 3 and 2 d after incubation at 27°C. The Kruskal-Wallis test showed no significant differences between the two experimental repetitions ($P > 0.05$, n = 6 replicates per experiment) and data from the two experiments were pooled. Means and standard errors for 12 replicates (dishes) from the two experiments are shown for each treatment. Different letters indicate significant differences (Kruskal-Wallis test; $P \leq 0.05$).

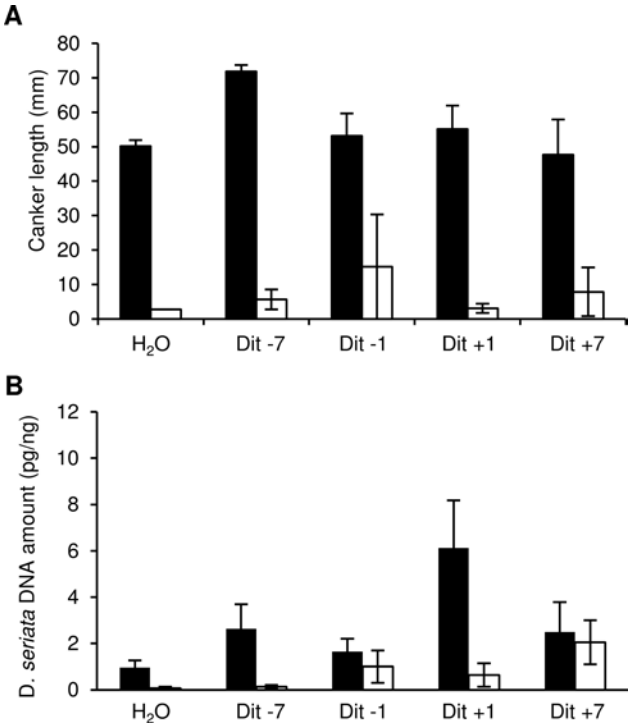

Figure 6. Disease severity a) caused by *Diplodia seriata* S and amount of pathogen DNA for 3-y-old trunks of apple plants inoculated with a mycelium plugs (black) or conidium suspensions (white) 60 d after incubation under greenhouse conditions. Plants were treated with water 1 d before inoculation (H_2O) or with dithianon 7 d before inoculation (Dit -7), 1 d before inoculation (Dit -1), 1 d after inoculation (Dit +1) or 7 d after inoculation (Dit +7). Kruskal-Wallis test showed no significant differences between the two experiments ($P > 0.05$, n = five replicates per experiment), and data from the two experiments were pooled. Means and standard errors for ten replicates (plants) from the two experiments are shown for each plant organ and inoculation procedure. No significant differences among treatments of mycelium plug- or conidium suspension-inoculated plants were found (Kruskal-Wallis test; $P > 0.05$).

and conidia were incubated in the fungicide solution for 5 h and then plated on PDA in our assays, while mycelium plugs were plated on PDA supplemented with fungicide in the protocol of Pitt *et al.* (2012), possibly leading to greater exposure to penconazole. Due to its frequent applications in IPM apple orchards (Blommers, 1994), dithianon was selected for efficacy tests in the present study. This fungicide gave no reduction in *D. seriata* severity, incidence or amounts of *D. seriata* DNA, after mycelium plug- or conidium suspension inoculations of apple plants. Discrepancies between *in vitro* and *in planta* efficacy tests have also been described for Botryosphaeria dieback agents (Mondello *et al.*, 2017), indicating that fungicide deposition, adhesion and/or penetration in host tissues can influence their efficacy (Zabkiewicz, 2007). Moreover, topical wound treatments of benomyl, kresoxim-methyl and trifloxystrobin reduced the incidence of *D. seriata* trunk canker under field conditions (Brown-Rytlewski and McManus, 2000), indicating that the mode of fungicide application and the active ingredient affected efficacy.

In the present study, methods for *D. seriata* inoculation and quantification were optimised on a scab-resistant apple cultivar (cv. Fujion), and efficacy tests of commercial fungicides were carried out *in vitro* and with conventional spray application under greenhouse conditions. Although *in vitro D. seriata* mycelium growth and conidium viability were inhibited by three fungicides (dithianon, captan and fluazinam), trunk canker symptoms were not affected by spray applications of dithianon under greenhouse condi-

tions, suggesting that application of this fungicide did not significantly contribute to indirect *D. seriata* control in IPM. The optimised methods described in this paper can be used to accurately assess fungicide efficacy against *D. seriata*, and to precisely estimate the impacts of reduced fungicide applications in low-input disease management systems for scab-resistant apple cultivars.

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Research Papers

Characterization and pathogenicity of *Colletotrichum* spp. causing citrus anthracnose in Tunisia

Haïfa BEN HADJ DAOUD[1], Elena BARALDI[2], Mirco IOTTI[3], Pamela LEONARDI[2], Naima BOUGHALLEB-M'HAMDI[1,*]

[1] *Department of Biological Sciences and Plant Protection, High Institute of Agronomy of Chott Mariem, 4042 Sousse, UR13AGR03, University of Sousse, Tunisia*
[2] *Department of Agricultural and Food Sciences, DISTAL, University of Bologna, Via le Fanin 46, 40127, Bologna, Italy*
[3] *Department of Life, Health and Environmental Sciences, University of L'Aquila, via Vetoio, Coppito 1, 67100 L'Aquila, Italy*
*Corresponding author: n.boughalleb2017@gmail.com

Abstract. In the winter and spring of 2014–2015, typical anthracnose symptoms were detected on different citrus varieties in Cap-Bon and Morneg-Tunis regions of Northern Tunisia. Surveys were conducted to determine the casual agents of these symptoms. A total of seven monosporic isolates were obtained from dark lesions on fruits, flowers, leaves and twigs of citrus from six orchards. One *Colletotrichum karstii* (from the *C. boninense* species complex) and six *C. gloeosporioides* isolates were identified through morphological analysis and sequencing of their ITS rDNA sequences. Pathogenicity tests with the seven isolates were performed on symptomless, detached citrus fruits (Valencia orange and Eureka lemon). All tested isolates caused anthracnose lesions after 1 week of incubation. Koch's postulates were fulfilled by re-isolation of pathogens from the inoculated fruits. This report elucidates the diversity of anthracnose pathogens in Tunisia. This is the first report of *C. karstii* from citrus in Tunisia.

Keywords. Citrus anthracnose, *Colletotrichum karstii*, *Colletotrichum gloeosporioides*, PCR.

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INTRODUCTION

The citrus industry is one of the most important fruit industries worldwide. Mediterranean countries are second only to China for citrus production, and are the largest exporter after South Africa (FAO, 2016). In Tunisia, citrus orchards cover 27,000 ha and include approx. 7 million trees. During the last decade, citrus production has steadily increased from 300,000 MT in 2007/08 as new orchards have begun production, reaching 560,000 MT in 2016/17. The most important crops are Maltese and Navel oranges [*Citrus sinensis* (L.) Osbeck], clementines (*Citrus* x *Clementina*), lemons (*C. limon* L.) and mandarins (*C. reticulata* Blanco) (Haas, 2017).

Knowledge of the pathogens affecting citrus crops is important. Previous investigations have demonstrated that these crops are affected by many fungal diseases such as Alternaria brown spot (Haddad *et al*., 2013), gummosis (Boughalleb-M'Hamdi *et al*., 2018) and anthracnose (Ben Hadj Daoud, 2013; Rhaiem and Taylor, 2016).

In the present study, we focus on citrus anthracnose. *Colletotrichum* is among the most economically important genera of plant pathogenic fungi worldwide (Sutton, 1992; Cai *et al*., 2009; Phoulivong, 2011). This genus has been designated the eighth most important group of plant pathogenic fungi, based on perceived scientific and economic importance (Dean *et al*., 2012). Several species of *Colletotrichum* cause diseases (anthracnoses) of a wide range of important crops (Sutton, 1992; Hyde *et al*., 2009a). Two species complexes, *C. acutatum* and *C. gloeosporioides* encompass the main causal agents of citrus diseases. Three important diseases of citrus caused by *Colletotrichum* spp. are anthracnose, post-bloom fruit drop and key lime anthracnose (Timmer *et al*., 2000; Lima *et al*., 2011; McGovern *et al*., 2012).

Colletotrichum gloeosporioides is reported as pathogen of the main cultivated citrus species worldwide (Huang *et al.,* 2013). *Colletotrichum gloeosporioides sensu stricto* occurs more frequently in the Old World, contrasting with the greater importance of *C. acutatum* in the Americas (Lima *et al*., 2011). Before multi-gene analysis, *C. acutatum* was identified as the only species responsible for post-bloom fruit drop (PFD) (Peres *et al.,* 2008) and key lime anthracnose (KLA) (Brown *et al*., 1996). Similarly, *C. gloeosporioides* was reported as the only *Colletotrichum* species to cause citrus fruit anthracnose (Timmer *et al*., 2000). PFD of citrus was first described in Belize (formerly British Honduras) in 1979, caused by *C. gloeosporioides* (Penz.) Sacc. (Denham, 1979; Fagan, 1979). This disease has been detected in Argentina, Brazil, Mexico and the United States (Schwarz *et al*., 1978; Orozco Santos and Gonzalez Garza, 1986; McMillan and Timmer, 1989; Kuramae-Izioka *et al*., 1997). PFD was widespread in the western hemisphere in the 1990s, including in Belize, Bermuda, Brazil, Costa Rica, Mexico and the United States (Florida), and has become less of a problem in Florida in recent years. However, PFD could re-emerge as a severe citrus disease (McGovern and Rouse, 1994; Timmer and Brown, 2000). In Florida, the causal agents of PFD and KLA were initially identified as strains of *C. gloeosporioides* (Agostini et *al*., 1992). Genetic characterization of *Colletotrichum* isolates from Brazil suggested that PFD can be caused by both *C. acutatum* and *C. gloeosporioides* (Kuramae-Izioka *et al*., 1997). A report from south Brazil identified *C. acutatum* as the cause of PFD (Theodoro *et al*., 2003). *Colletotrichum gloeosporioides* was reported as a causal agent of PFD on sweet orange (Lima *et al*., 2011; McGovern *et al*., 2012). An extensive investigation in citrus orchards of São Paulo state (Brazil) revealed only *C. abscissum* and *C. gloeosporioides sensu stricto* associated with PFD disease (Da Silva *et al*., 2016). Key lime anthracnose, a disease complex on leaves, flowers and fruits of Key lime, was initially reported to be caused by *C. acutatum* (Brown *et al*., 1996; Peres *et al*., 2008; MacKenzie *et al*., 2009), but later classified as *C. limetticola* (Damm *et al*., 2012a).

Following adoption of the use of multi-gene phylogenetic analyses, the polyphasic protocols for studying the genus *Colletotrichum* significantly changed the classification and species concepts this genus (Cannon *et al*., 2012; Damm *et al*., 2012a; 2012b; 2013; 2014; Weir *et al*., 2012). In China, at least 13 species of *Colletotrichum* have been associated with citrus (Peng *et al*., 2012). Huang *et al.* (2013) reported the presence of four important species complexes of *Colletotrichum* in citrus in China, namely the complexes of *C. gloeosporioides*, *C. boninense* and *C. acutatum,* and a group including *C. truncatum*. The species in the *C. gloeosporioides* species complex comprised *C. gloeosporioides* and *C. fructicola*, the *C. boninense* complex included *C. karstii* and a new species *C. citricola*, and the *C. acutatum* complex included a new species, *C. citri*. The most important causal agent is *C. gloeosporioides*, and *C. gloeosporioides sensu lato* was also reported to cause pre-harvest symptoms such as wither-tip on twigs, tear-stain (Klotz and Fawcett, 1941; Benyahia *et al*., 2003) and stem-end rot on fruit (Kaur *et al*., 2007).

Colletotrichum acutatum sensu stricto, as member of *C. acutatum* species complex, was found on citrus for the first time in Europe by Guarnaccia *et al.* (2017). This fungus was isolated in many citrus growing areas surveyed during 2015 and 2016, including Spain, Italy, Portugal, Greece and Malta, and from several citrus species showing typical anthracnose symptoms on leaves fruits and twigs. *Colletotrichum novaezelandiae* (from the *C. boninense* species complex) was also first reported on citrus. Infections caused by *Colletotrichum* spp. Have strongly compromised citrus production in Mediterranean countries. In Portugal, since 2003, anthracnose symptoms were detected in lemon and orange orchards. Ramos *et al.* (2006) found isolates with characteristics that did not fit those commonly described for *C. acutatum* and *C. gloeosporioides*, raising the hypothesis of other species of *Colletotrichum* were involved on preharvest anthracnose of citrus. Ramos *et al.* (2016) demonstrated that many isolates collected from symptomat-

ic leaves, twigs and fruits of several citrus cultivars in Portugal were *C. gloeosporioides sensu stricto* predominated in flowers and fruits, and *C. karstii* was found in leaves and branches. In Italy, pre-harvest anthracnose symptoms appeared on orange fruits, caused by *C. gloeosporioides* and *C. karstii* (Aiello *et al.*, 2015). *Colletotrichum karstii*, a member of the *C. boninense* species complex, has been reported on many host plants with wide geographical distribution (Damm *et al.*, 2012b). In Europe, this species has been detected on tropical fruits, cotton and lupin plants (Damm *et al.*, 2012b; Ismail *et al.*, 2015). *Colletotrichum karstii* was previously found to be an important pathogen on *Orchidaceae* hosts (Yang *et al.*, 2011), and has also been isolated from citrus plants in South Africa, New Zealand (Damm *et al.*, 2012b) and China (Peng *et al.*, 2012). Peng *et al.* (2012) showed that this species causes citrus leaf anthracnose. Guarnaccia *et al.* (2017) reported the presence of three major species complexes in surveyed citrus regions in European countries namely, *C. gloeosporioides sensu stricto* and two new species (*C. helleniense* and *C. hystricis*) were identified in the *C. gloeosporioides* species complex. *Colletotrichum karstii*, *C. novaezelandiae* and two new species (*C. catinaense* and *C. limonicola*) in the *C. boninense* species complex, and *C. acutatum sensu stricto* was also identified as member of *C. acutatum* species complex.

Anthracnose diseases of citrus in Tunisia had not been well researched. Since early 2009, wither-tip symptoms of twigs, resembling anthracnose, were observed on cultivated citrus trees in Northern Tunisia. Using morphological and molecular characteristics and pathogenicity tests on isolates collected from withered twigs, Rhaiem and Taylor (2016) reported for the first time the presence of *Colletotrichum gloeosporioides* as the causal agent of anthracnose disease of citrus in Tunisia. This fungus was also identified on olive by Rhouma *et al.* (2010). Species of *C. acutatum sensu stricto* were found by Chattaoui *et al.* (2016) on olives fruits from Northern Tunisia, showing symptoms of anthracnose since November 2011. The *C. acutatum sensu stricto* group was recorded for the first time in the country as the causal agent of olive anthracnose. Because of the commercial yield losses in citrus orchards caused by *Colletotrichum* infections in Tunisia, further research was required to identify *Colletotrichum* spp. related to citrus anthracnose. In our surveys commencing in April 2014, severe anthracnose symptoms, such as the typical dieback and wither-tip of twigs and dark lesions on fruits, leaves and flowers, were observed on different citrus varieties in orchards of Cap-Bon and Morneg-Tunis (Northern Tunisia).

The objectives of the present investigation were to: (i) evaluate symptoms of anthracnose in surveyed citrus orchards; (ii) characterize the *Colletotrichum* spp. causing anthracnose on citrus in Tunisia, using morphological and molecular techniques; and (iii) evaluate the pathogenicity of the species on Valencia orange and Eureka lemon.

MATERIALS AND METHODS

Fungus isolations

Ten citrus fruit, twig and leaf samples with typical anthracnose symptoms were collected from orange varieties Thomson, Malti and Meski and clementine variety Cassar during spring 2014 and winter 2015. Small pieces of tissue (5 mm × 5 mm) were sterilized in sodium hypochlorite solution for 5 min, followed by several rinses with sterile distilled water (SDW), and then placed on Potato Dextrose Agar (PDA, BD DIFCO) in Petri dishes for one week at 27°C.

Monoconidial cultures were generated from each sample and stored on dry filter paper at -20°C, and were later identified morphologically. The conidia were taken from actively growing colonies and suspended in sterile water. Lengths and widths of 50 conidia from each isolate were measured under an ECLIPSE TE 2000-E microscope (×1,000 magnification) (Nikon) using differential interference contrast optics. Measurements were taken using a Nis-Elements AR (v 3.10) software (Zeiss) from images captured with a DXM1200F digital camera (Nikon). Q-value of conidia (the ratio of length to width in side view) was also calculated. The growth rate of all isolates was determined on PDA in 90 mm diam. Petri dishes, using three plates for each isolate, incubated at 25°C. Colony diameters were measured for the first 7 d after inoculation, and mean daily colony growth rates were calculated as described by Huang *et al.* (2013).

Molecular characterization and phylogeny of isolates

The identity of the isolates were examined by amplification and sequencing of ITS rDNA regions by a direct PCR approach, without DNA isolation (Iotti and Zambonelli, 2006; Mari *et al.*, 2012). Direct PCR was applied to cultures grown on PDA for 7 d at 25°C. Under a dissecting microscope, a few hyphae were taken from the aerial mycelium of each culture using a sterile needle, and were placed in a 0.2 mL capacity PCR tube containing 20 µL of sterile water. Tubes with hyphae were always maintained on ice until PCR amplification. ITS

regions were amplified as described by Iotti and Zambonelli (2006), using the universal fungal primers ITS5 and ITS4 (White *et al.*, 1990). The amplifications were carried out with an initial denaturation at 94°C for 6 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 6 min. PCR products were purified using Nucleospin PCR cleanup kit (Macherey Nagel) and sequenced using both the amplification primers by the Eurofin Genomics sequencing service (https://www.eurofinsgenomics.eu/en/custom-dna-sequencing.aspx). The sequences were analysed using the BLASTn program with default parameters (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1990), and they were aligned using the ClustalW software (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Bayesian inference and maximum likelihood were applied to confirm the phylogenetic affiliation of isolates from this study. Two separate phylogenies were constructed for *C. boninense* and *C. gloeosporioides* species complexes. Sequences generated in this study were used in conjunction with those of Damm *et al.* (2012b) and Weir *et al.* (2012). One or more sequences for each *Colletotrichum* sp. were selected for tree construction, by giving priority to those from type specimens (NR GenBank accession prefix) when available. The best fit model of nucleotide substitution (TrNef + I) was selected by jModelTest 2.1.4 (Darriba *et al.*, 2012). Bayesian analysis was performed by two independent runs, with four chains each, and were carried out for 5 million generations, sampling every 100 generations. A 50% majority-rule consensus tree was generated after exclusion of the first 250 trees. Maximum likelihood (ML) analysis was performed in Raxml GUI 1.1.3 (Silvestro *et al.*, 2012). The ML search included 100 random addition replicates and 1,000 nonparametric bootstrap replicates.

Pathogenicity tests

Seven *Colletotrichum* isolates were grown on PDA for 1 week at 27°C, and were used in pathogenicity tests to compare their virulence and evaluate the susceptibility of citrus varieties Valencia orange and Eureka lemon. Ten fruits of each variety were inoculated with each isolate. The fruits were washed, disinfected by immersion for 10 min in sodium hypochlorite solution , rinsed twice in SDW, and were each inoculated by placing a 10 µL droplet of spore suspension (10^5 conidia mL^{-1}) on the surface. Inocula were obtained from cultures grown on PDA for 10 d at 25°C. Conidia were washed from the dishes, passed through four layers of cheesecloth, and diluted in sterile distilled water to 10^5 conidia mL^{-1}. Fruits were previously injured using a sterile tip (wounds of 2 mm diam.). Control fruits were inoculated with SDW. Fruits were each labelled with a permanent marker, and the inoculation points were circled on each fruit. The fruits were then incubated in plastic containers at 25°C and 100% relative humidity, and were examined for lesion development 7 d after inoculation (Aiello *et al.*, 2015). After 14 d, spores from infected fruits were aseptically transferred and sub-cultured onto PDA plates, which were incubated at 25°C. The resulting cultures were checked for colony and spore morphology to confirm Koch's postulates.

Statistical analyses

Data of conidium size, colony growth rate and isolate pathogenicity were compared using one way ANOVA, and mean values among treatments were compared by the Tukey's *post hoc* test ($P < 0.05$).

RESULTS

Surveys, and Colletotrichum *species isolation*

Surveyed orchards located in major citrus production areas in Northern Tunisia showed typical symptoms of anthracnose. These consisted of typical dieback and wither-tip of twigs showing orange conidium masses, which were observed on different citrus varieties (Figure 1). On fruits, symptoms were small, light to dark irregular and sunken lesions (Figure 2). On leaves, circular, light tan, flat areas with purple margins, containing black acervuli, were observed (Figure 3). Seven *Colletotrichum* spp. isolates were obtained from surveyed orchards of citrus growing areas from Northern Tunisia. Four isolates were from orange Malti, one was from orange Meski, one was from orange Thomson and one was from clementine Cassar. Three isolates were from fruits, two were from leaves, one was from a flower and one was from a twig (Table 1).

Morphological and cultural characteristics of isolated fungi

The seven isolates were initially characterized by morphological analysis of mycelium and conidia. Colonies of most of the isolates (six) closely resembled those typically formed by *C. gloeosporioides*, with dense, raised, cottony mycelium, which, after 7 d, turned from white or pale white (for isolate C21) and pink to orange (for isolates C2, C9, C31 and C32) (Figure 4). Conidia

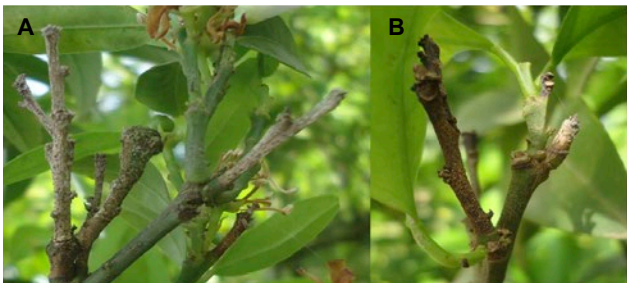

Figure 1. Symptoms on citrus twigs caused by *Colletotrichum* spp. A) wither-tip of twigs of clementine Cassar; B) orange conidiu masses of *Colletotrichum* spp. on twigs of clementine Cassar.

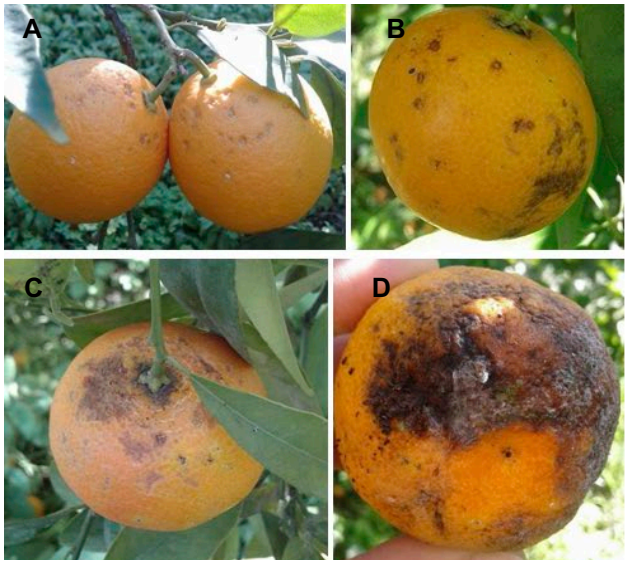

Figure 2. Symptoms on citrus fruits caused by *Colletotrichum* spp. A) Brown and dark lesions on orange, and B) on clementine. C) Stem-end rot on clementine. D) Typical anthracnose on fallen clementine fruit.

Table1. Characteristics of *Colletotrichum* species used in this study.

Isolate	Date of isolation	Location	Host citrus variety	Host tissue	GenBank[a]
C35	11/02/2015	Morneg	Malti	Fruit	MF581072
C2	14/04/2014	Hammamet	Meski	Flower	MF581073
C5	14/04/2014	Hammamet	Cassar	Leave	MF581074
C9	23/04/2014	BouArgoub	Malti	Leave	MF581075
C21	11/02/2015	Morneg	Malti	Fruit	MF581076
C31	11/02/2015	Morneg	Thomson	Twig	MF581077
C32	11/02/2015	Morneg	Malti	Fruit	MF581078

[a] Accession number of ITS sequences.

Figure 3. Anthracnose symptoms on leaves of clementine (A, B and C).

were consistently cylindrical with rounded or obtuse ends, and measured 7 to 21 × 2 to 5 µm (average = 10.9 × 3.7 µm) (Table 2). Some cultures developed black acervuli and bright orange spore masses, which were produced in the colony centres (Figure 4), as typical for *C. gloeosporioides*. In contrast, colonies of isolate C35 were similar to those produced by *C. karstii,* with moderately dense white-orange early mycelium, which, after 7 d, turned pink/orange (Figure 5). Conidia were cylindrical, hyaline and with round apices, and measured 11 to 16 × 4 to 6 µm (average = 13.1 × 4.7 µm) (Table 2). Statistical analyses of conidium dimension data revealed significant differences in length, width and Q-values. The conidia of C35 isolate were longer and their Q-value was greater than those of the other six isolates (Table 2). Statistically significant differences of conidium dimensions were also found among these six isolates.

The mean daily growth colony rate of the isolates, measured at 25°C, ranged from 4.1 to 6.9 mm (Table 2). Colonies of C35 grew more rapidly than the other isolates, and differences in colony growth rates were found between the isolates (Table 2).

PCR amplification and sequence analysis

Sequencing of the ITS regions of the seven isolates was performed in order to allow species identification. PCRs generated amplicons of 599 bp (isolates C2, C5, C9, C21, C31, and C32) and 618 bp (C35). BLASTn search against GenBank revealed that the ITS sequences of seven isolates were identical to those of *C. gloeosporioides* (for isolates C2, C5, C9, C21, C31, C32) and *C. karstii* (isolate C35). Sequence similarity ranged between 99.8 and 100% among *C. gloeosporioides* isolates (one variable position in ITS1) and 91.1 and 91.3% between *C. gloeosporioides* and *C. karstii* isolates (37 to 38 variable positions in ITS1 and six in ITS2). Phylogenetic reconstruction of the *C. boninense* and *C. gloeosporioides* species complexes placed the

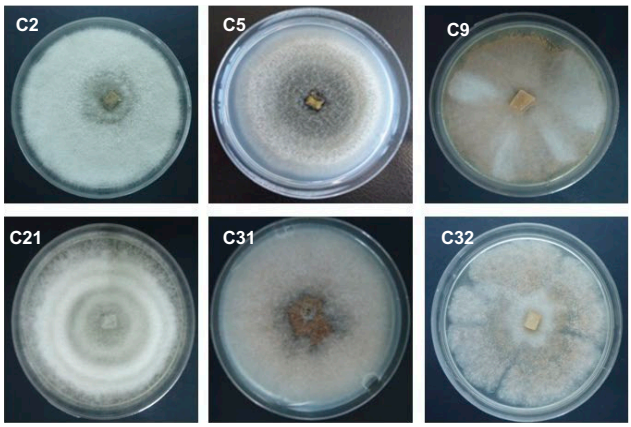

Figure 4. Morphology of *Colletotrichum gloeosporioides* isolates C2, C5, C9, C21, C31 and C32 on PDA plates incubated at 25 °C for 7 d.

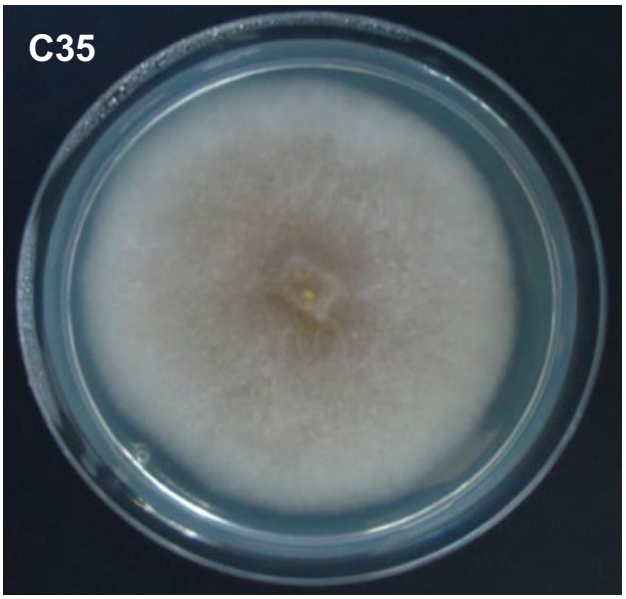

Figure 5. Morphology of *Colletotrichum karstii* (*C. boninense* complex) isolate (C35) on PDA plates incubated at 25 °C for 7 d.

isolates from this study into the clade *C. gloeosporioides sensu stricto* (isolates C2, C5, C9, C21, C31, C32) and the clade *C. karstii* (isolate C35) (Figure 6). Unlike other clades in the genus, both of these clades were well supported, by posterior probabilities and by ML bootstrap values. Two distinct subclades were also evident within the *C. gloeosporioides* clade, and respective Tunisian isolates were present in both. No correlations were found between ITS types and the metadata of the respective isolates (e.g. host tissue and variety, collection site).

Pathogenicity tests

The seven studied isolates induced symptoms on Valencia orange and Eureka lemon, which were identical to those observed in citrus orchards. One week post-inoculation, brown sunken and dark lesions appeared surrounding the inoculation points. After 14 d, the lesions contained dense mycelium, orange conidium masses and black acervuli (Figure 7). No lesions developed on fruits inoculated with water.

The data obtained from this assay were analysed to compare virulence of the studied isolates, taking into consideration the different susceptibilities of orange and lemon. Pathogen virulence and host species susceptibil-

Table 2. Conidium dimensions and growth rates of seven *Colletotrichum* isolates grown on PDA at 25°C. Isolates C2, C5, C9, C21, C31 and C32 are *C. gloeosporioides* and isolate C35 is *C. karstii*.

Character	C2	C5	C9	C21	C31	C32	C35
Length of conidia (µm)							
Mean ± SE[a]	9.6[b] ± 0.2 CD[c]	9.1 ± 0.2 D	10.9 ± 0.2 B	10.3 ± 0.2 BC	11.1 ± 0.2 B	8.9 ± 0.2 D	13.1 ± 0.2 A
Range	7.4-13.8	7.4-11.0	8.3-21.1	8.3-15.7	9.4-14.6	7.4-10.5	10.8-16.1
Width of conidia (µm)							
Mean ± SE	3.7 ± 0.1 D	3.9 ± 0.1 CD	4.1 ± 0.1 BC	4.1 ± 0.1 BC	4.2 ± 0.1 B	3.8 ± 0.1 D	4.7 ± 0.1 A
Range	2.6-5.3	2.9-5.0	2.4-4.9	2.9-4.9	3.4-5.0	2.7-4.6	4.0-5.9
Q-value of conidia							
Mean ± SE	2.6 ± 0.1A	2.4 ± 0.1B	2.7 ± 0.1A	2.6 ± 0.1AB	2.7 ± 0.1A	2.4 ± 0.1B	2.8 ± 0.1A
Growth rate (mm)							
Mean ± SE	4.6 ± 0.5B	4.3 ± 0.5 B	4.5 ± 0.5 B	4.1 ± 0.5 B	5.1 ± 0.5 B	4.3 ± 0.5 B	6.9 ± 0.5 A

[a]SE = standard errors of means.
[b]Each value represents the mean of 50 measurements.
[c]Values derived from the average of 50 measurements. Different letters accompanying means in each row indicate statistically significant differences ($P < 0.05$) according to Tukey's *post hoc* test.

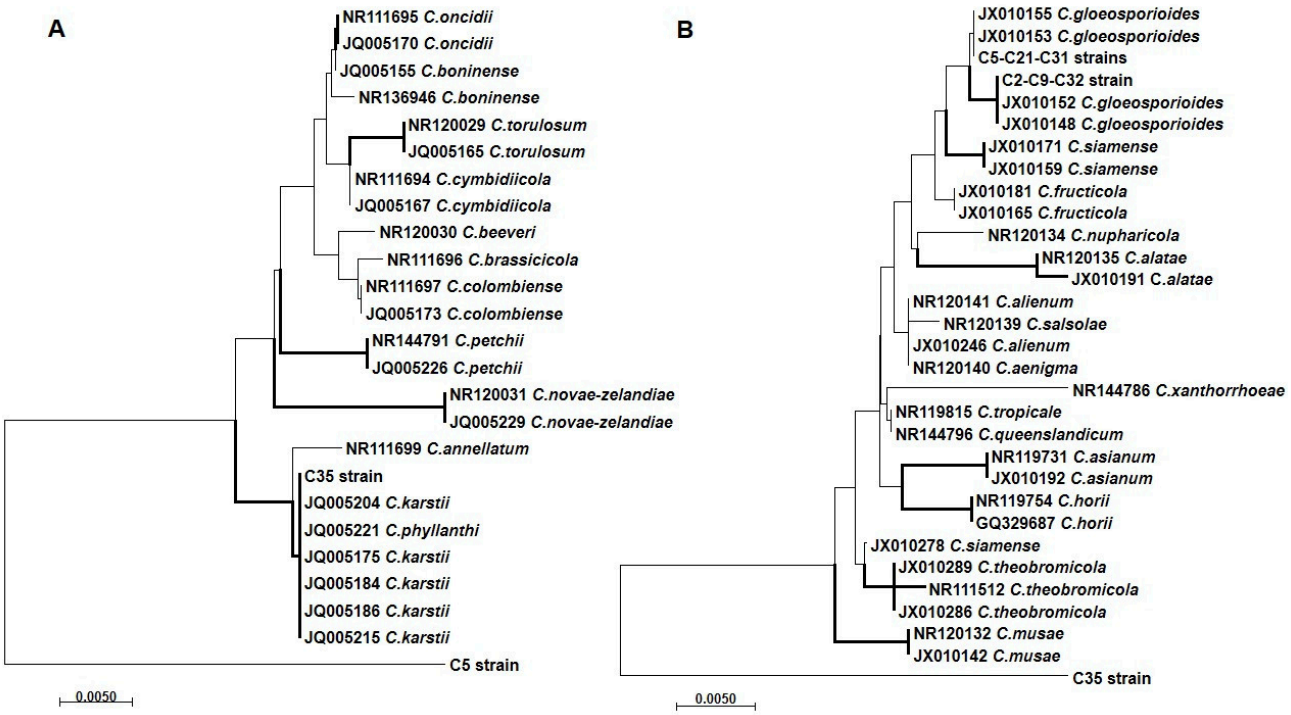

Figure 6. Maximum likelihood trees of *Boninense* (A) and *Gloeosporioides* (B) species complexes, based on the ITS1-5.8S-ITS2 region. Topologies resulting from maximum likelihood and Bayesian inference were congruent and the most likely trees are showed. Thickened branches are supported by likelihood bootstrap values of >70% and by Bayesian posterior probabilities of >95%. Taxa are labelled by their GenBank code (NR codes identify the type vouchers) or isolate code. Sequences from GenBank were selected based on results after Dumm *et al.* (2012) and Weir *et al.* (2012). Sequences of isolates C5 and C35 were differentially used as outgroups. The tree was edited using FigTree v 1.4.3.

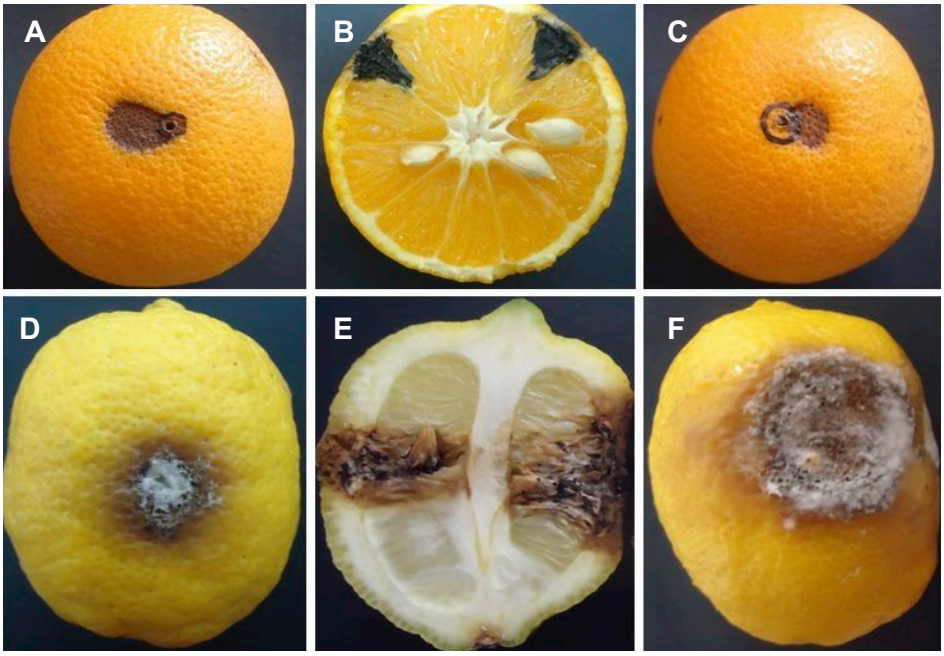

Figure 7. Lesions caused by *Colletotrichum karstii* (isolate C35) on artificially inoculated fruits of Valencia sweet orange (A and B) and on fruits of Eureka lemon (F). Lesions caused by *C. gloeosporioides* (isolate C31) on artificially inoculated fruit of Valencia sweet orange (C) and on fruits of Eureka lemon (D and E).

Table 3. Mean lesion diameter caused by *Colletotrichum* isolates on Valencia sweet orange and Eureka lemon.

Citrus variety	Isolate						
	C2	C5	C9	C21	C31	C32	C35
Valencia orange							
Inoculated fruit	5.4[a] ± 0.1[b] AB	0 ± 0.1 C	6.7 ± 0.1AB	5.3 ± 0.1 AB	8.2 ± 0.1 A	3.4 ± 0.1 BC	8.0 ± 0.1 A
Control fruit	0	0	0	0	0	0	0
Eureka lemon							
Inoculated fruit	24.0 ± 0.3 B	24.3 ± 0.3 B	22.6 ± 0.3 B	20.4 ± 0.3 B	36.8 ± 0.3 A	19.0 ± 0.3 B	13.6 ± 0.3 B
Control fruit	0	0	0	0	0	0	0

[a] Each value represents the mean of five replicates.
[b] SE = standard errors of means.
[c] Different letters accompanying means in each row indicate statistically significant differences for ($P < 0.05$), Tukey's *post hoc* test.

ity were statistically different as demonstrated in ANOVA ($P < 0.05$). For mean lesions diameter, on Valencia orange, the most aggressive isolates were C35 and C31 (Table 3), whereas no lesions were obtained with the isolate C5. On Eureka lemon, the most aggressive isolate was C31, which produced lesions three times larger than those of isolate C35. The data also showed that Eureka lemon was more susceptible to all of the *Colletotrichum* isolates compared to Valencia orange. For example, isolate C35 caused smaller lesions than the other isolates (Table 3). Isolations from lesions of inoculated fruits of the two citrus types yielded colonies identical to the original inoculated isolates, confirming Koch's postulates for these pathogenic fungi.

DISCUSSION

Symptoms of anthracnose, such as wither-tip of twigs, have been observed on cultivated citrus crops in Tunisia since 2009. *Colletotrichum gloeosporioides* was confirmed as the principal pathogen responsible for wither-tip of twigs and lesions on fruits and leaves of Encore mandarin and Minneola tangelo in Northern Tunisia (Rhaiem and Taylor, 2016). The present study has confirmed that *C. gloeosporioides* is the main causal agent of anthracnose also in clementine and sweet orange, and records for the first time in Tunisia that *C. karstii* is an anthracnose pathogen on citrus. Although ITS regions are not variable enough to separate closely related species in *Colletotrichum* (Crouch *et al.*, 2009), phylogeny obtained in the present study clearly showed that the strains were monophyletic in the *C. gloeosporioides sensu stricto* and *C. karstii* clades. Isolate C35 could also belong to *C. phyllanti*, a sister species of *C. karstii*, with identical ITS sequence, but *C. phyllanti* has only been recorded found in India on *Phyllanthus acidus*, *Bahuinia variegate* and *Bougainvillea glabra* (Damm *et al.*, 2012b; Sharma and Shenoy, 2013). *Colletotrichum karstii* could represent a species complex, although more isolates need to be analysed to validate this hypothesis (Sharma and Shenoy, 2013).

Severe symptoms of anthracnose were observed on fruits, flowers, leaves and twigs in different citrus types and varieties in Northern Tunisia. These included Malti, Meski, Thomson oranges and clementine Cassar. *Colletotrichum gloeosporioides* was isolated from citrus fruits, flowers, leaves and twigs, while the unique isolate of *C. karstii* was found on fruits.

Colletotrichum karstii is a morphologically diverse species (Alizadeh *et al.*, 2015). Colour of the upper surfaces of the colonies varies from white to grey, and pink on the reverse sides (Velho *et al.*, 2014), or also white to grey, usually with pink conidium masses, and reverse sides yellow to dark brown (Yang *et al.*, 2011). According to Taheri *et al.* (2016), the surfaces of colonies vary from white to grey with orange conidium masses and entire margins, and with pale orange reverse sides. Orange conidiomata are seen on PDA with filter paper. These variations have also been observed in other *Colletotrichum* spp. (Taheri *et al.*, 2016). In *C. karstii*, conidia also have different size ranges, including: (11.5–14.5) × (5–6.5) µm (Damm *et al.*, 2012b), (13.5–15) × (4–5) µm (Aiello *et al.*, 2015) or (10–16.5) × (4–6.5) µm (Taheri *et al.*, 2016).

During the last 2 to 3 decades, *Colletotrichum* spp. associated with citrus anthracnose have attracted considerable attention from researchers (Ramos *et al.*, 2016). Post-bloom Fruit Drop (PFD) and Key Lime Anthracnose (KLA) caused by *C. acutatum sensu lato* were known to affect citrus production in many countries (Peres *et al.*, 2008). However, these diseases were not previously reported in Tunisia. *Colletotrichum* fungi belonging to the *C. gloeosporioides* species complex include a number of economically important posthar-

vest pathogens (Gan *et al*., 2017). This species is very common on *Citrus* spp. in many countries, where this fungus is responsible for KLA and postharvest diseases. It also occurs on other hosts (Weir *et al*., 2012). In Southern Italy, *C. gloeosporioides* is commonly isolated from citrus, especially from *Citrus sinensis* (Aiello *et al*., 2015; Cannon *et al*., 2008; Peng *et al*., 2012). *Colletotrichum gloeosporioides* was also reported to cause preharvest symptoms in Southern Italy, including severe lesions on fruits of sweet orange (Aiello *et al*., 2015), and wither-tip of twigs and tear stain and stem-end rot of fruits (Benyahia *et al*., 2003; Huang *et al*., 2013). *Colletotrichum karstii* has been isolated from citrus plants in South Africa, New Zealand (Damm *et al*., 2012b) and China (Peng *et al*., 2012). In Iran, five species of *Colletotrichum*, including *C. gloeosporioides sensu stricto* and *C. karstii*, were found from leaves, fruits and stems of citrus species (Taheri *et al*., 2016). Recently, *C. karstii* was described as a causal agent of citrus anthracnose in Italy (Aiello *et al*., 2015). In Portugal, *C. gloeosporioides* and *C. karstii* were identified from symptoms on leaves, branches, flowers and fruit, with *C. karstii* frequently occurring in branches and leaves of lemon in specific geographic locations (Ramos *et al*., 2016).

The pathogenicity tests carried out in the present study showed that *C. karstii* and *C. gloeosporioides* isolates were virulent for the two studied citrus fruits. In Portugal, Ramos *et al.* (2016) reported that the importance of *C. karstii* was greater in lemons compared to other citrus types, although *C. karstii* can be as virulent as *C. gloeosporioides* for some citrus organs and varieties. In Italy, pathogenicity tests with isolates of *C. gloeosporioides* and *C. karstii* clearly showed their ability to cause lesions on fruits (Aiello *et al*., 2015). However, these data demonstrated that *C. gloeosporioides* was more aggressive than *C. karstii*. Our study has shown that in Tunisia, *C. karstii* was one of the most virulent isolates on fruits of Valencia orange but it caused less severe symptoms in fruits of Eureka lemon.

Anthracnose caused by different *Colletotrichum* species could become severe threats for citrus production and for other crops when they establish in particular horticulturally important areas. Further studies on the development and spread of anthracnose caused by *C. karstii* in Tunisia are necessary, to provide knowledge of the implications of anthracnose for citrus production, and to define suitable disease management strategies.

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Research Papers

An integrative approach for the selection of *Pochonia chlamydosporia* isolates for biocontrol of potato cyst and root knot nematodes

Maria Clara VIEIRA DOS SANTOS[1,3,*], Joana HORTA[1], Luísa MOURA[2], David V. PIRES[2], Isabel CONCEIÇÃO[1], Isabel ABRANTES[1], Sofia R. COSTA[2,3]

[1] *Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Calçada Martim de Freitas, P 3000 456 Coimbra, Portugal*
[2] *Mountain Research Centre (CIMO)/ Escola Superior Agrária (ESA), Instituto Politécnico de Viana do Castelo, Refóios, 4990-706 Ponte de Lima, Portugal*
[3] *CBMA- Centre of Molecular and Environmental Biology, Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal*
*Corresponding author: mcvs@sapo.pt

Abstract. The nematophagous fungus *Pochonia chlamydosporia*, a natural enemy of plant parasitic nematodes (PPN), has been exploited in the development of sustainable management strategies for PPN control. The intrinsic variation among *P. chlamydosporia* isolates affects biocontrol potential, so sound characterisation is required. Biological, molecular and metabolic analyses can be determinant in the screening and selection of these potential biological control agents. This study aimed to provide integrative characterisation of *P. chlamydosporia* isolates that can support isolate selection for biological control purposes. Eight Portuguese isolates, associated with *Meloidogyne* spp., were used as a case study. Isolates were identified and characterised: i) at the biological level through standard bioassays to evaluate their ability for rhizosphere colonisation, to produce chlamydospores, and to parasitise *Globodera pallida* and *Meloidogyne incognita* eggs; ii) at the molecular level, examining genetic variation using ERIC-PCR; and iii) at the metabolic level, assessing their metabolic profiles using Biolog FF MicroPlates, with concurrent reads of fungal utilisation of 95 different carbon sources. Molecular data and metabolic characterisation were reduced using Principal Component Analysis and compared with the biological characteristics. Molecular profiles could only be related to isolate geographical origin, but the original substrate (eggs or roots), parasitism of *M. incognita* eggs and ability for rhizosphere colonisation were correlated with metabolic profiles, indicative of utilisation of specific carbon sources. *Meloidogyne* egg parasitism and rhizosphere colonisation were related to each other. This integrative characterisation offers novel perspectives on the biology and biocontrol potential of *P. chlamydosporia*, and, once tested on a broader set of isolates, could be used to assist rapid isolate selection.

Keywords. Biolog, isolate selection, metabolic characterisation, nematophagous fungus, rhizosphere interactions.

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INTRODUCTION

Plant-parasitic nematodes (PPN) are widespread pests and are severe threats to agricultural crops (Nicol *et al*., 2011). The most economically important PPN, which have impacts on crops worldwide, are root knot nematodes (RKN), *Meloidogyne* spp., and potato cyst nematodes (PCN), *Globodera* spp. (Jones *et al*., 2013). The facultative nematophagous fungus *Pochonia chlamydosporia* is an endophyte that parasitises sedentary females and eggs of economically important PPN, including cyst nematodes and RKN, and has been found associated with nematode suppressive soils (Manzanilla-Lopez *et al*., 2013).

Growth and proliferation of *P. chlamydosporia* in soil can be slow, and only achieved after multiple applications of the fungus (Vieira dos Santos *et al*., 2014). However, impact of the fungus on nematode reproduction is more affected by the ability to colonise the rhizosphere rather than to proliferate in soil (Manzanilla-Lopez *et al*., 2013). The fungus is rhizosphere competent and proliferates in this niche using nutrients released in root exudates. Although mostly confined to the rhizosphere, an endophytic behaviour has been reported (Maciá-Vicente *et al*., 2009). When the colonising *P. chlamydosporia* contacts nematode egg masses in the rhizosphere, the fungus ceases its saprophytic stage and switches to parasitism. Appressoria formed in response to contact with the egg surfaces enable the attachment to, and penetration of, the eggs, which involves mechanical and enzymatic actions. Secreted enzymes destroy the outer vitelline egg membranes exposing the chitin layers, which are then penetrated by infection pegs. These grow into hyphae destroying egg contents and degrading the egg shells from the inside (Mauchline *et al*., 2004, Monteiro *et al*., 2017). Since RKN and PCN egg shells are constitutionally different, nematode host preference may be involved in the switch between fungal saprophytic and parasitic phases, but nutrient availability, either released by plants or available in nematode eggs, may play an important role (Mauchline *et al*., 2004; Esteves *et al*., 2009). Readily metabolised carbon sources and pH could influence the trophic phase switch as they interfere with the production of an alkaline serine protease (VCP1), known to be involved in the early stages of nematode egg infection (Segers *et al*., 1994; Ward *et al*., 2012).

Isolates of *P. chlamydosporia* differ markedly in their rhizosphere competence and ability to parasitise nematode eggs. The outcome of these variations in the regulation of nematode populations is difficult to predict, increasing the need for discriminative screening and selection of isolates. Standard screening encompasses *in vitro* assays for three criteria evaluating the ability of isolates to produce chlamydospores, colonise the rhizosphere, and parasitise nematode eggs (Abrantes *et al*., 2002).

Chlamydospores are resistant structures that can be produced using different solid substrates. These spores can withstand harsh conditions in soil for long periods without additional nutrients, and initiate fungal growth when in favourable environmental conditions. Chlamydospores are usually regarded as the preferred source of inoculum for biocontrol (Kerry, 2001). Isolates that successfully parasitise nematode eggs are not always competent in establishing in soil, as idiosyncratic results have been obtained for soil establishment (Vieira dos Santos *et al*., 2014). Inability to colonise the rhizosphere may hinder contact with nematode galls and egg masses, and thus affect biocontrol potential of particular isolates (Manzanilla-López *et al*., 2013).

Variation in *P. chlamydosporia* isolates has been related to host nematodes and the soil environment from which particular isolates originated (Zavala-González *et al*., 2015). Field biocontrol efficacy of isolates depends on several factors, including the nematode host, soil biotic and abiotic factors, and geographical origin (Nagesh *et al*., 2007). Molecular studies, required to identify isolates and provide fingerprinting, have been used to characterise isolates and detect genetic variation (Medina-Canales and Rodríguez-Tovar, 2017).

Enterobacterial Repetitive Intergenus Consensus sequence (ERIC)-PCR as a technique for PCR-based DNA fingerprinting provides rapid evaluation of isolates genetic variation. ERIC primers act randomly and amplify repetitive sequences in fungus genomes, and produce PCR products with multiple band profiles, and this allows isolate discrimination (Morton *et al*., 2003). However, ERIC-PCR profiling has only been informative for geographical origin and host preference of isolates.

The Biolog FF MicroPlate system (Biolog Inc.) has been used to characterise filamentous fungi according to their metabolic profiles. These profiles are obtained from fungus utilisation of 95 carbon sources from different chemical groups (amines/amides, amino acids, carbohydrates, carboxylic acids, polymers and miscellaneous compounds). This method has been used to identify and aid the definition of new species of fungi with small morphological variation, when molecular techniques are of limited application (Rice and Currah, 2005). In parallel, Biolog FF MicroPlates have been used to assess fungal functional diversity in environmental samples (Lucas *et al*., 2013), and to support fungal isolate characterisation, particularly if they are closely related (Pawlik *et al*., 2015; Singh, 2009). The method can also complement bioassays

in the selection and characterisation of isolates for biological control (Lopes *et al*., 2012). To our knowledge, *P. chlamydosporia* has not been characterised using this system.

The main goal of the present study was to provide an integrative methodology that can assist the selection of *P. chlamydosporia* isolates for the biocontrol of PPN. Here, we focus on *G. pallida* and *M. incognita* as, respectively, representative species of PCN and RKN. We anticipate that knowledge gained by the integration of molecular techniques, metabolic profiling and standard *in vitro* bioassays will support isolate selection, and provide new insights into the isolate functional characters that predict their success as biological control agents.

MATERIALS AND METHODS

Pochonia chlamydosporia isolates

Root knot nematode females from infected tomato (*Solanum lycopersicum* L.) roots, obtained in a greenhouse in Alcochete, Setúbal, Portugal, were identified by esterase phenotyping according to Esbenshade and Triantaphyllou (1985) and Pais *et al.* (1986). *Pochonia chlamydosporia* isolation was carried out by plating nematode eggs and host roots on semi-selective medium (De Leij and Kerry, 1991). After 14 d incubation at 25°C, colonies morphologically similar to *P. chlamydosporia* were transferred onto 1.7% corn meal agar (CMA) (Oxoid) for observation of characteristic diagnostic features (Abrantes *et al*., 2002).

The resulting isolates, from either RKN eggs (PE1, PE2, PE3, PE4, PE5) or tomato roots (PR1, PR2, PR3), were identified by PCR using specific primers. Isolate Pc2, from *G. rostochiensis* eggs (Vieira dos Santos *et al*., 2013), and a non-native isolate, Vc10 (IMI 331547) originally from *M. incognita* eggs from Brazil supplied by Rothamsted Research, United Kingdom, were also included (Table 1). Isolate Vc10 was used as a standard for *P. chlamydosporia* var. *chlamydosporia*. DNA was extracted from mycelium of *P. chlamydosporia* isolates grown for 10 d on 1.7% potato dextrose agar (PDA) (Difco™) using the E.Z.N.A. Fungal DNA miniKit (OMEGA bio-tek) according to the manufacturer's instructions. The identities of all the isolates were confirmed by PCR. DNA was amplified with the ***β***-tubulin primers specific for *P. chlamydosporia* var. *chlamydosporia* (Hirsch *et al*., 2000). PCR products were separated in 1% agarose gels stained with GreenSafe Direct Load (Nzytech genes & enzymes), and visualised in a transilluminator (Vilbert Lourmat). The relative mobilities of the PCR bands were calculated with the Bio-print Mega software version 12.15 for Windows (Vilbert Loumart).

Table 1. *Pochonia chlamydosporia* var. *chlamydosporia* isolates, geographic origin, substrate and associated nematode species.

Pc isolate	Geographic origin	Substrate	Nematode species associated
PE1	Setúbal, Portugal	Eggs	*Meloidogyne hispanica*
PE2		Eggs	*M. javanica*
PE3		Eggs	*M. javanica*
PE4		Eggs	*M. incognita*
PE5		Eggs	*M. incognita*
PR1		Roots	*M. hispanica*
PR2		Roots	*M. javanica*
PR3		Roots	*M. javanica*
Pc2	Guarda, Portugal	Eggs	*Globodera rostochiensis*
Vc10 (IMI 331547)	Brasil	Eggs	*M. incognita*

Biological characterisation

The abilities to produce chlamydospores, to colonise the rhizosphere of tomato plants, and to parasitise PPN eggs were tested, based on the methods reviewed by Abrantes *et al.* (2002), with modifications.

Chlamydospore production

Production and viability of chlamydospores produced by the eight isolates and of isolates Pc2 and Vc10 were assessed using the method described by De Leij and Kerry (1991). Five conical flasks per isolate, each containing 100 g of sterilised barley:sand substrate (1:1), were inoculated, each with three agar plugs from 10 d old colonies on CMA. After 30 d incubation at 25°C, the number of chlamydospores per three 1 g sub-samples of the colonised medium per flask was estimated using a haemocytometer. Chlamydospore viability was assessed in sorbose agar with antibiotics (12 g L^{-1} technical agar, 2 g L^{-1} of sorbose and 50 mg L^{-1} each of streptomycin sulphate, chlortetracycline and chloramphenicol). After 2 d at 25°C, the percentage of germinated chlamydospores was estimated using a stereomicroscope (Abrantes *et al*., 2002). Three biological replicates were carried out.

Rhizosphere colonisation

To study the ability of isolates to colonise the rhizosphere, roots of tomato cv. Tiny Tim roots were used,

since tomato is considered to be a good host for *P. chlamydosporia* (Manzanilla-López *et al*., 2013). Tomato seeds were surface sterilised with a sterilisation solution (1.6% sodium hypochlorite with 0.02% Tween) for 10 min, and rinsed three times with sterile distilled water. The seeds were then germinated in Petri dishes containing Gamborg's B5 medium supplemented with 15 g L^{-1} sucrose 1.5% and 8 g L^{-1} agar, adjusted to pH 6.4. The plates were incubated in the dark, at 25°C. Fungus inoculum was obtained by washing 10-d-old colonies grown on 1.7% CMA with 10 mL Gamborg's B5 medium. The number of spores (chlamydospores and conidia) was counted using a haemocytometer. Five 25 mL capacity flasks per isolate, containing 15 ml of autoclaved sand amended with 5 mL of half-strength Gamborg's B5 medium, were each inoculated with 10^5 spores, and planted with a 3 d-old single germinated tomato seed. The flasks were covered with transparent adhesive film and incubated for 21 d at 25°C and 12 h photoperiod. The plants were then up-rooted, shaken free of sand and placed onto 0.8% water agar for 3 d at 25°C. The percentage of roots colonised by each isolate was estimated using a stereomicroscope. Experimental controls consisted of flasks without fungal inoculum. These assays were conducted twice.

Nematode egg parasitism

The ability of each isolate to parasitise nematode eggs was assessed using standard *in vitro* bioassays (Abrantes *et al*., 2002). Ten-d-old colonies of each isolate, growing on 1.7% CMA, were washed with 10 mL of sterile distilled water. The numbers of chlamydospores and conidia per mL were quantified using a haemocytometer. Plates containing 0.8% technical agar and antibiotics (streptomycin sulphate, chloramphenicol and chlortetracycline, 50 mg L^{-1} of each) were each inoculated with a 0.2 mL of a 10^5 spores mL^{-1} suspension of each isolate, and were then incubated for 2 d at 25°C. Five plates per isolate were prepared.

A *G. pallida* population, originally from infected potato fields in Cantanhede, Coimbra, was selected from the collection at the NEMATO-lab, Centre for Functional Ecology (CFE), Department of Life Sciences, University of Coimbra (UC), and was propagated on roots of potato cv. 'Desirée' grown in 900 cm^3 plastic pots with a sterilised mixture of sandy loam soil and sand (1:2). Pots were inoculated with a small polyester bag containing cysts, to give approx. 5 eggs g^{-1} of soil. The number of eggs per cyst was assessed by counting five replicates of a suspension of eggs obtained by crushing 50 cysts in water. The pots were placed in a glasshouse at 20°C, with a 16 h photoperiod, and were watered regularly. After three months, new cysts were extracted from the soil using a modified Fenwick can (Shepherd, 1986). Eggs within cysts were released using forceps, and then suspended in water.

A RKN population of *M. incognita*, originally from potato roots and maintained in pot cultures of tomato cv. 'Coração de Boi' at the NEMATO-lab, CFE, UC, was selected. The population was propagated on susceptible tomato cv. 'Tiny Tim', grown in autoclaved sandy loam soil and sand mixture (1:1) in a greenhouse. The culture was transferred periodically to new tomato seedlings inoculated with ten egg masses per plant, placed beneath the tomato roots. Handpicked egg masses were mechanically disrupted, and the released eggs were suspended in water by agitation.

Egg suspensions were sieved through 75 µm and 20 µm sieves to separate eggs from debris, and were concentrated to obtain 2 eggs per µL. Two hundred and fifty eggs of each nematode species were transferred to each Petri plate colonised by the fungus, and were gently spread using a sterilised glass rod. Plates were incubated at 25°C for a further 3 d, after which the number of parasitised eggs was counted using standard methods (De Leij and Kerry, 1991). Experimental controls consisted of plates inoculated with nematode eggs, without the fungus. The bioassays were repeated three times.

Molecular characterisation

All the *P. chlamydosporia* isolates were characterised by ERIC-PCR, as described by Arora *et al.* (1996), and all reactions were performed three times to assess PCR pattern reproducibility. DNA was extracted from mycelium as described above. PCR products were separated in 2% agarose gels stained with GreenSafe Direct Load and visualised in a transilluminator. ERIC-PCR fingerprints produced distinct bands that were visually scored for presence (1) or absence (0) to produce a binary matrix from gel photographs recorded with the Bio-print Mega software version 12.15 for Windows.

Metabolic characterisation

The global phenotypes and utilisation by each of the isolates of 95 low molecular weight carbon sources (plus a negative control) were evaluated using the Biolog FF MicroPlate (Biolog Inc.), following manufacturer instructions. Pure cultures of each isolate were grown on CMA at 25˚C in the dark for 3 weeks, until spore formation was visible. The inoculum for the 96 well FF Micro-

Plates was prepared by first soaking a sterile swab in inoculating fluid and then gently rolling over the surface of the agar plates. The obtained spores were suspended in FF inoculating fluid supplied by Biolog in glass tubes (Cat. Nº 1006), mixed gently by hand and adjusted to approx. 75 % transmittance at 590 nm using a Biolog Turbidimeter, previously calibrated using a FF Biolog Turbidity standard (Cat. Nº 3426). Spore suspension (100 µL) was added to each well, and the FF MicroPlates were then incubated at 25˚C in the dark. The optical density at 490 nm (mitochondrial activity) was determined using an ASYS UVM 340 microplate reader (Hitech GmbH) for each plate at 24 h intervals over the next 7 d. Carbon sources were considered not utilised in wells in which colour development was less than, or equal to, that of negative controls.

Statistical analyses

Data of rhizosphere colonisation, numbers and viability of chlamydospores and nematode parasitism variables were analysed using IBM SPSS Statistics v.22. Descriptive statistics were used to explore data and identify extreme outliers that were, in most cases, due to technical error, and five data points were subsequently removed from analyses. Generalised linear mixed models (GLMM) were run for all variables using a normal distribution and an identity link function to establish statistical differences between treatments ($P < 0.05$). 'Isolate' was a fixed factor, and biological repeats and model hierarchy were both included as random factors. Satterthwaite approximations were used due to the small number of data points. Pairwise contrasts between estimated means per isolate adjusted for multiple comparisons were obtained by Least Significant Differences. A similar model structure was used to compare the parasitism of PCN eggs with the parasitism of RKN eggs by each isolate, combining results and adding parasitised PPN egg genus as a fixed factor. Estimated means per isolate for each variable resulting from the GLMM were explored with paired-data Pearson correlation analysis using PAST v.3.18. The highly significant correlation between rhizosphere colonisation and parasitism of *M. incognita* eggs was further analysed using Reduced Major Axis (RMA) analysis.

Multivariate analyses were performed in PAST v.3.18 (Hammer *et al.*, 2001) to reduce the number of variables resulting from the molecular (ERIC band matrix) and metabolic (Biolog- carbon source utilisation) profiles, using Principal Component Analysis (PCA). For the metabolic profiles, the average well colour development (AWCD) of the different isolates were calculated over the 7 d incubation, where AWCD equals the sum of the difference between the OD of the blank well (control) and substrate wells, divided by 95 (the number of substrate wells in the FF MicroPlates). The AWCD values were stabilised only at 7 d, therefore OD readings obtained in each of the 95 carbon sources at this incubation time were then reduced to a smaller, easily interpretable, number of explanatory variables with PCA.

The relationships between synthetic gradients on molecular and metabolic profiles obtained through PCA and the GLMM-estimated means of biological data were assessed through correlation analysis in PAST v.3.18. Pearson correlations were used for the comparison between the PC axes containing over 5% of the variance, and the number and viability of chlamydospores produced, rhizosphere colonisation and parasitism of PCN and RKN eggs. Spearman correlations were used for the comparisons of the same axes with nominal data on isolate geographical origin, substrate (eggs or roots) and nematode species associated.

RESULTS

Pochonia chlamydosporia *isolates*

Eight *P. chlamydosporia* isolates (PE1, PE2, PE3, PE4, PE5, PR1, PR2 and PR3) were obtained, associated with three RKN species: *M. hispanica*, *M. incognita* and *M. javanica* (Table 1).

All isolates presented a specific band (ca. 270 bp) for *P. chlamydosporia* var. *chlamydosporia* (data not shown).

Biological characterisation

Chlamydospore production

The ability to produce chlamydospores *in vitro* was variable among the isolates (Figure 1a). Isolates PR1 and Pc2 produced significantly greater numbers of spores (respectively, 171×10^5 g^{-1} and 187×10^5 g^{-1}) when compared with isolates PE1, PE3, PE5, PR2, PR3 and Vc10 (Figure 1a). Chlamydospore viability was less than 85% for isolates PE3, PR1 and PR3 (Figure 1b).

Rhizosphere colonisation

Root colonisation varied from 60% (PR3) to 92% (Pc2). Root colonisation by isolates PE1, Pc2 and Vc10 was significantly greater compared with root colonisation by isolate PR3 (Figure 2).

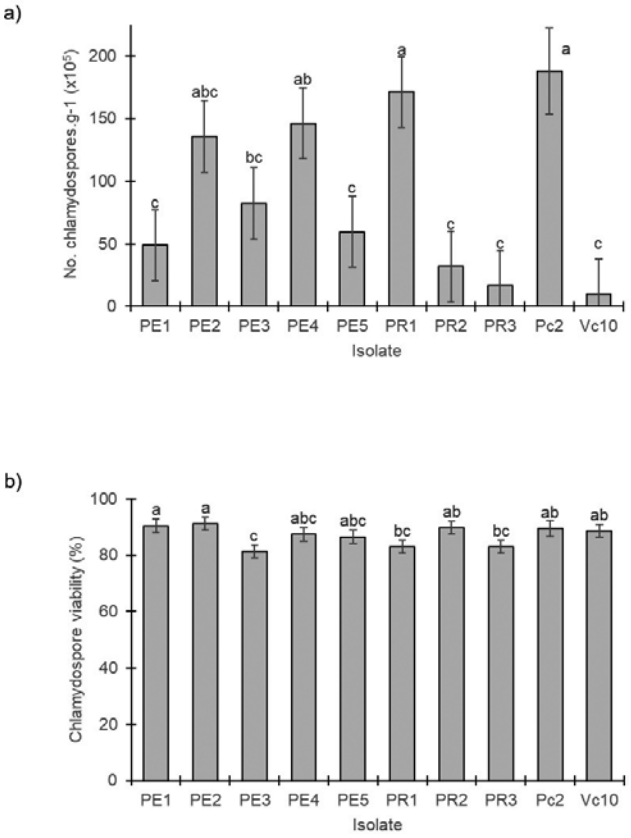

Figure 1. Number of chlamydospores (a) and chlamydospore viability (b) of ten *Pochonia chlamydosporia* isolates (PE1, PE2, PE3, PE4, PE5, PR1, PR2, PR3, Pc2 and Vc10) grown for 30 d on barley:sand substrate (1:1) (refer to Table 1 for isolate information). Bars represent standard errors of means. Columns accompanied by the same letter are not significantly, different (LSD test; $P < 0.05$).

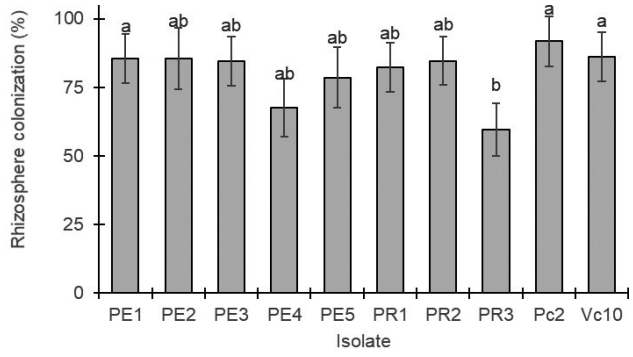

Figure 2. Mean percentages of colonised tomato roots for ten *Pochonia chlamydosporia* isolates (PE1, PE2, PE3, PE4, PE5, PR1, PR2, PR3, Pc2 and Vc10) grown for 21 d on sterilised sand (refer to Table 1 for isolate information). Bars represent standard errors of means. Columns accompanied by same letter are not significantly different (LSD test; $P < 0.05$).

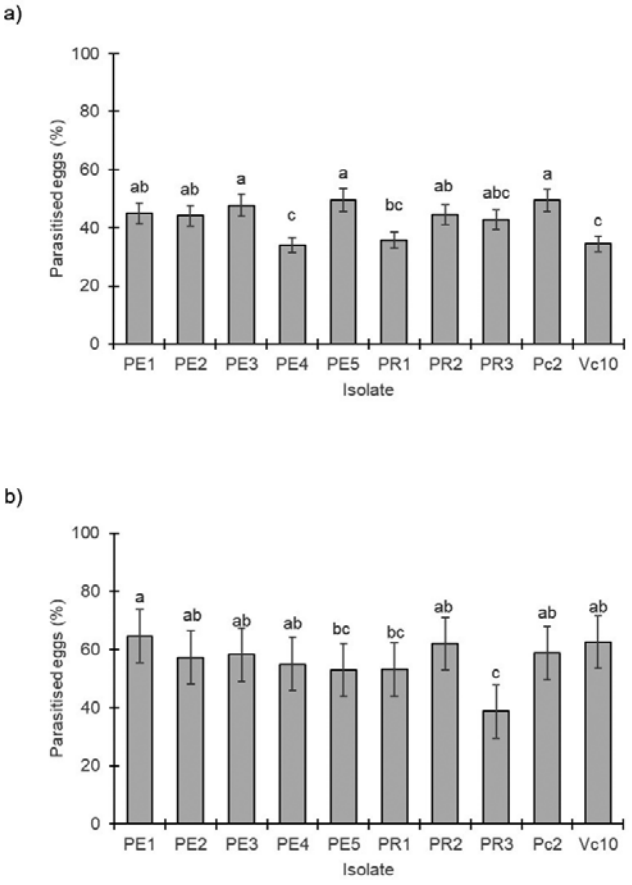

Figure 3. Mean percentages of parasitised eggs of *Globodera pallida* (a) and *Meloidogyne incognita* (b) by ten *Pochonia chlamydosporia* isolates (PE1, PE2, PE3, PE4, PE5, PR1, PR2, PR3, Pc2 and Vc10) after incubation at 25°C for 3 d (refer to Table 1 for isolate information). Bars represent standard errors of means. Columns accompanied by the same letter are not significantly different (LSD test; $P < 0.05$).

Nematode egg parasitism

After 3 d at 25°C, the percentages of parasitised eggs of PCN and RKN were low for all isolates (Figure 3). Pathogenicity of the isolates varied between 34 and 49 % against *G. pallida* eggs (Figure 3a) and 38 to 65 % against *M. incognita* eggs (Figure 3b). Parasitism of PCN eggs was less than parasitism of RKN eggs ($P < 0.01$), with the exception of isolate PR3, which had a greater parasitism rate of PCN eggs than of RKN eggs. Statistically significant differences in egg parasitism between PPN genera were not found for isolates PE1 and PE4. RMA analysis revealed a highly significant positive linear relationship ($r = 0.818$; $P < 0.01$) between parasitism of RKN eggs and rhizosphere colonisation by the fungus.

Molecular characterisation and integration with biological characterisation

Isolates associated with *Meloidogyne* spp. had very similar ERIC patterns. A different band pattern was observed for isolate Pc2 associated with *G. rostochiensis* eggs (Supplementary Material). The PCA analysis separated the fungal isolates into four quadrants, with isolates Pc2 and Vc10 clearly separated from other isolates along PCERIC2 and from each other along PCERIC1 (Figure 4). Isolates PE1, PE2 and PR3 were also separated by synthetic gradient PCERIC1 from isolates PE3, PE4, PE5, PR1 and PR2. Although significant associations were not found between biological characteristics and PC axes PCERIC1 and PCERIC2 (loadings presented in Table 2), the geographical origins of the isolates were correlated with PCERIC1 ($r = 0.707$, $P < 0.05$).

Metabolic characterisation and integration with biological characterisation

Altogether, the ten isolates used the 95 substrates, but only 72 substrates were used by all the isolates: these included four out of six amines/amides; nine of 13 amino acids, 38 of 44 carbohydrates, 12 of 17 carboxylic acids, three of five polymers and six of ten miscellaneous compounds. Six of the substrates, namely n-acetyl-d-mannosamine, sedoheptulosan, d-lactic acid methyl ester, α-cyclodextrin and β-cyclodextrin, were used by fewer than half of the isolates. Isolate PE5 was the only isolate able to utilise all 95 carbon sources, followed by Vc10 (92 carbon sources), isolate PR2 (91) and isolate Pc2 (90). Isolate PR3 utilised the least, with only 77 wells developing colour, as corrected to the water control (Figure 5). The different profiles of carbon source utilisation positioned

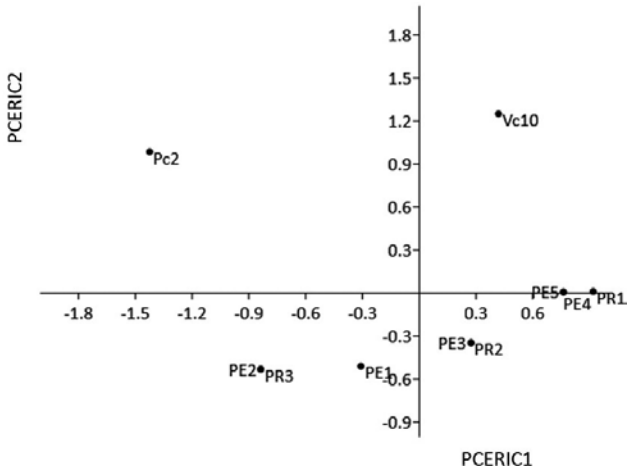

Figure 4. Principal Component Analysis of Enterobacterial Repetitive Intergenus Consensus sequence (ERIC) PCR profiles obtained from *Pochonia chlamydosporia* isolates (PE1, PE2, PE3, PE4, PE5, PR1, PR2, PR3, Pc2 and Vc10; refer to Table 1 for isolate information). PCERIC1 eigen value 0.651; PCERIC2 eigen value 0.400.

Table 2. Synthetic gradients generated by Principal Component Analysis of molecular and metabolic profiling of *Pochonia chlamydosporia* isolates.

Axis	Variance (%)	Positive loading	Negative loading
PCERIC1	49.22	ERIC bands 2, 4 and 6	---
PCERIC2	30.25	ERIC band 8	ERIC band 7
PCBLG1	45.484	Utilisation of most carbon sources; prominent: 2-aminoethanol (amines/amides) glycyl-L-glutamic acid L-glutamic acid L-ornithine L-serine L-threonine (amino acids) β-hydroxy-butyric acid succinic acid (carboxylic acids) adenosine-5'-monophosphate amygdalin succinic acid mono-methyl ester uridine (miscellaneous)	D-ribose (carbohydrates)
PCBLG2	28.58	α-methyl-D-glucoside β-methyl-D-galactoside n-acetyl-D-galactosamine n-acetyl-D-mannosamine (carbohydrates) D-galacturonic acid D-glucuronic acid L-lactic acid (carboxylic acids) D-lactic acid methyl ester (miscellaneous)	2-aminoethanol (amines/amides) L-aspartic acid L-glutamic acid (aminoacids) α-D-glucose L-rhamnose maltose maltotriose (carbohydrates) L-malic acid (carboxylic acids) glycerol succinic acid mono-methyl ester (miscellaneous)

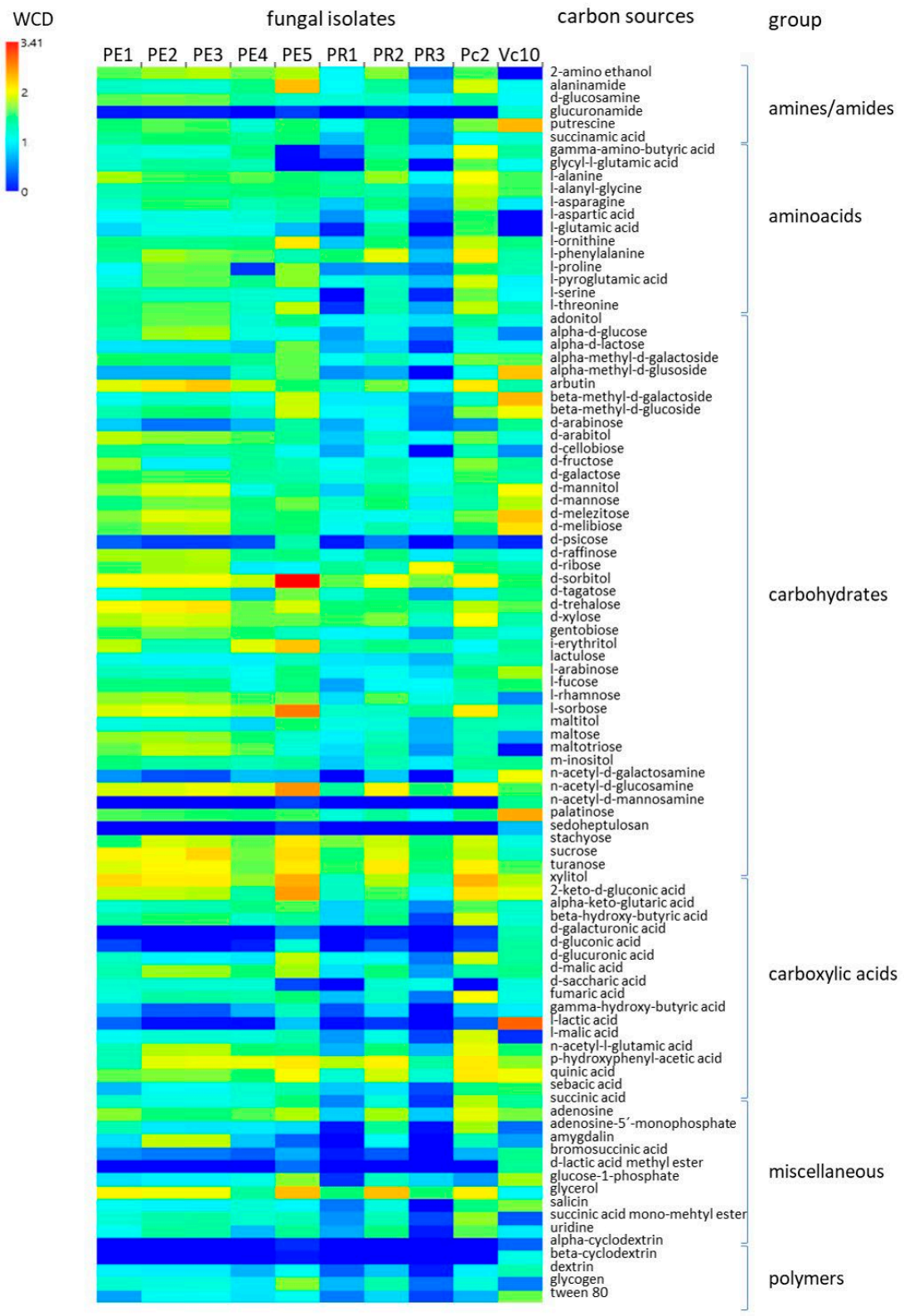

Figure 5. Heat map of carbon source utilisation by *Pochonia chlamydosporia* isolates (PE1, PE2, PE3, PE4, PE5, PR1, PR2, PR3, Pc2 and Vc10; refer to Table 1 for isolate information), assessed through Well Colour Development (WCD) at 7 d, corrected to control, of each of 95 carbon sources/chemical group, using Biolog FF MicroPlates.

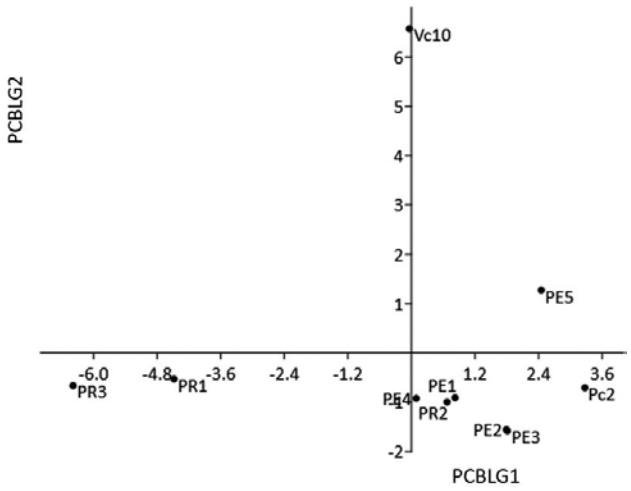

Figure 6. Principal Component Analysis of carbon utilisation profiles obtained through Biolog FF MicroPlates for *Pochonia chlamydosporia* isolates (PE1, PE2, PE3, PE4, PE5, PR1, PR2, PR3, Pc2 and Vc10, refer to Table 1 for isolate information). PCBLG1 eigen value 9.470; PCBLG2 eigen value 5.950.

isolates Vc10 and PE5 in different quadrants to all other isolates, along the two PCA axes of the metabolic profile (PCBLG1 and PCBLG2; Table 2 and Figure 6). This was particularly evident for isolate Vc10, depending mainly on separation obtained by synthetic gradient PCBLG2. Isolates PR1 and PR3 also each had a distinct metabolic profile from all other isolates, being separated by PCBLG1. Significant correlations were detected between the synthetic gradient PCBLG1 and the isolate substrate of origin (eggs or roots; $r = -0.646$, $P < 0.05$), and also with parasitism of RKN eggs ($r = 0.700$, $P < 0.05$) and rhizosphere colonisation ($r = 0.666$, $P < 0.05$).

DISCUSSION

The potential of eight *P. chlamydosporia* isolates associated with *Meloidogyne* spp. as biological control agents was evaluated using integrative characterisation, based on biological, molecular, and metabolic analyses.

Although few statistically significant differences were detected between the evaluated isolates, molecular identification and characterisation allowed evaluation of their variability, affected by different geographic substrate origins (Table 1). This information could be helpful for the potential registration of a specific isolate as a biological control agent (Abrantes *et al.*, 2002). Detection and quantification of particular fungi in soil can be achieved with the use of specific primers, which distinguish the two varieties of *P. chlamydosporia*, var. *chlamydosporia* and var. *catenulata* (Hirsch *et al.*, 2000; Mauchline *et al.*, 2002). The β-tubulin gene allowed design of specific primers for *P. chlamydosporia* var. *chlamydosporia*, and since it has an intron that is not present in other fungi, specific primers do not amplify DNA from other species of *Pochonia* or from other fungal genera (Mauchline *et al.*, 2002; Kerry and Hirsch, 2011). This confirmed the identity of all Portuguese isolates used in this study as *P. chlamydosporia* var. *chlamydosporia*.

Rapid *in vitro* bioassays are necessary for precise selection before introduction of a biological control agent in the soil. Although few statistical differences were found, variation between eight isolates was detected using three standard *in vitro* bioassays, including chlamydospore production, rhizosphere colonisation, and nematode egg parasitism. The inherent variation of *P. chlamydosporia* isolates has been previously described, and this variation stresses the need for precise isolate selection (Abrantes *et al.*, 2002).

Ability to produce chlamydospores *in vitro* was variable among the eight isolates. Standard bioassays are usually performed using agar plugs colonised with fungus (Abrantes *et al.*, 2002). However, the amount of chlamydospores, conidia and hyphal fragments in each plug may vary with the isolates, depending on individual growth rates and sporulation. Therefore, in order to assess differences among *P. chlamydosporia* isolates, equal amounts of spores should be used as inoculum (Vieira dos Santos *et al.*, 2013). The results obtained for reference isolates Pc2 and Vc10 were less than those reported by Vieira dos Santos *et al.* (2013). Additionally, fewer chlamydospores were reported for isolate Vc10 by Medina-Canales *et al.* (2014). Differences in chlamydospore production between isolates may be due to the type of substrate used, as chlamydospore production is greater in substrates with low available carbon and nitrogen, and no sulphur (Mo *et al.*, 2005). Although ability to produce chlamydospores is assessed in the standard characterisation, to ensure success in large-scale inoculum production, this may be variably affected by culture conditions, including carbon and nitrogen contents of the culture substrate (Liu and Chen, 2003; Mo *et al.*, 2005; Vieira dos Santos *et al.* 2012). Moreover, the success of a commercially developed product based on an oil emulsion containing chlamydospores, mycelium and conidia was recently reported (Sellitto *et al.*, 2016). Regarding viability, some isolates have low germination proportions (< 85%), which can be due to the incubation time (3 weeks). Some isolates may not withstand this incubation period, and although they continue to produce chlamydospores, the viability tends to decrease with time (Abrantes *et al.*, 2002). In the

present study, all isolates produced adequate numbers of chlamydospores for potential commercial scale production ($\geq 1 \times 10^6$ g^{-1} of substrate) (Medina-Canales *et al.*, 2014). Therefore, we anticipate that, in isolates that produce numbers of chlamydospores greater than accepted threshold of 10^6 g^{-1} of substrate, the ability to produce viable chlamydospores, rather than the exact number of chlamydospores produced, may be an important characteristic for the selection of these biological control agents.

Differences among isolates were detected in their abilities to colonise tomato rhizospheres. The best coloniser of tomato roots was the standard isolate Pc2 with 92% of colonisation. Isolates PE4, PE5 and PR3 colonised less than 80% of tomato roots, failing to achieve the 80% colonisation threshold for selection for biocontrol (Abrantes *et al.*, 2002). Rhizosphere colonisation is a key feature for these biocontrol agents, as it is critical for the control of nematodes by *P. chlamydosporia* (Bourne *et al.*, 1996). The biocontrol efficacy of the fungus depends on fungal rhizosphere colonisation to facilitate colonisation of galls (Manzanilla-López *et al.*, 2013). This assumption has now gained experimental support by our finding of a strong relationship between rhizosphere colonisation and parasitism of RKN eggs. A link between tomato rhizosphere colonisation and parasitism of PCN eggs was not detected. The proportions of colonisation of tomato roots by reference isolates Pc2 and Vc10 were greater than previous results for colonisation of barley roots (Vieira dos Santos *et al.*, 2013). It has been established that plant species differ in their ability to support the fungus (Bourne *et al.*, 1996; Kerry and Bourne, 1996). Whether this can be further related to fungal parasitism of eggs of nematodes that are able to parasitise those plant species is unknown. We speculate that the fungal isolates might emulate nematode host plant preferences, i.e. a virulent parasite of a given nematode population could be a good root coloniser of the host plant preferred by the nematode. This, to our knowledge, has not been investigated.

The low proportion of egg infection obtained through the standard bioassay has also been observed by other authors. This may be related to changes in the mucilage surrounding the eggs, which are considered as important sources of nutrition for the fungi and ensure high rates of egg colonisation. Additionally, isolates parasitise immature eggs more actively than mature eggs containing second-stage juveniles (Irving and Kerry, 1986). Spontaneous hatching detected in both nematode species during the bioassays could also contribute to the low parasitism levels observed. Our results do not support previous findings suggesting that *P. chlamydosporia* isolates have host preferences for host species from which the isolates were initially isolated (Mauchline *et al.*, 2004). Although isolate PR3 was originally associated with RKN, it was more virulent to PCN than RKN; conversely, Pc2, isolated from PCN, was more virulent to RKN eggs than to those of PCN. Seven of the ten isolates tested were more virulent to RKN eggs than to PCN eggs.

Principal Component Analysis of ERIC profiles obtained for *P. chlamydosporia* isolates separated them according to their geographic origin (Figure 4). Isolate Pc2 was the only one from PCN eggs, and was here compared with nine isolates associated with *Meloidogyne* spp. This may have hindered the detection of relationships among ERIC-PCR profiles and nematode origins. Genetic variability among isolates revealed that isolates can be grouped according to their geographical and the host nematode species. The importance of this genetic variation in the regulation of a host population is, however, unknown (Morton *et al.*, 2003).

Isolates PE5, Vc10, PR2 and Pc2 were able to utilise most of the carbon sources (≥ 90), whereas isolate PR3 utilised the least (77) (Figure 5). Isolate PR3 clearly had different carbon requirements as it produced the fewest chlamydospores in barley. Growth and sporulation are influenced by carbon and nitrogen sources, but are also determined by isolate preferences (Mo *et al.*, 2005; Liu and Chen, 2003). Isolate original substrate (eggs or roots), parasitism of *Meloidogyne* eggs, and rhizosphere colonisation ability were significantly correlated with metabolic profiles, indicating that specific carbon sources (amines/amides, amino acids, carboxylic acids and miscellaneous carbon sources) could be used to predict isolate ability for rhizosphere colonisation and for RKN egg parasitism. High proline contents are characteristic of RKN egg shells (Bird and McClure, 1976). For PCN, although proline was the most abundant, 64% of the total amino acids were aspartic acid, glycine and serine (Clarke *et al.*, 1967). Molecular studies have also found that genes encoding proteases known to be involved in nematode egg penetration processes are also highly expressed during endophytic colonisation of barley roots (Lopez-Moya *et al.*, 2017).

The inherent variation of *P. chlamydosporia* isolates has been widely described, and this variation increases the need to carefully select potential biocontrol isolates. Laboratory *in vitro* tests, that evaluate the three biological characteristics considered fundamental for a good fungus performance as biological control agents, enable rapid and economical selection of isolates, before testing their soil activity in pot tests ahead of field trials (Abrantes *et al.*, 2002).

Pochonia chlamydosporia isolate variability was assessed using Biolog FF MicroPlates for the first time.

It is not clear how the metabolic profiles of fungal isolates, or their ability to use particular carbon sources, ultimately affect biocontrol performance in the field. Nevertheless, use of this metabolic approach may facilitate future isolate selection, as the use of specific carbon sources provides complete information on isolate ability for rhizosphere colonisation, and for nematode egg parasitism. These studies may lead to new perspectives for understanding the intricate multitrophic life styles characteristic of *P. chlamydosporia*.

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Short Notes

Pleurostomophora richardsiae associated with olive tree and grapevine decline in Southern Brazil

Maria Cristina CANALE[1,*], Cristiano NUNES NESI[1], Bianca Ramos FALKENBACH[2], Cesar Arthur HUNHOFF DA SILVA[2], Eduardo Cesar BRUGNARA[1]

[1] *Agricultural Research and Rural Extension Company of Santa Catarina State (Epagri), 89803-904 Chapecó, Santa Catarina, Brazil*
[2] *Agronômica, Laboratory of Phytosanitary Diagnosis and Consultancy, 91530-000 Porto Alegre, Rio Grande do Sul, Brazil*
*Corresponding author: M.C. Canale. E-mail: cristinacanale@epagri.sc.gov.br

Abstract. Olive trees showing decline symptoms were reported in an orchard in Santa Catarina, Southern Brazil. The symptoms included foliar browning and leaf drop, die-back of twigs and branches, dark streaks in internal crown wood and necrotic lesions in bark. A grapevine sample exhibiting browning streaks in the vascular tissue of branches, trunk and roots, was also analysed. A *Phialophora*-like fungus was isolated from symptomatic tissues of both hosts, and monosporic cultures were established. Based on morphology and analysis of ITS, *tub2* and *tef* sequences, the fungus was identified as *Pleurostomophora richardsiae*. Isolates inoculated onto young nursery olive trees and grapevines produced brown streaking in the wood in most of the inoculated plants. This is the first report of *P. richardsiae* occurring in olive trees and grapevines in Brazil.

Keywords. Fungal pathogenicity, molecular systematics, *Olea europaea*, *Vitis* spp. wood decay fungus.

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INTRODUCTION

Olive cultivation is emerging in Brazil as an option for farmers. The production and cultivated area is restricted to the southeast and south of the country where the established olive groves are young and have been in production for only a few decades. In 2016, national production was 647 t from 573 ha of harvested area, having increased 138% since 2014 (FAO, 2018). Government agencies estimate that approx. 110,000 L of olive oil were produced in 2017. Viticulture is mainly carried out in the south and northeast of Brazil, and approx. 0.8 million t of grapes were produced in 2014 (FAO-OIV, 2016). The total area of vineyards in Brazil is 86,000 ha, and the south region is responsible for 75.3% of the production of table grapes and wines (IBGE, 2017). Olive orchard and vineyard production supply mostly to the Brazilian domestic markets.

Studies of pathogens associated with grapevine decline in Brazil are few, and reports of pathogens in olive trees are even fewer (Correia *et al.*, 2013; Chliyeh *et al.*, 2014). During a survey of an olive orchard in Santa Catarina (SC), a state in Southern Brazil, a decline disease of olive trees was noted. At the same time, a grapevine plant brought to our laboratory for diagnosis showed brown streaking in the vascular tissues of the branches. The purpose of the study reported here was to determine the cause of these diseases.

MATERIALS AND METHODS

Sampling and fungal isolation

Symptomatic olive trees, showing foliar browning and leaf drop, wilting of apical shoots, dieback of twigs and branches, dark streaks in the internal wood, and crown and necrotic bark lesions (Figure 1) were noted during a survey in an experimental olive orchard in Itá, a municipality in SC (-27.273913, -52.339676), in 2015. The orchard was approx. 2.5 ha and contained 3-year-old olive trees of cv. Arbosana. Symptomatic trees were in a 1,000 m² area where the soil was poorly drained. Samples were taken from two diseased plants. At the same time (in 2016), a grapevine plant, aged approx. 3 years, with dieback symptoms, was received by our laboratory for disease diagnosis. The branches showed brown discolouration in the vascular tissue and the bark was cracking (Figure 1). The grapevine was from Riqueza municipality, also in SC (-27.064126, -53.327396). Small pieces of the necrotic and discoloured tissues were removed, surface-disinfected for 3 min in 1.5% sodium hypochlorite solution, washed twice in sterile water and then incubated on 2% potato dextrose agar (PDA, Merck) amended with 100 mg L^{-1} streptomycin sulphate (Calbiochem). One fungal isolate was obtained from each sampled plant, *i.e.*, two isolates from the two sampled olive tree and one from the diseased grapevine. After sporulation, 20 µL of a spore suspension were plated on PDA, and after 2 d of incubation individual germinated spores were transferred to fresh PDA plates. The resulting cultures produced *Phialophora*-like fungi (Vijaykrishna *et al.*, 2004). One isolate from olive (pr_OLIV) and one from the grapevine (pr_GRAP) were used for further analyses.

Morphological characterization

Morphological characterization followed the procedures of Vijaykrishna *et al.* (2004) and Carlucci *et al.* (2015) with some modifications. Colony morphology was determined after 21 d of incubation on 2% malt extract agar (MEA) at 25 ± 2°C in the dark. Dimensions and morphology of conidia, conidiophores and phialides were determined from microscopic examination of fungal tissues in lactic acid preparations. For each structure, 30 measurements were made with a ×100 objective on a Zeiss Scope A1 Axio microscope equipped with AxioCam MRC, operated through Zen Lite Software 2012 (Zeiss). Radial growth of the isolates was determined by placing individual 3 mm diam. mycelial discs on MEA in Petri dishes. Two perpendicular measurements of colony diameters were made with a digital caliper after 8 d incubation in the dark at 4°C, 25°C or 37°C, with five replicates at each temperature.

DNA isolation and sequencing

Genomic DNA of the two isolates was extracted using the Wizard Genomic DNA Purification Kit (Promega) following the manufacturer's instructions. The 5.8S rDNA gene and flanking internal transcribed spacers 1 and 2 (ITS), partial β-tubulin (*tub2*) and translation elongation factor 1-α (*tef1*) genes were sequenced to confirm fungal identification, using, respectively, the primer pairs ITS4 and ITS5 (White *et al.*, 1990), Bt2a and Bt2b (Glass and Donaldson, 1995), and EF1 and EF2 (O'Donnell *et al.*, 1998). After amplification, PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen). Sequencing was performed by ATCGene (Alvorada, Rio Grande do Sul, Brazil) in forward direction. Sequences of pr_OLIV and pr_GRAP isolates were deposited in GenBank. Matches with sequences in GenBank was performed with BLAST searches.

Pathogenicity of P. richardsiae *isolates*

The two isolates obtained in this study were inoculated onto the same plant species from which they were each isolated. Isolate pr_OLIV was also inoculated onto grapevine cultivars and isolate pr_GRAP was inoculated onto an olive cultivar. One-year-old nursery plants of cv. Arbosana olive trees (*Olea europaea*) and two rootstock varieties of grapevines (*Vitis vinifera*), 'Paulsen' (*V. berlandieri* Planch. × *V. rupestris* Scheele) and 'VR04343' (*V. rotundifolia* Michx. × *V. vinifera* L.) approx. 50 cm tall, were growing in 6 L capacity plastic bags of potting mix. The plants were each wounded to about 3 mm depth with a histological needle at 7 cm above soil level. Colonized mycelial agar plugs (3 mm diam.) were placed on the wounds, and each covered with a wet

Figure 1. (a–c) Disease symptoms in olive trees. (a) Severe dieback of twigs and branches, with browning of leaves, and premature plant death. (b) Transversal cross section of an affected trunk showing dark brown to black streaking in the crown. (c) Dark streaking in the core of wood and necrotic lesions affecting the bark. (d–e) Grapevine branches showing cracks and vascular discoloration. Scale bar = 1 cm. (f–g) Longitudinal sections showing acropetal and basipetal wood discolouration from inoculation points in (e) olive tree and (f) grapevine. Scale bar = 1 cm. (h) *Pleurostomophora richardsiae* colony, on MEA after 21 d in the dark at 25°C. (i–j) Micrographs of *P. richardsiae* structures. Scale bar = 10 µm. Arrows in (i) show a phialide, a conidiophore and hyphal bundles. Arrows in (j) indicate subglobose and oblong ellipsoid conidia, and coiled hyphae.

piece of cotton and sealed with Parafilm. Mycelial plugs were taken from fungal colonies grown on PDA for 21 d at 23°C (± 2°C). Non-colonised sterile PDA plugs were used for non-inoculated controls. Five non-inoculated or inoculated plants were prepared for each isolate. Pots containing the plants were randomly arranged on a bench in a greenhouse covered with an anti-aphid net, with temperature around 25°C, and watered three times each week. The inoculation points were analysed 4 months after inoculation. A longitudinal cut was made in the wood tissue of each plant through the point of inoculation, and the length of the discoloured wood above and below the point of inoculation was measured with a digital caliper. Re-isolations were made from all of the inoculated pieces of wood to recover the isolates, in attempts to fulfil Koch's postulates.

RESULTS AND DISCUSSION

The symptoms of disease on olive trees were foliar browning and leaf drop, wilting and dieback of twigs and branches, dark streaking in the inner wood tissues and necrosis, leading to a generalized decline of the trees. The grapevine received for diagnosis showed advanced symptoms of dieback, with severe discolouration of the vascular tissues affecting the whole plant from the roots to the branches, where the bark was cracking (Figure 1). Isolates of a *Phialophora*-like fungus was isolated from the diseased plants. Detailed morphological characterization and molecular analyses identified the isolates as *Pleurostomophora richardsiae* (Nannf.) Réblová & Jaklitsch. Voucher cultures of these isolates were deposited in the culture collection of the Centre of Biological Sciences, Federal University of Pernambuco (UFPE), with access numbers 7857 (pr_OLIV) and 7858 (pr_GRAP).

Pleurostomophora richardsiae is frequently found when searching for other pathogenic fungi in necrotic lesions in the periderms of plants (Vijaykrishna *et al*., 2004; Mostert *et al*., 2006; Olmo *et al*., 2015; Réblová *et al*., 2015). This fungus is an emerging plant pathogen and is gaining recognition as an important vascular pathogen involved in Esca disease complex of grapevine, and, more recently, associated with decline of olive trees (Carlucci *et al*., 2013; Varela *et al*., 2016). Pathogenicity of *P. richardsiae* in grapevine was confirmed by Halleen *et al.* (2007) in South Africa, and this pathogen has been recorded in California (Rolshausen *et al*., 2010), Italy (Carlucci *et al*., 2015), Spain (Varela *et al*., 2016) and Turkey (Özben *et al*., 2017). Reports of *P. richardsiae* causing decline in olive trees are more recent. Carlucci *et al.* (2013) found *P. richardsiae* associated with olive tree decline in surveys conducted in Italy, and remarked on the aggressiveness of the fungus to this host, since it can frequently be the only pathogen isolated from affected trees.

After 21 d in the dark on MEA, colonies of pr_GRAP and pr_OLIV were cottony, light brown and paler towards the periphery, with entire margins (Figure 1), which agrees with the description provided by Carlucci *et al.* (2015). The mycelium is composed of branched septate hyphae, sometimes in bundles or forming coils (Figure 1). Neither of the isolates grew in cultures at 4°C. According to Carlucci *et al.* (2015) the minimum temperature for growth of *P. richardsiae* is 10°C. Mycelia of *P. richardsiae* developed at 25°C (mean colony diam. = 32.7 cm for pr_ OLIV and 30.2 cm for pr_GRAP), and at 37°C (mean colony diam. + 11.4 cm for pr_ OLIV and 8.5 cm for pr_GRAP). Morphology of the isolates agreed with the previous description of *P. richardsiae*, producing cylindrical and oblong ellipsoidal conidia, short, unbranched conidiophores and predominantly type II phialides. The phialides were elongated, ampulliform and constricted at the bases (Vijaykrishna *et al*., 2004; Mostert *et al*., 2006; Carlucci *et al*., 2015).

ITS sequences of pr_GRAP and pr_OLIV were lodged in GenBank (NCBI), under accession numbers, respectively, MG966406.1 and MG966416.1. BLAST searches showed that these isolates had 100% identity with several *P. richardsiae* ITS sequences already deposited in GenBank, including the isolate that was associated with grapevine decline in Spain, access number JX258852.1 (Varela *et al*., 2016). The ITS sequences also presented 99% identity with the ex-type isolate CBS 270.33 (GenBank accession number NR135933; Mostert *et al*., 2003). Sequences of partial *tub2* and *tef1* genes were also deposited in GenBank. The accession numbers for pr_GRAP *tub2* and *tef1* sequences are MH053437 and MH053438. For pr_OLIV *tub2* and *tef1* sequences, the accession numbers are MH053439 and MH053440.

In the pathogenicity tests, both isolates produced brown streaking in the wood in most of the inoculated plants (Figure 1). Despite of the spread of the brown streaking, the isolates did not kill the inoculated nursery plants. Both isolates were re-isolated from inoculated plants.

This is the first report of *P. richardsiae* associated with diseased olive trees and grapevine in Brazil. With this report, we hope to contribute to knowledge of fungal phytopathogens that can be found in these plant species in Brazil. Further epidemiological studies are required to investigate the incidence of *P. richardsiae* in the south and other regions of Brazil, where olive trees and grapevines are cultivated, and determine

the impacts of the pathogen on productivity of olive orchards and vineyards.

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Short Notes

Phytotoxins produced by *Lasiodiplodia laeliocattleyae* involved in Botryosphaeria dieback of grapevines in Brazil

Pierluigi REVEGLIA[1,2,*], Marco MASI[2], Alessio CIMMINO[2], Sami MICHEREFF[3], Tamara CINELLI[3], Laura MUGNAI[3], Antonio EVIDENTE[2]

[1] *National Wine and Grape Industry Centre, School of Agricultural and Wine Sciences, Charles Sturt University, Locked Bag 588 Wagga Wagga NSW 2678, Australia*
[2] *University of Naples Federico II, Department of Chemical Sciences Complesso Universitario Montesant'Angelo, Via Cintia 4, 80126, Naples, Italy*
[3] *Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente, Sez. Patologia vegetale ed entomologia, Università di Firenze, Piazzale delle Cascine 28, 50144 Firenze, Italy*
Corresponding author: pierluigi.reveglia@unina.it

Abstract. Botryosphaeria dieback (BD) is an important trunk disease affecting grapevines. Several *Lasiodiplodia* species have been shown to be involved in BD affecting the perennial organs of grapevine, mainly causing cankers. (*R*)-(-)-mellein and tyrosol, two well-known fungal phytotoxins, were isolated from the organic extract of culture filtrate of *Lasiodiplodia laeliocattleyae* (syn. *egyptiacae*), which had been isolated from grapevines affected by BD in Brazil. This increases knowledge of the secondary metabolites produced by *Lasiodiplodia* species, confirming that (*R*)-(-)-mellein is a toxin typically produced by Botryosphaericeae species.

Keywords. Grapevine wood disease, phytotoxins, (*R*)-(-)-mellein, tyrosol.

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INTRODUCTION

Botryosphaeria dieback (BD) is a grapevine trunk disease that causes serious problems for grape production, including emerging wine-producing countries such as Brazil. The economic impacts of grapevine trunk diseases result from significant yield reductions from diseased vines and increased production costs for application of control measures. Many efforts have been made to find new and effective management practices for the disease (Mondello *et al.*, 2018). BD also affects many fruit tree crops, including mango, olive, walnut and almond (de Oliveira Costa *et al.*, 2010; Olmo *et al.*, 2016; Rodríguez-Gálvez *et al.*, 2017).

BD of grapevine is caused by several Botryosphaeriaceae species, associated with decline symptoms including dieback, wood canker and spur dieback (Úrbez-Torres, 2011; Billones-Baijens and Savocchia 2018; Gramaje *et al.*, 2018; Mondello *et al.*, 2018).

Like other trunk disease pathogens, Botryosphaeriaceous species can produce toxic metabolites belonging to different compound classes including aromatic compounds, isocoumarins, jasmonates, naphthalenones, polyketides, and phenols (Martos *et al*., 2008; Masi *et al*., 2018). In particular, *Lasiodiplodia* species were investigated for production of phytotoxic metabolites and other substances (including jasmonic acid, mellein, lasiodiplodin, theobroxide, butyrolactones, botryosphaeran, botryorodines, lasiodiplodan) are produced *in vitro* by different isolates of *L. theobromae* and other *Lasiodiplodia* spp. such as *L. mediterranea*. These toxic metabolites have also been purified from *in vitro* cultures of strains isolated from host pants other than grapevine, and tested for their toxic activity (Aldridge *et al.* 1971, Husan *et al.* 1993, Matsuura *et al*., 1998, He *et al*., 2004, Miranda *et al*., 2008, Kitoaka *et al*., 2009, Andolfi *et al*., 2014).

In a recent study, phytotoxic metabolites produced in liquid cultures by six species of *Lasiodiplodia* isolated in Brazil, and causing Botryosphaeria dieback of grapevine, were chemically identified. As determined by LC-MS, *L. brasiliense, L. crassispora., L. iraniensis, L. pseudotheobromae* produced jasmonic acid, while *L. brasiliense* synthesized jasmonic acid and (3*R*,4*S*)-4-hydroxymellein. *Lasiodiplodia euphorbiaceicola* and *L. hormozganensis* produced some low molecular weight lipophilic toxins, that were isolated and identified (Cimmino *et al*., 2017). In particular, from culture filtrate of *L. euphorbiaceicola*, (*R*)-(-)-mellein, (3*R*,4*R*)-(-)- and (3*R*,4*S*)-(-)-4-hydroxymellein, and tyrosol were isolated, and identified. Tyrosol and *p*-hydroxybenzoic acid were also isolated from culture filtrates of *L. hormozganensis* (Cimmino *et al*., 2017).

Knowledge of the non-specific phytotoxins produced by Botryosphaeriaceous species is increasing (Masi *et al*., 2018), and it is increasingly important to determine the full spectrum of these metabolites to understand their roles in disease and symptom development (Meh *et al*., 2013). This note reports the isolation of phytotoxins produced *in vitro* from *L. laeliocattleyae* (syn. *egyptiacae*) (Jayawardena *et al*., 2018), a pathogen of grapevine (Correia *et al*., 2016) and agent of mango dieback and fruit rot (Rosado *et al*., 2016). To our knowledge this is the first report of phytotoxic metabolites isolated from *L. laeliocattleyae*.

MATERIALS AND METHODS

Fungal isolates and culture conditions

The strain of *L. laeliocattleyae* (CMM0206) used in this study was obtained from the collection of Universidade Federal Rural de Pernambuco, Recife, Brazil. It was inoculated and grown in stationary culture, as reported for other strains of *Lasiodiplodia* (Cimmino *et al*., 2017), in modified Difco Czapek Dox medium (Benton), with 0.5% yeast and 0.5% malt extract (Difco). The cultures were grown for 21 d at 25 °C in the dark. The mycelium was removed and the liquid cultures were lyophilized prior to the extraction procedure.

Extraction of low molecular weight phytotoxic metabolites

The lyophilized residues of the culture filtrates (2.85 L) were dissolved in 300 mL of water and extracted with EtOAc (3 × 300 mL) at the same pH as the original culture (pH 8). The organic extracts were then combined, dried (Na_2SO_4), filtered, and evaporated under low pressure. The organic residue (264.0 mg) was purified by silica gel column chromatography using $CHCl_3$-*i*-PrOH (95:5, v/v), and six fractions of homogenous groups were collected. The residue (21.9 mg) of fraction #1 was purified by preparative TLC on silica gel using $CHCl_3$ as eluent. This yielded a white solid, which was identified as *R*(-)- mellein (**1**, Figure 1, 13.7 mg). The residue of fraction #3 was purified on preparative TLC on silica gel, using $CHCl_3$-i-PrOH (97:3, v/v) as eluent, yielding a white solid, which was identified as tyrosol (**2**, Figure 1, 12.4 mg).

Chemical analyses and characterization

Optical rotations were measured in MeOH on a Jasco P-1010 digital polarimeter (Jasco). ^{1}H NMR spectra were recorded at 400 or 500 MHz in $CDCl_3$ on Bruker and Varian instruments, with MeOH as an internal standard. ESI MS and LC/MS analyses were performed using the LC/MS TOF system AGILENT (Agilent Technologies) 6230B, HPLC 1260 Infinity. Analytical and preparative TLC was carried out on silica gel plates (Kieselgel 60, F254, 0.25 mm and 0.5 mm) (Merck). TLC spots were visualized by exposure to UV radiation, or by spraying first with 10% H_2SO_4 in MeOH, and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110°C for 10 min. Column chromatography was performed using silica gel (Kieselgel 60, 0.063-0.200 mm) (Merck). Standard sample of (*R*)-(-)-mellein was obtained from the culture filtrates of *Sardiniella urbana* (Cimmino *et al*., 2018), and of tyrosol from *Lasiodiplodia euphorbiaceicola* (Cimmino *et al*., 2017).

Phytotoxicity bioassay

The phytotoxic activity of chromatographic organic extract fractions was assayed on lemon fruit, using pre-

viously reported protocol (Andolfi *et al*., 2014b; Cimmino *et al*., 2017).

RESULTS

The culture filtrates of *L. laeliocattleyae* were exhaustively extracted with ethyl acetate. The organic extract and the resulting aqueous phases were assayed for phytotoxic activity, and both showed some phytotoxicity in the bioassay conditions. The phytotoxicity results were essentially the same as previously outlined (Cimmino et al, 2017). The organic extract was purified by column chromatography. When assayed on lemon fruit, the residue of fractions #1 and #3 produced intense necrotic spots. These were further purified by preparative TLC, obtaining two pure metabolites. The purified compounds were identified as (*R*)-(-)-mellein and tyrosol (**1** and **2**, Figure 1) by comparison with standard samples (Cimmino *et al*., 2017; 2018). The identity of metabolite **1** was confirmed comparing the $[a]^{25}_{D}$, ^{1}H NMR and ESIMS(+) data with those reported in previous studies (Djoukeng *et al*., 2009; Evidente *et al*., 2010). Metabolite **2** was identified by comparing its ^{1}H NMR and ESIMS data with those reported in literature (Kimura *et al*., 1973; Evidente *et al*., 2010).

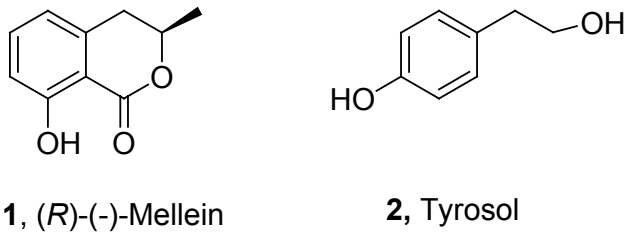

Figure 1. Structures of *R*(-)-mellein **1** and tyrosol **2** isolated from *Lasiodiplodia laeliocattleyae* H141a.

DISCUSSION

Melleins are metabolites produced by many fungi in different genera which are involved in numerous plant diseases. These compounds give different phytotoxic, zootoxic and moderate antifungal effects. (*R*)-(-)-mellein (**1**), produced by different Botryosphaeriaceae, produces toxic effects on grapevine leaves and grapevine calli (Vankatasubbaiah *et al*., 1991; Djoukeng *et al*., 2009,; Evidente *et al*., 2010). Furthermore, this compound was detected in infected wood samples and in green shoots of grapevines affected with Botryosphaeria dieback (Abou-Mansour *et al*., 2015). *R*-(-)-mellein was produced *in vitro* by several different species of Botryosphaeriaceae such us *Diplodia mutila, Neofusicoccum parvum, Neofusiccoum australe, Neofusicoccum luteum* isolated from grapevine in different grape-growing areas in the world, posing questions about its involvement in the virulence of Botryosphaeria dieback pathogens (Reveglia *et al*., 2018).

Melleins are isocoumarins, and together with jasmonic acid, its esters, dihydrofuranones and closely related compounds, these are specifically related to Botryosphaeriaceous pathogens. Eutypine and analogues are only produced by *Eutypa* species, the cause of Eutypa dieback (Masi *et al*., 2018).

Tyrosol (**2**), is a ubiquitous phytotoxic secondary metabolite that has been isolated from *N. parvum* (Evidente *et al*., 2010) and *N. australe*, both of which are well-known Botryosphaeria dieback agents (Andolfi *et al*., 2012). Furthermore, metabolite **2** was produced by some *Lasiodiplodia* species such as *L. euphorbiaceicola* and *L. hormozganensis* (Cimmino *et al*., 2017). More recently, it was also isolated from *Diplodia seriata*, *N. luteum* and *D. mutila* associated with grapevine wood infections (Reveglia *et al*., 2018). The phytotoxic activity of tyrosol was shown on tomato cuttings (Evidente *et al*., 2010), but other results point to activity of tyrosol as an active quorum sensing compound in *Candida albicans* (Chen *et al*., 2004), controlling of this organism. Tyrosol was also shown to have a synergistic inhibitory effect on radish and grain sorghum when tested with vanillic acid (Einhelling *et al*., 1978,;Yu *et al*., 1994; Evidente *et al*., 2010).

This first report on phytotoxic metabolites produced by *L. laeliocattleyae*, one of the pathogens associated with cankers and diebacks on grapevine in Brazil, adds further information on the complex interactions between virulence factors produced by the internationally common pathogens involved in Botryosphaeria dieback of grapevine and the diseases they cause.

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New or Unusual Disease Reports

Leaf rot caused by *Rhizopus oryzae* on pak choy (*Brassica campestris* ssp. *chinensis*) in China

Samiah ARIF[1], Muhammad Farooq Hussain MUNIS[2,3], Fiza LIAQUAT[4], Iftikhar Hussain SHAH[1], Yidong ZHANG[1,*]

[1] *Department of Plant Sciences, School of Agriculture and Biology, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai, China*
[2] *Department of Plant Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, 45320, Pakistan*
[3] *Department of Plant Pathology, University of California, Davis, 95616, United States of America*
[4] *School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, 200240, P. R. China*
*Corresponding author: zhyd@sjtu.edu.cn

Abstract. Pak choy (*Brassica campestris* ssp. *chinensis* L.) is the major vegetable crop cultivated in China. During December 2017, leaf rot was frequently observed on leaves of pak choy in greenhouses of Shanghai, China. Diseased leaf samples were plated onto acidified potato dextrose agar and fungal cultures were isolated and identified as *Rhizopus oryzae*, based on morphological features and molecular identification. Definitive identification as *R. oryzae* was based on the comparative molecular analysis of rRNA gene sequences. Blast analysis revealed 99% similarity with *R. oryzae*. Pathogenicity was determined by inoculating healthy pak choy leaves and plants with hyphal suspensions of *R. oryzae*. The fungus was re-isolated from developing similar lesions on the inoculated plants and identified as similar to the inoculated fungus, thus fulfilling Koch's postulates. This is the first report of fungal leaf rot caused by *R. oryzae* on pak choy in China.

Keywords. ITS sequence, Shanghai.

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INTRODUCTION

Pak choy (*Brassica campestris* subsp. *chinensis* L.) is a leafy green Chinese cabbage which does not form heads. It is a popular vegetable crop, with smooth dark green leaves and is grown widely in southern China and Southeast Asia (Khalid *et al*., 2017). Compared to other Brassicaceae members, pak choy does not have well-developed root systems, producing sessile leaves with a short stems. Due to high water and soil fertility requirements, this plant cannot tolerate dry conditions for long periods (Ajisaka *et al*., 2001). A 100 g portion of fresh pak choy supplies 13 calories and is rich in vitamins A, C and K (Noia, 2014).

Rhizopus oryzae is fungus in the Zygomycetes, and is a ubiquitous soil pathogen (Battaglia *et al*., 2011) causing worldwide losses in crop yields (Bassanezi *et al*., 2011). It causes diseases on many economically important crops, including citrus (Hakim *et al*., 2015), sweet potato (Wang *et al*., 2017), rice (Lanoiselet *et al*., 2007) and yellow oleander (Arif *et al*., 2017). *Rhizopus oryzae* is found globally but has wide distribution in tropical and sub-tropical regions. The fungus causes small water-soaked spots on plant tissues which become soft, pulpy and ultimately rot. Whitish mycelia with many spore producing sporangia are usually evident near infected tissues (Shtienberg 1997; Kwon *et al*., 2012a).

In the winter of 2017, a leaf rot disease of pak choy was observed in green houses in Shanghai, China. Early foliar symptoms were characterized by yellow to grey, irregular-shaped lesions on the affected leaves. As these spots increased in size, they developed to irregular round spots that turned to sunken whitish grey lesions surrounded by brownish borders. After approx. 2 weeks, the lesions expanded to cover entire leaves. Infected leaves turned dark brown and withered. Random disease distribution was observed on 79% of plant population. This disease was observed in more than 20 greenhouses in Shanghai area. The objective of the present study was to isolate and identify the causal agent of leaf rot of pak choy.

MATERIALS AND METHODS

For isolation of the disease causing pathogen, 3-5 diseased leaves were randomly collected from 12 different greenhouses. Small portions (<1 cm^2) of symptomatic leaf tissues were surface sterilized in 75% ethanol for 30 s, then rinsed three times with sterile distilled water, and were then were then placed on acidified potato dextrose agar (APDA) for incubation at 28°C in the dark. Single conidiospore cultures were made from 36 isolated colonies, and these were incubated on APDA.

To perform the morphological observations of fungal isolates, samples from 7-d-old cultures of all 36 isolates were mounted with water on glass slides and examined under a light microscope (Leica DM750, Leica Microsystems,) to observe culture and fungus morphological characters. As all isolates had similar form one isolate was randomly selected and used for molecular identification and pathogenicity testing.

Genomic DNA was extracted from mycelia of the isolate using the CTAB method (Cullings 1992). The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified using ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers. The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd, and the resulting products were sequenced. The PCR was carried out sequentially in a volume of 25 µL containing Master Mix (10 µL), 10 mM forward primer (1 µL), 10 mM reverse primer (1 µL), DNA template (1 µL) and sterile distilled water (12 µL). The cycling parameters were 94 °C for 3 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and final extension of 72°C for 1 min. The PCR product was sequenced by TSINGKE Biological Technology and analyzed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast). Nucleotide sequences were aligned with MEGA version 6.0 (Tamura *et al*., 2013). Phylogenetic analysis was performed using the maximum likelihood with 1,000 bootstrap replications.

Pathogenicity tests were carried out on detached, young pak choy leaves. Twenty non-infected detached 20-d-old were surface-sterilized and each inoculated by placing 10 µL of conidium suspension (10^6 conidia mL^{-1}) of the isolated fungus using either non-wound/drop or wound/drop methods. The same inoculation pattern was used for control leaves while distilled water was used instead of conidium suspension (Ford, 2004). The infected leaves were incubated at 28°C and 80 to 85% humidity with a 12 h photoperiod. The experiment was repeated twice. Koch's postulates were assessed using 20-day-old healthy pak choy plants. Ten plants were sprayed with spore suspensions (10^6 conidia mL^{-1}), while plants sprayed with water served as negative controls. The plants were then covered with plastic bags for 48 h and kept in a greenhouse. After symptom development, the pathogen was re-isolated from the inoculation induced lesions, and was morphologically identical to the original isolates obtained from diseased pak choy plants.

RESULTS AND DISCUSSION

Thirty-six isolates of similar morphology were obtained from 40 diseased leaf samples (Figure 1a), and the frequency of isolation from diseased leaves was 90%. After incubation at 28°C on APDA, white mycelium initially appeared then became heavily speckled with sporangia and finally became blackish-gray and spread rapidly with stolons attached at various points to the substrate by rhizoids (Figure 1b). The reverse sides of Petri plate colonies were light brown (Figure 1c). The mycelial appearance, colour and growth pattern was similar to previously described features of *Rhizopus oryzae* (Zhang *et al*., 2013). Sporangiophores were mostly erect, subhyaline to brown, single or in groups, and 180 to 1400 µm long and 8 to 14 µm wide (Figure 1d). Sporangia were globose to subglobose, greyish brown to black, and 60

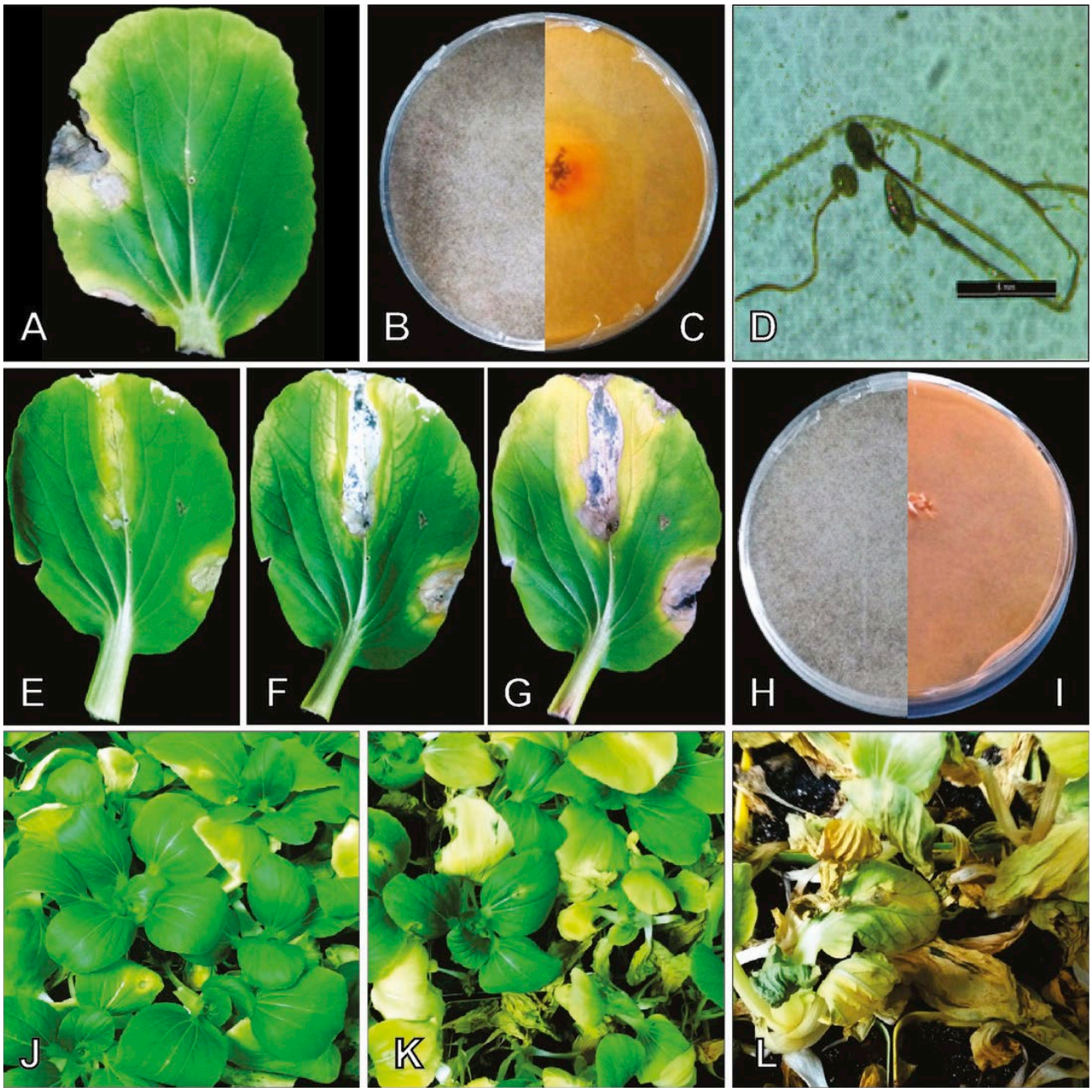

Figure 1. Disease symptoms on a leaf of pak choy (A). Disease causing pathogen was isolated on APDA and observed from front side (B) and back side (C) of Petri plates. Fungus morphology was observed under a compound microscope (D). Typical disease symptoms were observed after 4 d (E), 6 d (F) or 8 d (G) after inoculation. The inoculated pathogen was isolated from diseased leaves (H and I). After foliar inoculation of fresh leaves (J), lesions appeared in the 1st first week after innoculation (K) and completely damaged plants in the 3rd week (L).

to 180 µm in diameter (Figure 1d). Columella were subglobose to ovoid, subhyaline to greyish brown, and 35 to 100 × 50 to 120 µm. Sporangiospores were ovoid, angular, striate, pale greyish to brown, and 5 to 8 × 3 to 5 µm (Figure 1d). Similar features have been described for *R. oryzae* by Park *et al*., (2014).

Whitish grey lesions surrounded by brownish borders, similar to those observed on naturally infected pak choy leaves in the commercial greenhouses, appeared on all inoculated leaves, then rotted within 7 d. No lesions developed on the control leaves (Figure 1; e, f and g). All the inoculated pak choy plants also showed the typi-

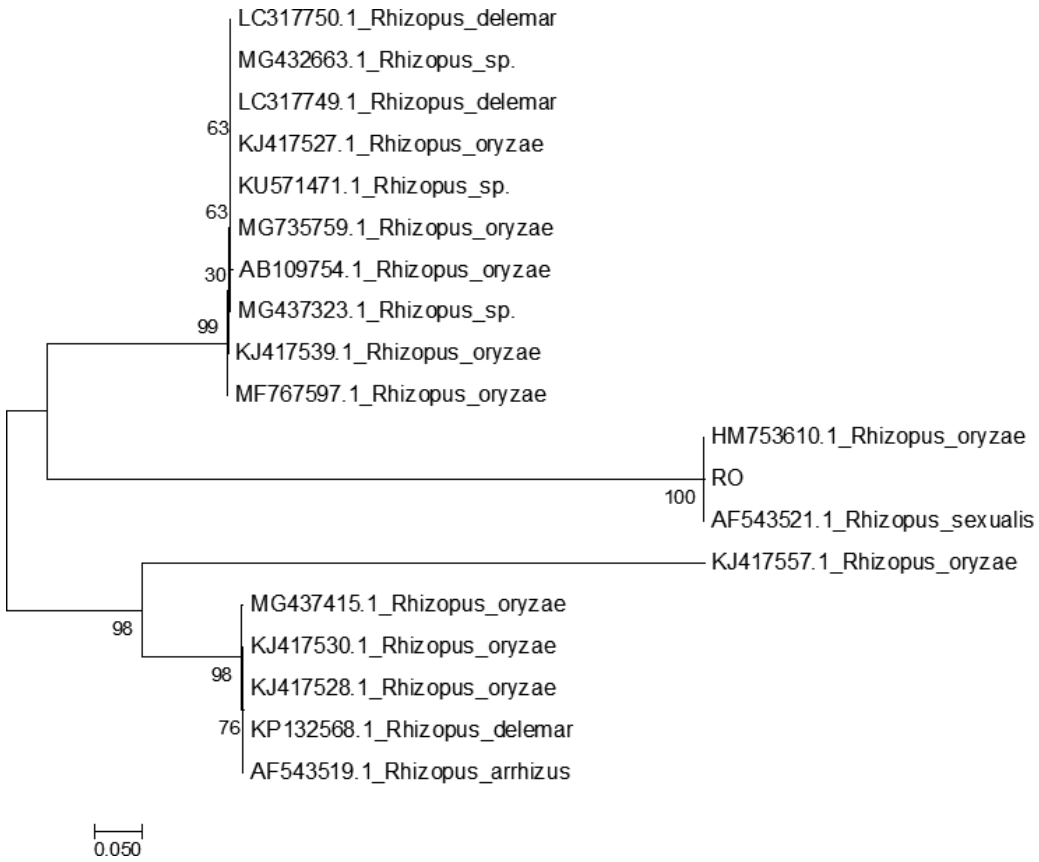

Figure 2. Phylogenetic analysis of a *Rhizopus oryzae* isolate from pak choy, with 18 related sequences from GenBank. Resulting from 1,000 replicates, bootstrap values are shown at the branch points.

cal symptoms within 10 d, whereas the control plants remained healthy (Figure 1; k, l and m). *Rhizopus oryzae* was re-isolated from the lesions of inoculated leaves, fulfilling Koch's postulates (Figure 1; h, i and j).

BLAST analysis indicated that the ITS sequence was 99% similar to *R. oryzae* (accession number HM753610). Phylogenetic analysis showed the isolated sample was conspecific with other members of *R. oryzae* (Figure 2).

Morphological, microscopic and molecular analyses, coupled with fulfillment of Koch's postulates, proved *R. oryzae* to be the casual pathogen of leaf rot disease of pak choy in this study. This fungus has been isolated from soil, decaying vegetation, vegetables, fruits, seeds and dung (Domsch *et al*., 1980). *Rhizopus oryzae* has been reported to cause infection and decay of tobacco leaves (Kortekamp 2006). Previous studies showed that the pathogen also causes soft or fruit rots of peach (Kwon *et al*., 2012a), jackfruit (Nelson 2005), and banana (Kwon *et al*., 2012b). However, there is no prior report of *R. oryzae* causing leaf rot on pak choy. To our knowledge, this is the first report of *R. oryzae* causing leaf rot on pak choy in China, and elsewhere in the world. Our future research will be focused primarily on management of this disease.

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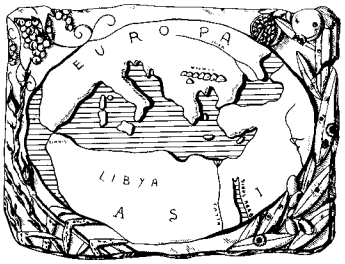

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