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Research Paper

Alternaria brown spot on new clones of sweet orange and lemon in Italy

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Summary. Since 2013 an Alternaria disease has been observed in Italy on new clones of sweet orange and lemon, during pre-harvest stages. Isolations were made from diseased leaves and fruit collected from these hosts and from other known *Alternaria* hosts (*Citrus* x *clementina*, *Citrus reticulata* 'Mandalate' and *Citrus sinensis* 'Valencia'). The typical small-conidium *Alternaria* species was consistently isolated from symptomatic tissues. One hundred and sixty-two *Alternaria* isolates were recovered from 15 citrus orchards in Catania, Syracuse and Palermo provinces, and 148 of these were used for multigene phylogenetic analyses based on the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and translation elongation factor 1-α (*EF-1α*) genes. Most of the characterized isolates (97.3%) belonged to the *A. alternata* clade, and the remainder were *A. arborescens*. Pathogenicity tests of 49 isolates of *A. alternata* and one of *A. arborescens* were performed on young detached leaves and on detached immature fruit of orange, lemon, calamondin, and rangpur lime. Toxicity of culture filtrates of representative isolates on detached leaves was also evaluated. In addition, pathogenicity tests were carried out on immature fruit *in planta* under controlled environment conditions. Most of the isolates (72%, including *A. alternata* and *A. arborescens*) were pathogenic on all inoculated citrus species and caused brown spot symptoms identical to those induced on tangerine. This study demonstrates the ability of *A. alternata* to cause damage in the field on new and popular clones of sweet orange and lemon. The hypothesis is supported that secondary metabolites, in addition to the ACT-toxin or ACR-toxin, could play roles in *Alternaria* spp. pathogenicity to citrus hosts.

Keywords. Multigene analysis, pathogenicity, disease symptoms.

INTRODUCTION

Alternaria species cause four diseases of citrus, including Alternaria brown spot of tangerine (*Citrus reticulata* Blanco) and their hybrids, Alternaria leaf spot of rough lemon (*C. jambhiri* Lush.) and rangpur lime (*C. limonia* Osbeck), Alternaria black rot of many citrus cultivars, and Mancha foliar of Mexican lime (*C. aurantifolia* Swingle) (Timmer *et al.*, 2003; Peever *et al.*, 2004). Black rot of citrus is considered distinct from Alternaria brown spot, and is a significant postharvest disease that may appear in the field prior to harvest on navel oranges (*Citrus sinensis* L. Osbeck) and in storage on lemons, tangerines and their hybrids (Peever *et al.*, 2004, 2005). Brown spot is caused by *Alternaria alternata* in all citrus producing countries. The pathogen attacks young leaves, twigs and fruit causing brown to black lesions surrounded by yellow haloes. Under appropriate environmental conditions, severe infections may cause leaf and fruit drop and significant losses of yield and marketable fruit (Peever *et al.*, 2002; Akimitsu *et al.*, 2003; Timmer *et al.*, 2003). Alternaria brown spot is a serious disease in humid areas, and highly susceptible cultivars cannot be grown in some regions. In semiarid areas this disease can also be a significant problem (Akitimitsu *et al.*, 2003).

Two distinct pathotypes of *A. alternata* associated with citrus species have been described based on differences in host specificity and toxin production (Kohmoto *et al.*, 1993; Peever *et al.*, 1999; Ohtani *et al.*, 2009). The tangerine pathotype is specific to tangerines, tangelos (*C. reticulata* × *C. paradisi*), tangors (*C. reticulata* × *C. sinensis*) and hybrids, and produces a host-selective ACT-toxin. The rough lemon pathotype is specific to this host and rangpur lime, and produces a host specific ACR-toxin. On some citrus species the disease was reproduced only by artificial inoculation or by the toxin assay (Kohmoto *et al.*, 1979; Solel and Kimchi, 1997; Elena, 2006).

Despite the well-known role of ACTs as pathogenicity factors, Garganese *et al.* (2016) have shown the ability of 20 selected isolates collected in Spain and Italy to cause brown spot of tangerine on freshly detached citrus fruit and leaves (*C. reticulata* cv. Fortune) in the absence of ACTT1/ACTT2 expression.

During a survey conducted in December 2013 in an orchard located in Catania province (Sicily, southern Italy), symptoms resembling Alternaria brown spot were detected on sweet orange clone 'Tarocco Sciara' and lemon (*Citrus* × *limon* (L.) Burm. f.) clone 'Femminello Siracusano 2KR'. Severe brown spot symptoms were observed on leaves and fruit, similar to those caused by *A. alternata* (Polizzi and Azzaro, 2015). During the period from 2014 to 2019 the disease was much more widespread and was observed in different orchards in Catania, Syracuse and Palermo provinces. The symptoms were observed in the spring and summer on different cultivars and clones of sweet orange, lemon, bitter orange, mandarin, clementine, mandalate and 'Carrizo' citrange trees. Small brown necrotic leaf spots of various sizes appeared on leaves, with sometimes extension of necrosis into the leaf veins. Lesions were often surrounded by yellow halos. On immature fruit, slightly depressed brown to black lesions surrounded by yellow halo areas were the first symptoms. Summer infections in many cases caused leaf drop and considerable yield losses (up to 40%). Sometimes, the typical anthracnose symptoms on leaves and fruit associated with brown spot were also observed (Aiello *et al.*, 2015; Piccirillo *et al.,* 2018). Premature colouration and development of light brown to blackish discolouration of the rind at or near the stylar end of fruit were also observed.

After the discovery of severe symptoms on citrus species in orchards, the research reported here aimed to: i) determine the identity of the pathogens causing the infections; ii) evaluate pathogenicity of these organisms on different citrus hosts; and iii) determine the toxicity of culture filtrates from selected pathogen isolates.

MATERIALS AND METHODS

Fungus isolates

During surveys conducted from 2014 to 2019, infected leaves and fruit were collected from different citrus species with Alternaria disease symptoms (Figure 1).

Isolations were performed by disinfesting at least 1,200 fragments of symptomatic tissues (each approx. 5 mm^2) with 1% NaOCl for 1 min, rinsed in sterile distilled water (SDW), then placed on potato dextrose agar (PDA, Oxoid) amended with streptomycin sulphate (Sigma) at 100 mg/L, and then incubated at 25±1°C in the dark. After 7 d, single conidia were selected from resulting colonies and transferred into PDA plates. For long-term storage, each plate was covered with six pieces of sterilized filter paper (1 cm^2), which was then inoculated and incubated for 7 d in the dark. Colonized filter paper was gently removed and placed into sterile envelopes, which were dried overnight in a laminar flow hood (Peever *et al.*, 2002). All envelopes were stored at -20°C.

DNA extraction, amplification and sequencing

Fungus isolates were grown on malt extract agar (MEA) for 7 d at room temperature (20°C). Resulting mycelium of each isolate was harvested with a sterile scalpel, and the genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega Corporation), according to the manufacturer's protocol. The internal transcribed spacer of ribosomal DNA (rDNA-ITS) region was targeted for PCR amplification and sequencing. Two genomic regions, translation elongation factor 1-α (*EF-1α*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), were selected due to their highly informative combined to distinguish *A. alternata* from all the other *Alternaria* species of the section *Alternaria* (Woudenberg *et al.*, 2015). The primers used for these regions were: ITS5 and ITS4 for ITS (White *et al.*, 1990), EF1-728F and EF1-986R for *EF-1α* (Carbone and Kohn, 1999) and GPD1 and GPD2 for *GAPDH*. The PCR amplifications were performed on a GeneAmp PCR System 9700 (Applied Biosystems). The PCR mixtures for ITS, *GAPDH* and *EF-1α* contained 1 μL of genomic DNA, 2 μM $MgCl_2$, 40 μM of each dNTP, 0.2 μM of each primer and 0.5 Unit GoTaq® Flexi DNA polymerase (Promega), in a total volume of 12.5 μL. The PCR conditions were: initial denaturation at 94°C for 5 min; 35 cycles amplification at 94°C for 30 s; annealing at 48°C (ITS) or 52°C (*EF-1α*) for 50 sec, and extension at 72°C for 2 min; and a final extension at 72°C for 7 min. Following PCR amplification, the amplicons were visualized on a 1% agarose gel stained with GelRed™ (Biotium) and viewed under ultra-violet light. The sizes of resulting amplicons were determined against a HyperLadder 1 kb molecular marker (Bioline). The amplicons were sequenced in both directions using the same primers used for amplification and a BigDye® Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems Life Technologies), following the protocol provided by the manufacturer. DNA sequencing amplicons were purified through Sephadex G-50 Superfine columns (Sigma Aldrich) in 96-well MultiScreen HV plates (Millipore). Purified sequence reactions were analysed on an Applied Biosystems 3730 × l DNA Analyzer (Life Technologies). The DNA sequences generated were analysed and consensus sequences were determined using Seqman (DNA Star Inc.). All sequences were manually corrected, and arrangement of nucleotides in ambiguous positions was corrected using comparisons of the sequences generated from both the forward and reverse primers. Novel sequences were lodged in GenBank (Table 1). The sequences of *EF-1α* and *GAPDH* genes of 148 *Alternaria* isolates from citrus in Sicily and 25 representative strains of *Alternaria* sect. *Alternaria* (Wounderberg *et al.*, 2015) obtained from GenBank (http://www.ncbi.nlm.nih.gov) were subsequently aligned using the Muscle algorithm in MEGA V.6 (Tamura *et al.*, 2013). Phylogenetic analyses were performed in MEGA V.6 using both Neighbour-Joining (NJ) (Saitou and Nei, 1987) and Maximum Likelihood (ML). The phylogenetic trees were calculated, first singularly for each locus to verify the genealogical concordance phylogenetic species recognition (GCPSR), and subsequently in a concatenated tree. For the phylogenetic analysis, the evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992). An initial tree(s) for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and selecting the topology with the superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories (+G, parameter =0.1625)]. All positions containing gaps and missing data were eliminated.

Pathogenicity test on detached leaves

To evaluate the pathogenicity of *Alternaria* isolates on citrus species, 50 isolates collected in this study were selected and inoculated on detached 10- to 15-day-old leaves. The plants used for tests were grown under controlled environment conditions in a growth chamber, and included calamondin, rangpur lime, sweet orange (‘Moro’, ‘Valencia’, ‘Tarocco Nucellare’, ‘Tarocco Sciara’) and lemon (‘Femminello Siracusano 2KR’). Calamondin is susceptible to the tangerine pathotype of *A. alternata* but resistant to the rough lemon pathotype, and rangpur lime is sensitive to the rough lemon pathotype but not to the tangerine pathotype (Kohmoto *et al.*, 1991; 1993; Timmer *et al.*, 2003; Ohtani *et al.*, 2009). One isolate of profile I and one of profile II of tangerine pathotype of *A. alternata* (Vega and Dewdney, 2014) were also included.

Each isolate was grown on V8 agar in Petri plates to induce the production of conidia (Vega *et al.*, 2012). After 7–10 d, conidium suspensions from each isolate were prepared by adding SDW to the plates and gently rubbing the mycelia with a sterile loop and filtering the suspension through a triple layer of cheesecloth. Conidium suspensions (1×10^5 conidia mL^{-1}, adjusted using a microscope slide haemocytometer) were each sprayed on the adaxial side of three leaves of each citrus species. SDW was sprayed on young leaves as negative controls. Inoculated leaves were placed on sterile aluminium trays, placed into plastic bags covered with plastic film, and then incubated at 25±1°C in the dark. After 48-72 h, development of necrotic spots on the leaves was evaluated. If at least two of the three leaves showed these symptoms, the pathogenicity reaction was considered positive (Table 2). The experiment was conducted twice.

Pathogenicity test on detached fruit

The pathogenicity of eight representative isolates (AA2, AA19, AA22, AA27, AA37, AA66, AA144 and AA145), from different groups determined with patho-

Table 1. Isolates from citrus used in this study, and their GenBank accession numbers.

Strain number[1]	Host, cultivar, clone	Symptom	Location	GenBank accession number[2]		
				ITS	*GAPDH*	*EF-1α*
AA 1	*Citrus reticulata* 'Nova'	Leaf spot	Italy (Sicily, Catania province)	KX056429	KX019989	KX064063
AA 2	*Citrus reticulata* 'Nova'	Leaf spot	Italy (Sicily, Catania province)	KX056422	KX020143	KX064201
AA 3	*Citrus reticulata* 'Nova'	Leaf spot	Italy (Sicily, Catania province)	KX056423	KX020002	KX064064
AA 4	*Citrus reticulata* 'Nova'	Leaf spot	Italy (Sicily, Catania province)	KX056424	KX019990	-
AA 5	*Citrus reticulata* 'Nova'	Leaf spot	Italy (Sicily, Catania province)	KX056421	KX020134	KX064065
AA 6	*Citrus reticulata* 'Nova'	Leaf spot	Italy (Sicily, Catania province)	KX056318	KX020144	KX064202
AA 7	*Citrus reticulata* 'Nova'	Leaf spot	Italy (Sicily, Catania province)	KX056340	KX020065	KX064066
AA 8	*Citrus reticulata* 'Nova'	Leaf spot	Italy (Sicily, Catania province)	KX056408	KX019991	KX064067
AA 9	*Citrus reticulata* 'Nova'	Leaf spot	Italy (Sicily, Catania province)	KX056319	KX019992	KX064068
AA 10	*Citrus reticulata* 'Nova'	Leaf spot	Italy (Sicily, Catania province)	KX056404	KX020145	KX064203
AA 11	*Citrus reticulata* 'Nova'	Leaf spot	Italy (Sicily, Catania province)	KX056405	KX020006	KX064069
AA 12	*Citrus reticulata* 'Nova'	Leaf spot	Italy (Sicily, Catania province)	KX056320	KX019993	KX064070
AA 13	*Citrus reticulata* 'Nova'	Leaf spot	Italy (Sicily, Catania province)	KX056403	KX020007	KX064071
AA 14	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056321	KX020008	KX064072
AA 15	*Citrus reticulata* 'Nova'	Leaf spot	Italy (Sicily, Catania province)	KX056402	KX020110	KX064073
AA 16	*Citrus sinensis* 'Tarocco Sciara'	Leaf spot	Italy (Sicily, Catania province)	KX056322	KX020124	KX064074
AA 17	*Citrus sinensis* 'Tarocco Sciara'	Leaf spot	Italy (Sicily, Catania province)	KX056401	KX019994	KX064075
AA 18	*Citrus sinensis* 'Tarocco Sciara'	Leaf spot	Italy (Sicily, Catania province)	KX056323	KX020009	KX064076
AA 19	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056400	KX020111	KX064213
AA 20	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056324	KX020120	KX064204
AA 22	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056399	KX020044	KX064205
AA 23	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056325	KX020112	KX064077
AA 24	*Citrus sinensis* 'Tarocco Nucellare'	Fruit spot	Italy (Sicily, Catania province)	KX056398	KX020010	KX064078
AA 25	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056326	KX020051	KX064079
AA 26	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056397	KX020102	KX064080
AA 27	*Citrus sinensis* 'Navelina'	Fruit spot	Italy (Sicily, Catania province)	KX056327	KX020129	KX064081
AA 28	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056396	KX020103	KX064206
AA 30	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056328	KX020011	KX064082
AA 31	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056395	KX020041	KX064083
AA 32	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056329	KX020052	KX064084
AA 33	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056434	KX020088	KX064085
AA 34	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056394	KX020012	KX064086
AA 35	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056330	KX019995	KX064214
AA 36	*Citrus sinensis* 'Tarocco Nucellare'	Fruit spot	Italy (Sicily, Catania province)	KX056341	KX020074	KX064087
AA 37	*Citrus sinensis* 'Tarocco Nucellare'	Fruit spot	Italy (Sicily, Catania province)	KX056342	KX020118	KX064088
AA 38	*Citrus sinensis* 'Tarocco Nucellare'	Fruit spot	Italy (Sicily, Catania province)	KX056393	KX019996	KX064089
AA 39	*Citrus sinensis* 'Tarocco Nucellare'	Fruit spot	Italy (Sicily, Catania province)	KX056343	KX020119	KX064090
AA 40	*Citrus sinensis* 'Tarocco Nucellare'	Fruit spot	Italy (Sicily, Catania province)	KX056331	KX020013	KX064091
AA 41	*Citrus sinensis* 'Tarocco Nucellare'	Fruit spot	Italy (Sicily, Catania province)	KX056392	KX020132	KX064092
AA 42	*Citrus sinensis* 'Tarocco Nucellare'	Fruit spot	Italy (Sicily, Catania province)	KX056332	KX020014	KX064093
AA 43	*Citrus sinensis* 'Tarocco Nucellare'	Fruit spot	Italy (Sicily, Catania province)	KX056391	KX020121	KX064094
AA 44	*Citrus sinensis* 'Tarocco Nucellare'	Fruit spot	Italy (Sicily, Catania province)	KX056333	KX020045	KX064095
AA 45	*Citrus sinensis* 'Tarocco Nucellare'	Fruit spot	Italy (Sicily, Catania province)	KX056390	KX020046	KX064195
AA 46	*Citrus sinensis* 'Tarocco Nucellare'	Fruit spot	Italy (Sicily, Catania province)	KX056334	KX020047	KX064096
AA 47	*Citrus sinensis* 'Tarocco Nucellare'	Fruit spot	Italy (Sicily, Catania province)	KX056389	KX019997	KX064097
AA 48	*Citrus sinensis* 'Tarocco Nucellare'	Fruit spot	Italy (Sicily, Catania province)	KX056335	KX020048	KX064209
AA 50	*Citrus sinensis* 'Navelina'	Fruit spot	Italy (Sicily, Catania province)	KX056388	KX020015	KX064098

(Continued)

Table 1. (Continued).

Strain number[1]	Host, cultivar, clone	Symptom	Location	GenBank accession number[2]		
				ITS	*GAPDH*	*EF-1α*
AA 51	*Citrus sinensis* 'Navelina'	Fruit spot	Italy (Sicily, Catania province)	KX056336	KX020016	KX064099
AA 52	*Citrus sinensis* 'Navelina'	Fruit spot	Italy (Sicily, Catania province)	KX056387	KX020073	KX064100
AA 53	*Citrus sinensis* 'Navelina'	Fruit spot	Italy (Sicily, Catania province)	KX056278	KX020142	KX064210
AA 54	*Citrus sinensis* 'Navelina'	Fruit spot	Italy (Sicily, Catania province)	KX056344	KX020096	KX064211
AA 55	*Citrus sinensis* 'Navelina'	Fruit spot	Italy (Sicily, Catania province)	KX056406	KX020054	KX064101
AA 56	*Citrus sinensis* 'Navelina'	Fruit spot	Italy (Sicily, Catania province)	KX056279	KX020081	-
AA 57	*Citrus sinensis* 'Navelina'	Fruit spot	Italy (Sicily, Catania province)	KX056345	KX020109	KX064102
AA 58	*Citrus sinensis* 'Navelina'	Fruit spot	Italy (Sicily, Catania province)	KX056280	KX020106	KX064103
AA 59	*Citrus sinensis* 'Navelina'	Fruit spot	Italy (Sicily, Catania province)	KX056281	KX020125	-
AA 60	*Citrus sinensis* 'Navelina'	Fruit spot	Italy (Sicily, Catania province)	KX056282	KX020122	KX064104
AA 61	*Citrus sinensis* 'Navelina'	Fruit spot	Italy (Sicily, Catania province)	KX056346	KX020101	KX064105
AA 62	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056337	KX020017	KX064106
AA 63	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056386	KX019998	KX064107
AA 64	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056338	KX020130	KX064108
AA 65	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056385	KX019999	KX064109
AA 66	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056339	KX020138	KX064196
AA 67	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056283	KX020082	KX064110
AA 68	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056284	KX020003	KX064111
AA 69	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056285	KX020053	KX064112
AA 70	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056286	KX020083	KX064113
AA 71	*Citrus sinensis* 'Tarocco Sciara'	Leaf spot	Italy (Sicily, Catania province)	KX056287	KX020049	KX064215
AA 72	*Citrus sinensis* 'Tarocco Sciara'	Leaf spot	Italy (Sicily, Catania province)	KX056288	KX020084	KX064216
AA 73	*Citrus sinensis* 'Tarocco Sciara'	Leaf spot	Italy (Sicily, Catania province)	KX056407	KX020018	KX064114
AA 74	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056289	KX020104	KX064197
AA 75	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056290	KX020085	KX064115
AA 76	*Citrus sinensis* 'Tarocco Sciara'	Fruit spot	Italy (Sicily, Catania province)	KX056291	KX020126	KX064116
AA 77	*Citrus limon* 'Femminello Siracusano 2KR'	Twig lesion	Italy (Sicily, Syracuse province)	KX056420	KX020137	KX064117
AA 78	*Citrus limon* 'Femminello Siracusano 2KR'	Twig lesion	Italy (Sicily, Syracuse province)	KX056292	KX020019	KX064118
AA 79	*Citrus limon* 'Femminello Siracusano 2KR'	Leaf spot	Italy (Sicily, Syracuse province)	KX056293	KX020086	KX064119
AA 80	*Citrus limon* 'Femminello Siracusano 2KR'	Leaf spot	Italy (Sicily, Syracuse province)	KX056294	KX020123	-
AA 81	*Citrus limon* 'Femminello Siracusano 2KR'	Leaf spot	Italy (Sicily, Syracuse province)	KX056295	KX020100	KX064120
AA 82	*Citrus limon* 'Femminello Siracusano 2KR'	Leaf spot	Italy (Sicily, Syracuse province)	KX056296	KX020055	KX064121
AA 83	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056347	KX020114	KX064122
AA 84	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056433	KX020078	KX064123
AA 85	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056348	KX020066	KX064200
AA 86	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056349	KX020089	KX064124
AA 87	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056350	KX020067	KX064125
AA 88	*Citrus sinensis* 'Valencia'	Leaf spot	Italy (Sicily, Catania province)	KX056351	KX020090	KX064126
AA 89	*Citrus sinensis* 'Valencia'	Leaf spot	Italy (Sicily, Catania province)	KX056352	KX020115	KX064127
AA 90	*Citrus sinensis* 'Valencia'	Fruit spot	Italy (Sicily, Catania province)	KX056297	KX020099	KX064128
AA 91	*Citrus sinensis* 'Valencia'	Fruit spot	Italy (Sicily, Catania province)	KX056298	KX020056	KX064129
AA 92	*Citrus sinensis* 'Tarocco Nucellare'	Leaf spot	Italy (Sicily, Catania province)	KX056299	KX020076	KX064130
AA 93	*Citrus sinensis* 'Tarocco Nucellare'	Leaf spot	Italy (Sicily, Catania province)	KX056300	KX020057	KX064198
AA 94	*Citrus sinensis* 'Tarocco Nucellare'	Leaf spot	Italy (Sicily, Catania province)	KX056425	KX020004	KX064131
AA 95	*Citrus sinensis* 'Tarocco Nucellare'	Leaf spot	Italy (Sicily, Catania province)	KX056426	KX020058	KX064132
AA 96	*Citrus sinensis* 'Tarocco Nucellare'	Leaf spot	Italy (Sicily, Catania province)	KX056427	KX020020	KX064133
AA 97	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056428	KX020059	KX064134

(Continued)

Table 1. (Continued).

Strain number[1]	Host, cultivar, clone	Symptom	Location	GenBank accession number[2]		
				ITS	*GAPDH*	*EF-1α*
AA 98	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056430	KX020131	KX064135
AA 99	*Citrus sinesis* × *Poncirus trifoliata* 'Carrizo'	Leaf spot	Italy (Sicily, Catania province)	KX056432	KX020105	KX064136
AA 100	*Citrus sinensis* × *Poncirus trifoliata* 'Carrizo'	Leaf spot	Italy (Sicily, Catania province)	KX056431	KX020021	KX064137
AA 101	*Citrus sinensis* × *Poncirus trifoliata* 'Carrizo'	Leaf spot	Italy (Sicily, Catania province)	KX056303	KX020022	KX064138
AA 102	*Citrus* × *clementina*	Fruit spot	Italy (Sicily, Palermo province)	KX056353	KX020116	KX064139
AA 103	*Citrus* × *clementina*	Fruit spot	Italy (Sicily, Palermo province)	KX056384	KX020001	KX064140
AA 104	*Citrus* × *clementina*	Fruit spot	Italy (Sicily, Palermo province)	KX056354	KX020068	KX064141
AA 105	*Citrus* × *clementina*	Fruit spot	Italy (Sicily, Palermo province)	KX056383	KX020079	KX064142
AA 106	*Citrus* × *clementina*	Fruit spot	Italy (Sicily, Palermo province)	KX056355	KX020075	KX064143
AA 107	*Citrus* × *clementina*	Fruit spot	Italy (Sicily, Palermo province)	KX056304	KX020060	KX064144
AA 108	*Citrus reticulata* 'Nova'	Fruit spot	Italy (Sicily, Catania province)	KX056382	KX020139	KX064145
AA 109	*Citrus reticulata* 'Nova'	Fruit spot	Italy (Sicily, Catania province)	KX056356	KX020127	KX064146
AA 110	*Citrus reticulata* 'Nova'	Fruit spot	Italy (Sicily, Catania province)	KX056381	KX020097	KX064147
AA 111	*Citrus aurantium*	Leaf spot	Italy (Sicily, Catania province)	KX056305	KX020061	KX064148
AA 112	*Citrus sinensis* × *Poncirus trifoliata* 'Carrizo'	Twig lesion	Italy (Sicily, Catania province)	KX056357	KX020095	KX064149
AA 113	*Citrus sinensis* × *Poncirus trifoliata* 'Carrizo'	Twig lesion	Italy (Sicily, Catania province)	KX056306	KX020133	KX064150
AA 114	*Citrus sinensis* × *Poncirus trifoliata* 'Carrizo'	Twig lesion	Italy (Sicily, Catania province)	KX056316	KX020023	KX064151
AA 115	*Citrus sinensis* 'Valencia'	Fruit spot	Italy (Sicily, Catania province)	KX056317	KX020024	KX064199
AA 116	*Citrus sinensis* 'Valencia'	Fruit spot	Italy (Sicily, Catania province)	KX056380	KX020117	KX064152
AA 117	*Citrus aurantium*	Fruit spot	Italy (Sicily, Catania province)	-	-	-
AA 118	*Citrus aurantium*	Fruit spot	Italy (Sicily, Catania province)	KX056358	KX020038	KX064153
AA 119	*Citrus aurantium*	Fruit spot	Italy (Sicily, Catania province)	KX056301	KX020025	KX064154
AA 120	*Citrus sinensis* 'Moro'	Fruit spot	Italy (Sicily, Catania province)	KX056302	KX020107	KX064155
AA 121	*Citrus sinensis* 'Moro'	Fruit spot	Italy (Sicily, Catania province)	KX056379	KX020091	KX064156
AA 122	*Citrus sinensis* 'Moro'	Fruit spot	Italy (Sicily, Catania province)	KX056359	KX020128	KX064157
AA 123	*Citrus sinensis* 'Moro'	Fruit spot	Italy (Sicily, Catania province)	KX056378	KX020080	KX064158
AA 124	*Citrus sinensis* 'Moro'	Fruit spot	Italy (Sicily, Catania province)	KX056315	KX020062	KX064159
AA 125	*Citrus sinensis* 'Valencia'	Fruit spot	Italy (Sicily, Catania province)	KX056360	KX020098	KX064160
AA 126	*Citrus sinensis* 'Valencia'	Fruit spot	Italy (Sicily, Catania province)	KX056377	KX020092	KX064161
AA 127	*Citrus sinensis* × *Poncirus trifoliata* 'Carrizo'	Leaf spot	Italy (Sicily, Catania province)	KX056307	KX020026	KX064162
AA 128	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056409	KX020027	KX064163
AA 129	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056314	KX020087	KX064164
AA 130	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056419	KX020028	KX064165
AA 131	*Citrus limon* 'Femminello Siracusano 2KR'	Fruit spot	Italy (Sicily, Syracuse province)	KX056410	KX020136	-
AA 132	*Citrus reticulata* 'Ciaculli'	Fruit spot	Italy (Sicily, Catania province)	KX056418	KX020029	KX064166
AA 133	*Citrus reticulata* 'Ciaculli'	Fruit spot	Italy (Sicily, Catania province)	KX056308	KX020030	KX064167
AA 134	*Citrus reticulata* 'Ciaculli'	Fruit spot	Italy (Sicily, Catania province)	KX056313	KX020031	KX064168
AA 135	*Citrus reticulata* 'Ciaculli'	Fruit spot	Italy (Sicily, Catania province)	KX056309	KX020032	KX064169
AA 136	*Citrus reticulata* 'Ciaculli'	Fruit spot	Italy (Sicily, Catania province)	KX056411	KX020033	KX064170
AA 137	*Citrus reticulata* 'Ciaculli'	Fruit spot	Italy (Sicily, Catania province)	KX056312	KX020063	KX064171
AA 138	*Citrus reticulata* 'Mandalate'	Fruit spot	Italy (Sicily, Catania province)	KX056310	KX020141	KX064172
AA 139	*Citrus reticulata* 'Mandalate'	Fruit spot	Italy (Sicily, Catania province)	KX056417	KX020034	KX064173
AA 140	*Citrus reticulata* 'Mandalate'	Fruit spot	Italy (Sicily, Catania province)	KX056311	KX020042	KX064174
AA 141	*Citrus reticulata* 'Mandalate'	Fruit spot	Italy (Sicily, Catania province)	KX056412	KX020146	KX064207
AA 142	*Citrus reticulata* 'Mandalate'	Fruit spot	Italy (Sicily, Catania province)	KX056416	KX020035	KX064175
AA 143	*Citrus reticulata* 'Mandalate'	Fruit spot	Italy (Sicily, Catania province)	KX056361	KX020093	KX064208
AA 144	*Citrus reticulata* 'Mandalate'	Fruit spot	Italy (Sicily, Catania province)	KX056413	KX020108	KX064176

(Continued)

Table 1. (Continued).

Strain number[1]	Host, cultivar, clone	Symptom	Location	GenBank accession number[2]		
				ITS	*GAPDH*	*EF-1α*
AA 145	*Citrus reticulata* 'Mandalate'	Fruit spot	Italy (Sicily, Catania province)	KX056376	KX020069	KX064177
AA 146	*Citrus reticulata* 'Mandalate'	Fruit spot	Italy (Sicily, Catania province)	-	KX020036	KX064178
AA 147	*Citrus sinensis* 'Tarocco Scire'	Fruit spot	Italy (Sicily, Catania province)	KX056362	KX020077	KX064179
AA 148	*Citrus sinensis* 'Tarocco Scire'	Fruit spot	Italy (Sicily, Catania province)	KX056375	KX020005	KX064180
AA 149	*Citrus sinensis* 'Tarocco Scire'	Fruit spot	Italy (Sicily, Catania province)	-	KX020000	KX064181
AA 150	*Citrus sinensis* 'Tarocco Scire'	Fruit spot	Italy (Sicily, Catania province)	KX056363	KX020050	KX064182
AA 151	*Citrus sinensis* 'Tarocco Scire'	Fruit spot	Italy (Sicily, Catania province)	KX056374	KX020070	KX064183
AA 152	*Citrus sinensis* 'Tarocco Scire'	Fruit spot	Italy (Sicily, Catania province)	KX056364	-	KX064184
AA 153	*Citrus sinensis* 'Tarocco Scire'	Fruit spot	Italy (Sicily, Catania province)	KX056373	KX020039	KX064185
AA 154	*Citrus sinensis* 'Tarocco Scire'	Fruit spot	Italy (Sicily, Catania province)	-	KX020043	KX064186
AA 155	*Citrus sinensis* 'Tarocco Scire'	Fruit spot	Italy (Sicily, Catania province)	KX056365	KX020135	KX064187
AA 156	*Citrus sinensis* 'Tarocco Scire'	Fruit spot	Italy (Sicily, Catania province)	KX056372	KX020071	KX064188
AA 157	*Citrus limon*	Fruit spot	Italy (Sicily, Catania province)	KX056415	KX020064	KX064189
AA 158	*Citrus sinensis* 'Tarocco Scire'	Leaf spot	Italy (Sicily, Catania province)	KX056414	KX020037	KX064190
AA 159	*Citrus sinensis* 'Tarocco Scire'	Leaf spot	Italy (Sicily, Catania province)	KX056366	-	-
AA 160	*Citrus sinensis* 'Tarocco Scire'	Leaf spot	Italy (Sicily, Catania province)	KX056371	KX020094	KX064191
AA 163	*Citrus limon*	Fruit spot	Italy (Sicily, Catania province)	KX056367	KX020113	KX064212
AA 164	*Citrus limon*	Fruit spot	Italy (Sicily, Catania province)	KX056370	KX020140	KX064192
AA 165	*Citrus limon*	Fruit spot	Italy (Sicily, Catania province)	KX056368	KX020040	KX064193
AA 166	*Citrus limon*	Fruit spot	Italy (Sicily, Catania province)	KX056369	KX020072	KX064194
AA 167	*Citrus limon*	Fruit spot	Italy (Sicily, Catania province)	-	-	-

[1] AA: Cultures stored at the University of Catania, Italy.

[2] ITS: internal transcribed spacer region, *EF-1α*: translation elongation factor 1-α, *GAPDH:* glyceraldehyde-3-phosphate dehydrogenase.

Table 2. Pathogenicity of *Alternaria* isolates on detached leaves and immature fruit.

Isolate	Citrus host			
	Calamondin	Sweet orange	Rangpur Lime	Lemon
GROUP A				
AA1, AA3-11, AA15, AA17-AA24, AA26-31, AA36-38, AA40-41, AA48, AA55, AA57-58, AA62, AA74, AA80, AA86	+	+	+	+
GROUP B				
AA2	-	+	+	+
GROUP C				
AA12-14, AA16, AA25, AA32, AA39, AA52, AA59, AA70, AA144, AA145	+	+	-	+
GROUP D				
AA66	+	-	-	-

The isolates AA2, AA19, AA22, AA27, AA37, AA66, AA144, AA145 were inoculated on immature fruit.
+ positive reaction (necrotic spots) on leaves and fruit in two inoculation experiments.
– negative reaction on leaves and fruit in two inoculation experiments.

genicity tests on detached leaves, was tested on immature fruit of calamondin, rangpur lime, 'Femminello Siracusano 2KR' lemon and 'Tarocco Sciara' sweet orange. Each fruit was punctured (four punctures per fruit) with a sterile needle, and was then inoculated with 10 μL of a conidium suspension. Ten fruit from each host were inoculated, and ten fruit were inoculated without needle punctures. The inoculated fruit were placed into plastic bags, covered with plastic films and incubated at 25 ± 1°C in the dark. Negative controls were wounded fruit with 10 μL of SDW applied to each wound. Symptom development was evaluated after 4, 7 and 10 d. The experiment was conducted twice.

Pathogenicity test on fruit in planta

The pathogenicity of eight representative isolates (AA2, AA19, AA22, AA27, AA37, AA66, AA144 and AA145) from different groups was also tested *in planta* on fruit of calamondin, rangpur lime, 'Femminello Siracusano 2KR' lemon, 'Tarocco Sciara' sweet orange. Each fruit was punctured with a sterile needle and was inoculated with 10 μL of conidium suspension (four punctures per fruit). Ten fruit on each host were inoculated, and ten fruit were inoculated without needle punctures. Inoculated fruit were covered with plastic bags and incubated at 25 ± 1°C and 95% relative humidity (RH) in a 12 h fluorescent light/12 h dark regime in a growth chamber. Negative controls were fruit treated with SDW. Symptom development was observed after 4, 7 and 10 d. The experiments were conducted twice.

Toxins in culture filtrates

Toxicity of culture filtrates of the eight representative isolates (AA2, AA19, AA22, AA27, AA37, AA66, AA144 and AA145) was determined using a leaf necrosis assay (Kohmoto *et al*., 1993; Masunaka *et al*., 2000). Culture filtrates of profile I and II of tangerine pathotype were also assessed. The citrus species and clones used for pathogenicity tests on immature fruit (see above) were used. The isolates were grown on V8-juice agar plates. Small pieces of mycelium were inoculated into modified Richard's solution (Kohmoto *et al*., 1993), and incubated for 3 weeks at 25°C under cool-white fluorescent light. Each culture was then filtered through four layers gauze to retain the mycelium. Supernatant of these culture filtrates was harvested by centrifugation at 1,500 g for 5 min (Kohmoto *et al*., 1993; Masunaka *et al*., 2000). Host leaves were washed and disinfested for 1 min in 1.5 % NaOCl, rinsed twice in SDW, and then each punctured with a sterile needle on the lower surface (four punctures per leaf). Each leaf puncture was then treated with a 10 μL droplet of culture filtrate. The leaves were incubated in a moist chamber in the dark at 25°C to encourage transpiration. When toxin was present, dark necroses developed around the points of filtrate treatment. The experiment was conducted twice.

RESULTS

Fungus isolates

From symptomatic tissues, typical small conidium *Alternaria* species were consistently isolated. Occasionally, typical *Colletotrichum* spp. colonies were recovered in association with *Alternaria* species. One hundred sixty-two *Alternaria* isolates were collected from brown spot lesions from plants in 15 citrus orchards (Table 1). Disease incidence was from 5 to 100% of the trees in each orchard, depending on host species. The symptoms appeared on leaves and fruit of different cultivars and clones of sweet orange ('Moro', 'Valencia', 'Navelina', 'Tarocco Sciara', 'Tarocco Scirè', 'Tarocco Gallo', 'Tarocco Nucellare'), 'Femminello Siracusano 2KR' lemon, bitter orange (*Citrus aurantium* L.), 'Nova' mandarin, clementine (*Citrus clementina* Hort. ex Tan.), mandalate (*Citrus reticulata* × *Citrus deliciosa*) and 'Carrizo' citrange (*Citrus sinensis* Osbeck × *Poncirus trifoliata* Raf.) (Figure 1).

DNA extraction, amplification and sequencing

In total, 173 fungus isolates were included in the phylogenetic analysis. Of these, 148 were collected from citrus in Sicily and 25 were representative strains of *Alternaria* species (Wounderberg *et al*., 2015), the sequences of which were obtained from GenBank (http://www.ncbi.nlm.nih.gov). Four of the 148 strains collected from *Citrus* spp. clustered within the *A. arborescens* species complex, and the remaining strains clustered with *A. alternata*. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The tree with the highest log likelihood (-1624.2743) is shown (Figure 4). The analysis involved 173 nucleotide sequences. The final dataset consisted of 767 bases totally, represented by 530 from *GAPDH* and 237 from *EF-1α* locus.

Pathogenicity tests on detached leaves

All of the isolates inoculated gave positive pathogenicity reactions (Table 2). Typical brown spot lesions were observed 3-4 d after inoculation (Figure 2). Profile I and profile II isolates from tangelo 'Minneola' and tangor 'Murcott', known to be pathogenic to these hosts but not to rangpur lime (Peever *et al*., 1999; Vega and Dewdney, 2014), were also pathogenic to all inoculated cultivars and clones of sweet orange and lemon. Twelve isolates (group C) of 50 inoculated (24%) were pathogenic and showed the same behaviour of profile I and II of the *A. alternata* pathotype. Thirty-five isolates

Figure 1. Natural symptoms observed on fruit of (a) mandarin 'Nova'; (b) and (c), lemon 'Femminello 2KR'; (d) 'Tarocco Sciara'; and (e) 'Tarocco Gallo' sweet orange. (f), (g) and (h) show symptoms caused by artificial inoculation with *Alternaria alternata* on fruit of lemon, and (i) on sweet orange *in planta*.

of *A. alternata* and one isolate of *A. arborescens* (group A) of 50 isolates (72%) were pathogenic to all citrus species inoculated. One isolate of *A. alternata* (AA2) (group B) was pathogenic to all species, but not to calamondin, showing typical behaviour of the rough lemon pathotype. One isolate of *A. alternata* (AA66) (group D) was pathogenic only to calamondin. The negative controls remained symptomless. Re-isolation of the pathogens from symptomatic leaves confirmed the etiology of infection, fulfilling Koch's postulates. Very similar results were obtained in both experiments.

Pathogenicity tests on detached fruit

The isolates which were pathogenic on detached leaves were also pathogenic on immature fruit. Four

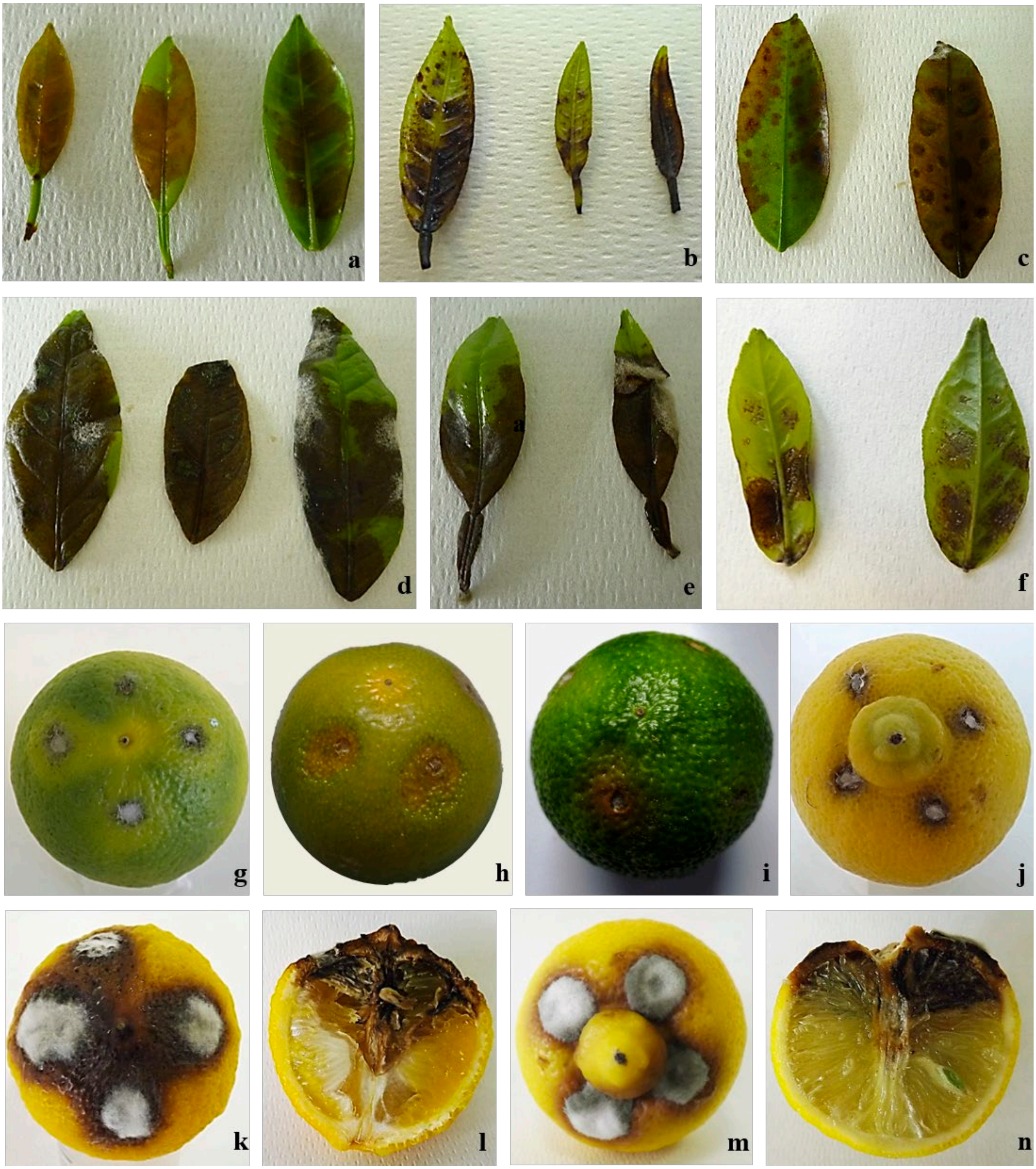

Figure 2. Symptoms caused by artificial inoculation of *Alternaria alternata* on detached leaves of calamondin (a), rangpur lime (b), lemon 'Femminello 2KR' (c), 'Moro' sweet orange (d), 'Valencia' sweet orange (e), or 'Tarocco Sciara' sweet orange (f); or on detached immature fruit of 'Tarocco Sciara' sweet orange (g); calamondin (h), rangpur lime (i), or lemon (j). Symptoms after 10 d on fruit of rangpur lime (k, l), lemon (m, n). Sporulation of *Alternaria alternata* on rangpur lime (k) and lemon (m).

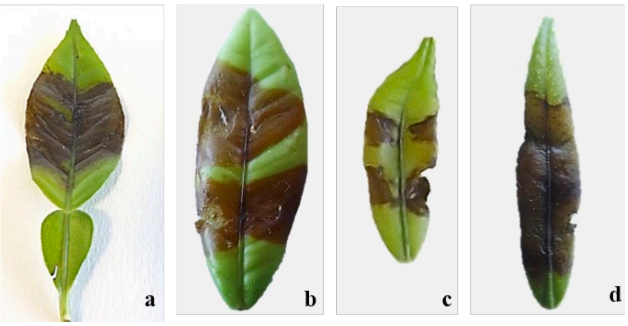

Figure 3. Toxicity of culture filtrates of *Alternaria alternata* on leaves of 'Tarocco Sciara' sweet orange (a), calamondin (b), rangpur lime (c), or lemon 'Femminello 2KR' (d).

d after inoculation, all fruit had started to show brown spots with small diameters. Seven days after inoculation, brown spots of 1 cm diam., covered by *Alternaria* mycelium, were observed on these fruit. The inoculated fruit were each cut in half at 10 d after inoculation, and those inoculated with isolates AA37, AA27 and AA22 had black rot symptoms (Figure 2). When inoculated without needle punctures, the isolates were not pathogenic on orange, lemon or rangpur lime, but were pathogenic to calamondin. Control fruit remained symptomless. Re-isolation of the pathogens from symptomatic fruit confirmed the etiology of infections, fulfilling Koch's postulates. Very similar results were obtained in both experiments.

Pathogenicity tests on fruit in planta

The isolates which were pathogenic on detached fruit were also pathogenic on fruit *in planta* when they were inoculated with needle punctures, confirming the results obtained on detached fruit. Small brown lesions were observed 4 d after pathogen inoculation. After 7 d, lesions varied from small specks to large pockmarks on all inoculated fruit. In some cases, the fruit rinds responded to infections by forming barriers of corky tissues that erupted from the fruit surfaces. These corky tissue sometimes falls out, forming craters or pockmarks on the fruit surfaces after 10 d (Figure 1). When inoculated without wounds, the isolates were not pathogenic on orange, lemon or rangpur lime but were pathogenic on calamondin. Control fruit remained symptomless. Re-isolation of the pathogens from symptomatic fruit confirmed the etiology of infections, fulfilling Koch's postulates. Very similar results were obtained in both experiments.

Toxicity of culture filtrates

Toxin assays on leaves revealed complete correlation between pathogenicity reactions and responses to toxins. Culture filtrates from group A isolates caused dark necroses on all host species 3 d after treatment (Figure 3). The isolates of group C caused symptoms on calamondin, sweet orange and lemon leaves identical to symptoms caused by profile I and II of the tangerine pathotype, but caused no visible effects on rangpur lime. In contrast, isolate AA2 (group B) produced symptoms on all the treated host species, but did not affect calamondin, while isolate AA66 (group D) showed symptoms only on calamondin. Similar results were obtained in both experiments.

DISCUSSION

In this study, brown spot symptoms were widely detected pre-harvest on sweet orange ('Tarocco Nucellare', 'Tarocco Sciara', 'Tarocco Scirè', 'Tarocco Gallo', 'Navelina', 'Moro') and lemon ('Femminello Siracusano 2KR') in Sicily (southern Italy). One hundred sixty-two *Alternaria* isolates were collected from symptomatic citrus species, and from other well-known Alternaria brown spot-susceptible citrus species from 2014 in Catania, Syracuse and Palermo provinces.

Alternaria is a cosmopolitan genus that consists of different saprophytic and pathogenic species. *Alternaria* was divided into 26 sections by Woudenberg *et al.* (2013). *Alternaria* sect. *Alternaria* contains most of the small-conidium *Alternaria* species with concatenated conidia, including important plant, human and postharvest pathogens. Species within sect. *Alternaria* have been mostly described based on morphology and/or host-specificity, and genetic variation among them is minimal. Based on genome and transcriptome comparisons and molecular phylogeny, *Alternaria* sect. *Alternaria* consists of only 11 phylogenetic species and one species complex. Thirty-five morphospecies, which cannot be distinguished based on multi-gene phylogeny, have been synonymized under *A. alternata* (Woudenberg *et al*., 2015). In the present study, we compared isolates of *Alternaria* with the isolates used by Wounderberg *et al.* (2015). Of 148 isolates used in phylogenetic analyses, four clustered in the *A. arborescens* species complex (AASC), while the majority of the isolates did not form clear phylogenetic clades and were grouped in *A. alternata*. *Alternaria arborescens* was reported in Italy from citrus by Garganese *et al.* (2016).

Lemon and sweet orange are reported to be resistant to Alternaria brown spot in field conditions. However,

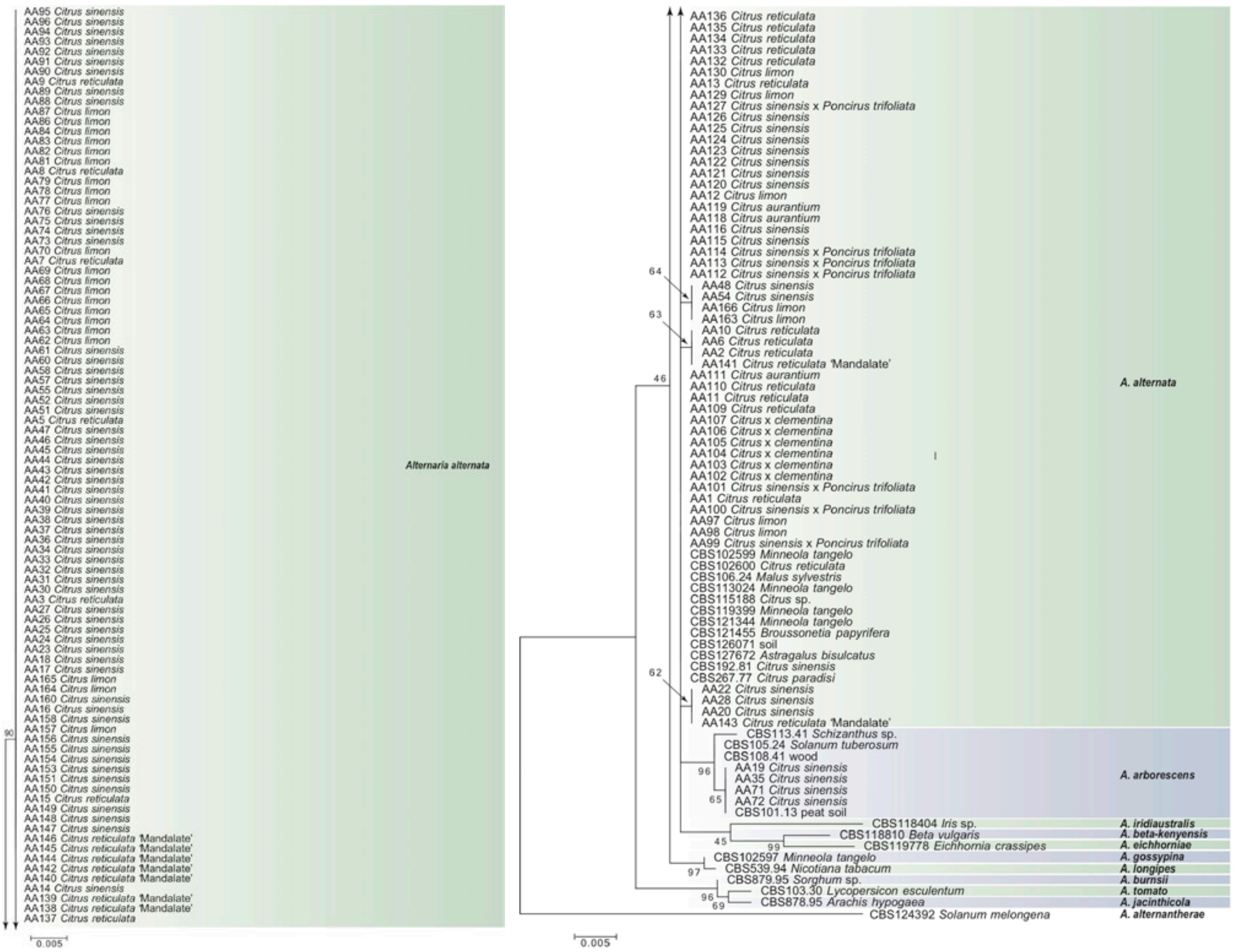

Figure 4. Multilocus glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and translation elongation factor 1-α (*EF-1α*) phylogenetic tree developed using the maximum likelihood (ML) method. The tree with the greatest obtained log likelihood (-1624.2743) is shown.

after artificial inoculation, some symptoms were induced in 'Valencia', 'Shamouti' and 'Washington Navel' sweet orange as well as in 'Volkameriano' lemon (Kohmoto *et al*., 1979; Solel and Kimchi, 1997), and on 'Moro' sweet orange in Greece (Elena, 2006) after inoculating *A. alternata* tangerine pathotype obtained from 'Minneola' tangelo.

Pathogenicity of *A. alternata* is controlled by the production of host-selective toxins (HSTs). HSTs can be defined as groups of chemically diverse and complex metabolites produced by plant pathogenic isolates of certain fungi during germination of spores on plant surfaces, which play important roles in host specificity and isolate virulence (Nishimura and Kohmoto, 1983; Gardner *et al*., 1986; Walton, 1996). ACR-toxin is an HST produced by the *A. alternata* rough lemon pathotype, and cultivars of rough lemon and rangpur lime are known to be sensitive to this toxin (Kohmoto *et al*., 1979, 1991; Gardner *et al*., 1985; Akimutsu *et al*., 2003). Tangerine, mandarin and grapefruit are completely insensitive to ACR-toxin. ACT-toxin is an HST produced by the *A. alternata* tangerine pathotype, and this toxin affects only this host and hybrids (Kohomoto *et al*., 1993; Timmer *et al*., 2000). The exclusive roles of ACTs as pathogenic factors have, however, been questioned by Garganese *et al*. (2016). They demonstrated the ability of 20 selected *Alternaria* isolates to cause brown spot of tangerine, even in the absence of ACTT1/ACTT2 expression. They also concluded that mycotoxin effects could be equivalent to phytotoxin effects in disease outcomes, so that in the absence of phytotoxins, some level of virulence may also be evident.

In the present study, artificial inoculations on detached leaves revealed that 24% of the isolates tested were pathogenic to calamondin, lemon and sweet orange but not to rangpur lime, showing behaviour of profile I and II of *A. alternata* and indicating that these isolates belong to the tangerine pathotype of *A. alternata*. Only one isolate (AA2) was pathogenic to all host species, calamondin excluded, indicating that this isolate belong to the rough lemon pathotype, whereas the isolate AA66 caused symptoms only on calamondin. Instead, 72% of our isolates (group A), including the *A. arborescens* isolate, were pathogenic to all the citrus species inoculated, and did not show any host specificity. This questions the unique role of phytotoxins as pathogenic factors. Nonpathogenic *Alternaria* isolates were not recognized among those tested, confirming that the presence of these on brown spot lesions occurs at low frequencies (Peever *et al.*, 1999).

Toxicity tests of culture filtrates revealed close relationship between pathogenicity reactions and responses to toxins. The symptoms caused by the culture filtrates of the profile I and II tangerine pathotype on sweet orange and lemon were the same as symptoms produced from culture filtrates of group C. No symptoms were observed on rangpur lime. In contrast, the isolate AA2 (group B) produced necrosis on all host species treated, except calamondin. The isolate AA66 (group D) produced symptoms only on calamondin, whereas the culture filtrates from group A caused necrosis on all host species treated.

On the basis of the data presented here, we hypothesise that the isolates belonging to group A (72% of those inoculated) produce two HSTs, ACT-toxin of the tangerine pathotype and ACR-toxin of the rough lemon pathotype. The presence of dual host specificity and toxin production is not common in populations of *A. alternata* on citrus. Masunaka *et al.* (2005) reported only one isolate that produced both toxins among hundreds of isolates examined in Florida. *Alternaria* species have no known sexual cycle in nature, and the populations of *A. alternata* on citrus are clonal. Therefore, the ability to produce both toxins is acquired when a dispensable chromosome carrying the gene cluster controlling biosynthesis of one HST is transferred horizontally and rearranged in another isolate of the fungus carrying gene for biosynthesis of the other HST (Masunaka *et al.*, 2005). Instead, the hypothesis that other toxins may have played roles in pathogenicity of selected isolates is more plausible, as reported by da Garganese *et al.* (2016). However, this needs to be confirmed by specific assays.

Our data demonstrate that most of *A. alternata* isolates and one of *A. arborescens* were pathogenic on detached leaves, detached fruit, and *in planta*, producing symptoms similar to those observed in the field. The artificial inoculation experiments with different host species gave symptoms the same as those observed in orchards on tangerine and their hybrids. Occasionally, fruit inoculated with isolates AA37, AA27 and AA22 developed symptoms similar to black rot. Black rot is a significant citrus postharvest problem that may appear in the field prior to harvest. This disease occurs most commonly on sweet orange in the field, and on tangerines and their hybrids and lemon in storage, and affects all citrus under the appropriate conditions (Brown and Eckert, 2000). Most small-conidium species of *Alternaria* have been reported as capable of causing black rot when inoculated into wounded citrus fruit. These fungi include saprophytic isolates, epiphytes and the tangerine and rough lemon pathotypes (Peever *et al.*, 2005).

Management of Alternaria brown spot includes the use of cultural practices such as disease-free nursery stock, wide tree spacing in orchards, reduction of nitrogen fertilization, and fungicide applications (Timmer and Peever, 1997; Dewdney, 2016). Chemical management is the main strategy to control disease on citrus. Quinone-outside inhibitors (QoIs) have been widely used in Florida over the last decade, but are classified as high risk for development of fungicide resistance. Currently in Italy, mancozeb and pyraclostrobin are the only fungicides approved in integrated citrus production, but chemical control strategies must be developed for the different orchard areas.

In conclusion, this study has demonstrated the high pre-harvest incidence of Alternaria disease on the common and widespread new clones of sweet orange and lemon in Sicily. These infections could be promoted by favourable local environmental conditions.

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