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RESEARCH PAPERS

Diversity and virulence of *Diaporthe* species associated with wood disease symptoms in deciduous fruit trees in Uruguay

Lucía SESSA[1], Eduardo ABREO[2], Lina BETTUCCI[1] and Sandra LUPO[1]

[1] Sección Micología, Facultad de Ciencias, Universidad de la República, Uruguay
[2] Laboratorio de Bioproducción, INIA Las Brujas, Uruguay

Summary. Several *Diaporthe* species are recognized as causal agents of many plant disease symptoms, including twig and branch cankers, dieback, shoot blight, and root and fruit rots. In Uruguay, the proximity between apple, pear and peach orchards offers the possibility to study the presence of different *Diaporthe* spp. associated with wood cankers across different deciduous fruit trees. Symptomatic twigs and branches of these orchard species were sampled, and isolates of *Diaporthe* were obtained. Selected isolates were used for cross inoculations in the three hosts. Seven *Diaporthe* spp. were identified, based on the internal transcribed spacer (ITS) region of rDNA and the translation elongation factor 1-alpha gene (EF1-α) phylogenies. The species were: *Diaporthe amygdali*, *D. foeniculina*, *D. infecunda*, *D. eres*, *D. terebinthifolii*, *D. oxe* and *D. phaseolorum*, while two isolates *Diaporthe* sp. 1 and *Diaporthe* sp. 4 could not be assigned to any species. *Diaporthe infecunda, D. eres, D. terebinthifolii, D. phaseolorum and D. oxe* on *Pyrus communis* and *D. foeniculina* on *Malus domestica* represent new records in these hosts in Uruguay, while *D. oxe* isolated from *Prunus persica* is a new record for this species. *Diaporthe eres* and *D. phaseolorum* were the most virulent species, posing the greatest risk due to their wide distribution and virulence in apple and peach trees. Although pear trees showed less symptomatic tissues and were less susceptible than peach and apple trees in the pathogenicity tests, they harboured seven of the species, and therefore should be considered as reservoirs of *Diaporthe* in Uruguayan orchards. Trees of the three hosts could be considered potential reciprocal sources of pathogenic *Diaporthe* spp.

Key words: *Malus domestica, Pyrus communis, Prunus persica, Diaporthe eres, Diaporthe oxe*.

Introduction

The genus *Diaporthe* Nitschke (syn. *Phomopsis* (Sacc.) Bubák) is a highly diverse group of fungi that includes several species known to be plant pathogens, endophytes and saprobes of a wide range of hosts worldwide (Rossman *et al*., 2007). Several *Diaporthe* spp. are recognized as the causal agents of many plant disease symptoms, including twig and branch cankers, dieback, shoot blight, root and fruit rots, leaf and pod blights and seed decay (van Niekerk *et al*., 2011; Udayanga *et al*., 2011; Lawrence *et al*., 2015).

Corresponding author: E. Abreo
E-mail: eabreo@inia.org.uy

Whereas many *Diaporthe* spp. have wide or rather specific host ranges (Udayanga *et al*., 2011, Gomes *et al*., 2013), the same host species may sometimes be colonized by diverse *Diaporthe* spp. at the same time (van Niekerk *et al*., 2005; Thompson *et al*., 2011). In fruit trees, *D. amygdali* (Delacroix) Udayanga, P.W. Crous & K.D. Hyde (syn: *Phomopsis amygdali* (Delacr.) J.J. Tuset & M.T. Portilla) has been associated with peach and almond trees showing cankers (Uddin *et al*., 1998; Farr *et al*., 1999), and has also been associated with symptomatic grapevines (van Niekerk *et al*., 2005). Other *Diaporthe* spp. associated with grapevines include: *D. ampelina* (Berk. & M.A. Curtis) R.R. Gomes, C. Glienke & Crous (syn: *Ph. ampelina* (Berk. & M.A. Curtis) Grove), *D. perjuncta* Niessl (Willison *et al*., 1965; Mostert *et al*., 2001; Schil-

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der *et al*., 2005; van Niekerk *et al*., 2005), *D. chamaeropis* (Cooke) R.R. Gomes, Glienke & Crous (syn: *Ph. chamaeropis* (Cooke) Petr.), *D. eres* Nitschke (syn: *Ph. velata* (Sacc.) Traverso), *D. foeniculina* (Sacc.) Udayanga & Castl. (syn: *Ph. foeniculina* (Sacc.) Câmara, *D. nobilis* Sacc. & Speg. (syn: *Ph. laurella* (Sacc.) Traverso), *D. novem* J.M. Santos, Vrandecic & A.J.L. Phillips (Lawrence *et al*., 2015; Cinelli *et al*., 2016) and *D. viticola* Nitschke (Baumgartner *et al*., 2013; Úrbez-Torres *et al*., 2013). *Diaporthe viticola* is also associated with olive trees affected by twig and branch dieback symptoms (Úrbez-Torres *et al*., 2013). *Diaporthe chamaeropis* was recently reported as the causal agent of pistachio shoot blight (Chen *et al*., 2014). *Diaporthe citri* F.A. Wolf, J. is a well-known pathogen associated with melanose and stem-end rot of citrus fruits (Mondal *et al*., 2007), and recently other *Diaporthe* spp. found from stem-end rot disease of *Citrus* spp. were *D. cytosporella* (Penz. & Sacc.) Udayanga & Castl. and *D. foeniculina* (Udayanga *et al*., 2014). *Diaporthe eres* was associated with *Pyrus communis* (Bai *et al*., 2015), and this species has been reported as associated with stem cankers on *Prunus persica* (Prencipe *et al*., 2017). *Diaporthe ambigua* Nitschke was isolated from cankers on rootstocks of apple, pear and plum (Smit *et al*., 1996), and it was also associated with stem canker of blueberry in Chile (Elfar *et al*., 2013). This species, together with *D. australafricana* Crous & Van Niekerk, has been found in association with cordon dieback, the most important disease on kiwifruit in Chile (Díaz *et al*., 2016). In Uruguay, only four species of *Diaporthe* have been identified, in association with disease symptoms on taxonomically unrelated crops. These are *D. amygdali*, causing twig and shoot blight in peach and nectarine (Alvarez *et al*., 2014), *D. neoviticola* (Sacc.) Udayanga, PW Cous & KD Hyde (syn: *Ph. viticola* (Sacc.) Sacc.) associated with grapevine trunk disease (Abreo, 2011), *D. eres* reported as *Ph. cotoneastri* causing reddish cankers in apple trunks (Abreo *et al*., 2012), and *D. phaseolorum* var. *sojae*, var. *caulivora*, and var. *meridionalis* associated with stem canker of soybean (Stewart, 2015).

In spite of these records, systematic studies of *Diaporthe* in deciduous fruit trees are scarce. In Uruguay, the proximity to each other of apple (*Malus domestica* Borkh.), peach (*Prunus persica* (L.) Batsch.) and pear (*Pyrus communis* (L.) Ehrh.) orchards provides opportunity to study the interactions among these hosts regarding the presence of pathogenic fungi they might have in common. Accordingly, the main aims of this study were to identify the species of *Diaporthe* associated with wood disease symptoms of apple, pear and peach trees in closely associated orchards, and to evaluate the susceptibility of these hosts in cross inoculations with the identified pathogenic fungal species.

Materials and methods

Sampling and isolation of fungi

Isolates were recovered from several cultivars of apple, pear and peach trees from five fruit producing areas in Uruguay during the same survey reported by Sessa *et al.* (2016). Most of the orchards were in the south of the country, and a few were in the warmer northern territories, and all were run using integrated pest management strategies. Wood samples with symptoms in shoots and branches such as cankers, gummosis, dead shoots and shoot blights were collected (Figure 1). In total 96 symptomatic trees were sampled from 21 orchards: 39 trees from eight apple orchards, 41 from nine peach orchards and 16 from four pear orchards. From each sample, bark was removed and symptomatic wood was surface-disinfected by sequential immersion in 70% ethanol for 60 s and 4% NaOCl for 90 s, then washed with distilled sterile water and dried with sterilized paper. Xylem pieces (2 × 2 mm) were excised from the margins between necrotic and healthy tissue and placed on 2% potato dextrose agar (PDA) amended with lactic acid (pH 4.5). Plates were incubated at 25°C under 12 h light/12 h dark periods until fungal growth was detected. Subcultures were made from the growing hyphae onto fresh 2% PDA plates and these were incubated under the same conditions. Isolates showing *Diaporthe* spp. cultural characteristics, including white, creamy or grayish aerial mycelia with dark pycnidia and presence of alpha and/or beta conidia, were selected for morphological and DNA sequence analyses. Morphotypes were assigned based on macro-morphology of the colonies, and the presence of alpha and/or beta conidia observed with an optical microscope (Table 1).

Molecular characterization and phylogeny of isolated fungi

Preliminary identification of isolates was performed using cultural, micro- and macro-morphological characteristics (Table 1). All isolates were

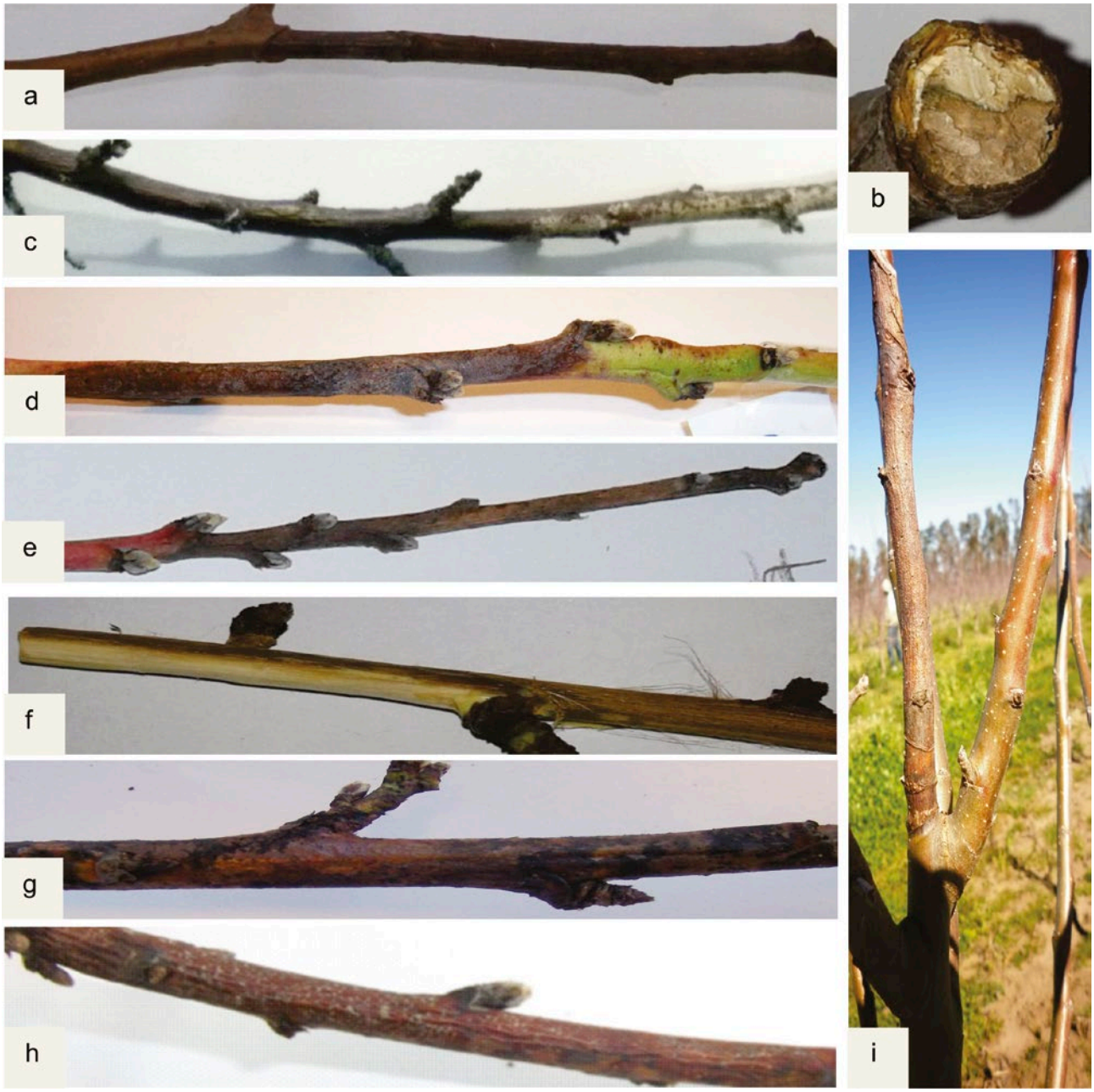


Figure 1. Disease symptoms observed on twigs and branches of pear (a-b), peach (c-h) and apple (i) trees in Uruguayan orchards. (a) apical dead shoot (*Diaporthe phaseolorum*, *D. terebinthifolii*); (b) Wedge-shaped necrosis (*D. infecunda, D. eres*); (c) whitish dead twig; (d) shoot blight with gummosis; (e) apical dead shoot (*D. amygdali*); (f) Internal necrosis (*D. oxe*); (g) black and reddish necrosis (*Diaporthe* sp. 1); (h) reddish necrosis (*Diaporthe* sp. 1); (i) reddish sunken canker (*D. eres*, *D. foeniculina*).

stored in the culture collection of the Mycology Laboratory of the Universidad de la República (UdelaR), in PDA plugs immersed in water and maintained at 4ºC.

Fungal genomic DNA was extracted from mycelium of pure fungal colonies after 10–14 d of growth on PDA, according to Lee and Taylor (1990). The integrity of the DNA was evaluated by electrophoresis

Table 1. Micro and macroscopic characteristics of *Diaporthe* morphotypes.

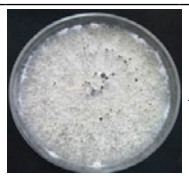
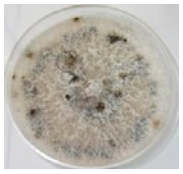
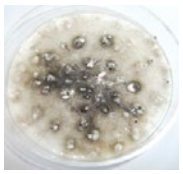
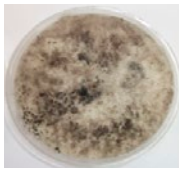
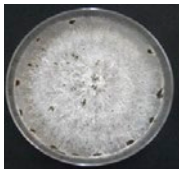
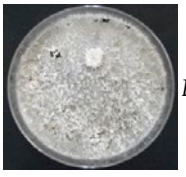
Morphotype		Species	Origin	Culture characteristics Top / reverse	Conidium morphology alpha and/or beta
Number	Colony morphology				
1		*D. foeniculina*	*Malus domestica*	White / white	alpha and beta
2		*D. infecunda*	*Pyrus communis*	grayish / pale brown	Sterile
3		*D. amygdali*	*Prunus persica*	creamy / pale brown	alpha
4		*D. eres*	*Pyrus communis* *Malus domestica*	grayish	alpha and beta
5		*D. terebinthifolii*	*Pyrus communis*	whitish brown / pale brown	beta
6		*D. oxe* *Diaporthe* sp. 1	*Pyrus communis* *Prunus persica*	white / pale brown	alpha and beta
7		*Diaporthe* sp. 4	*Pyrus communis*	white / white	alpha
8		*D. phaseolorum*	*Pyrus communis*	white / white	beta

Table 2. Isolates of *Diaporthe* spp. recovered in this study, their host origins, and species obtained from GenBank included in the phylogenetic analyses of this study.

Species	Isolate	Host		GenBank No.	
		Species	Origin	ITS	EF-1α
Diaporthe ampelina	STE-U2660*	*Vitis vinifera*	France	AF230751	
	V176	*Vitis vinifera*	Uruguay	KX833230	
	V211	*Vitis vinifera*	Uruguay	KX833231	
D. amygdali	CBS126679*	*Prunus dulcis*	Portugal	KC343022	KC343748
	CBS 111811	*Vitis vinifera*	South Africa	KC343019	
	Fi2302	*Prunus persica*	Uruguay	KR002808	
	Fi2305	*Prunus persica*	Uruguay	KR002811	
	Fi2307	*Prunus persica*	Uruguay	KR002812	
	Fi2308	*Prunus persica*	Uruguay	KR002813	
	Fi2304	*Prunus persica*	Uruguay	KR002810	
	Fi2303	*Prunus persica*	Uruguay	KR002809	
	Fi2310	*Prunus persica*	Uruguay	KR002814	KX986877
D. citri	CBS 230.52	*Citrus sinensis*	Suriname	KC343052	
D. eres	CBS439.82*	*Cotoneaster* sp.	United Kingdom	FJ889450	GQ250341
	Fi2333[a]	*Malus domestica*	Uruguay	KR023623	KX986881
D. foeniculina	CBS123208*	*Foeniculum vulgare*	Portugal	KC343104	KC343830
	CBS111554	*Foeniculum vulgare*	Portugal	KC343102	
	Fi2340	*Malus domestica*	Uruguay	KR023630	KX986880
D. infecunda	CBS133812*	*Schinus terebinthifolius*	Brazil	KC343126	KC343852
	Fi2335[a]	*Pyrus communis*	Uruguay	KR023625	na
Diaporthe oxe	CBS133186*	*Maytenus ilicifolia*	Brazil	KC343164	KC343890
	CBS 133187	*Maytenus ilicifolia*	Brazil	KC343165	
	LGMF945	*Maytenus ilicifolia*	Brazil	KC343168	
	Fi2337	*Pyrus communis*	Uruguay	KR023627	KX986874
	Fi2338[a]	*Pyrus communis*	Uruguay	KR023628	KX986875
	Fi2343	*Prunus persica*	Uruguay	KR023633	
D. phaseolorum	CBS127465	*Actinidia chinensis*	New Zeland	KC343177	KC343903
	CBS116019	*Caperonia palustris*	U.S.A	KC343175	
	Fi2334[a]	*Pyrus communis*	Uruguay	KR023624	KX986878
Diaporthe sp. 1	**Fi2341**	*Prunus persica*	Uruguay	KR023631	KX986873
	Fi2342[a]	*Pyrus communis*	Uruguay	KR023632	
Diaporthe sp. 4	**Fi2339**	*Pyrus communis*	Uruguay	KR023629	KX986879

(Continued)

Table 2. (Continued).

Species	Isolate	Host		GenBank No.	
		Species	Origin	ITS	EF-1α
D. terebinthifolii	CBS133180*	*Schinus terebinthifolius*	Brazil	KC343216	KC343942
	LGMF907	*Schinus terebinthifolius*	Brazil	KC343217	
	Fi2336	*Pyrus communis*	Uruguay	KR023626	KX986876
D. vaccinii	CBS160.32*	*Oxycoccus macrocarpos*	U.S.A	AF317578	KC343954

Bold letters indicate isolates from this study.
* Indicates ex-type
[a] Correspond to isolates used in the pathogenicity trials

in 1.0% agarose gels in 1× Tris-borate-EDTA (TBE) buffer, stained with EZ vision®One (Amresco®), and visualized under UV light transillumination. Polymerase chain reactions (PCR) were performed to amplify the internal transcribed spacer (ITS) regions ITS1 and ITS2, including the 5.8S subunit, using primers ITS5 and ITS4 (White *et al.*, 1990). Part of the translation elongation factor 1-alpha gene (EF1-α) was also amplified using the primers EF1-728F and EF1-986R (Carbone and Khon 1999). Conditions for each PCR reaction were as described by the authors cited above. PCR mix of 25 µL comprised 2 µL of genomic DNA (10 ng), 2.5 µL of 10× PCR buffer, 2.5 µL dNTPs (2.5 mM), 0.75 µL $MgCl_2$ (50mM), 0.5 µL of each primer (10 µM), 0.25 µL 1 U Taq DNA polymerase (Thermo Fisher Scientific) and 16 µL of DNAse-free sterile water.

PCR products were verified by electrophoresis in 1.0% agarose gels in TBE buffer, that were stained with EZ vision®One (Amresco®) and visualized under UV light transillumination. PCR products were purified and sequenced by Macrogen. Sequences were submitted to GenBank (Table 2).

Sequences obtained were assembled and manually corrected using MEGA version 6 software (Tamura *et al*., 2013). Phylogenetic analyses of the newly generated sequences and reference sequences from GenBank were performed by maximum parsimony with PAUP 4.0b10 (Swofford, 2002), using the heuristic search option with simple taxa addition and tree bisection, and with reconnection used as the branch-swapping algorithm.

The ITS phylogenetic analysis was performed for 18 representative isolate sequences of the eight morphotypes obtained. A concatenated analysis of ITS and EF1-α was included, which comprised the sequences of the strains whose identity could not be defined with ITS. The phylogenetic tree based solely on the EF1-α sequences is deposited in TreeBase with accession code 21669.

All characters were treated as unordered and of equal weight. Support of the nodes was determined by analysis of 1,000 bootstrap replicates (Hillis and Bull, 1993). Tree length (TL), consistency index (CI) and retention index (RI) were recorded. Alignments and trees were submitted to TreeBase (codes 20170 and 20171).

Pathogenicity trials

Isolates included in the preliminary pathogenicity screening trial on detached shoots in the laboratory are shown in Table 2. These include *D. infecunda* (Fi2335), *D. oxe* (Fi2338), *D. phaseolorum* (Fi2334), *D. eres* (Fi2333) and *Diaporthe* sp. 1 (Fi2341). *Diaporthe foeniculina* Fi2340 and *Diaporthe* sp. 4 Fi2339 were not included, as they were not identified until later. *Diaporthe amygdali* was not included as its pathogenicity is well documented. One isolate of each identified species was inoculated in 1-y-old detached shoots of apple (cv. Red Chief), peach (cv. Dixieland) and pear (cv. Williams). Each fungal isolate was used to inoculate five shoots of each host species. Shoots were cut into 20 cm long segments, rinsed with tap water, surface disinfected with 70% ethanol for 60 s and dried with sterile paper. A superficial wound was made on an intermediate internode of each shoot using a disinfected blade. A 4-mm mycelium/agar plug from a 1-week-old culture was placed in the wound and wrapped with parafilm. Non-colonized

sterile agar plugs were inoculated as controls. Shoots were placed into sterile nylon bags in a randomized block design consisting on three blocks or incubation chambers. Shoots were incubated at 25°C under black light in moist chambers (RH>90%). Twenty d post inoculation, the number of shoots with necrosis was recorded, necrotic bark was removed and the lesion lengths observed on xylem were measured. Fungi were re-isolated by plating surface disinfected symptomatic pieces of wood on 2% PDA amended with lactic acid (pH 4.5). Plates were incubated at 25°C under 12 h light/12 h dark periods until growth was detected. Fungal identity was confirmed by molecular identification, using the protocols for DNA extraction and PCR conditions described above.

A second pathogenicity test using selected *Diaporthe* isolates from the previous trial was conducted under field conditions on 1-y-old shoots of apple (cv. Red Chief), peach (cv. Dixieland) and pear (cv. Williams) trees, during their dormant period. Only those isolates that produced lesions in the preliminary laboratory test were selected for the field trial. *Diaporthe infecunda* and *D. phaseolorum* were not included in the pear trial, because they did not cause any symptoms in this host in the favourable conditions of the laboratory trial. *Diaporthe* sp. 1 was not included in the pear or the peach trial for the same reason. Ten dormant shoots from the last year growth (one shoot per tree) were inoculated with each fungal isolate during winter of 2014. For each inoculation, a superficial wound was made on the shoot internode using a disinfected blade. A 4 mm diam. mycelium agar plug from a 1-week-old culture was placed in the wound and wrapped with parafilm. Non-colonized agar plugs were inoculated as controls. Shoots were inspected for lesion development 30 d post-inoculation; peach shoots were removed at day 30 when shoot necrosis was evident, placed in nylon bags and brought to the laboratory for immediate analysis. Apple and pear shoots were collected 45 d after inoculation, when shoot necrosis was evident in these two species. The number of shoots with lesions was recorded, and the extension of the lesions was measured. Fungal re-isolation and identification were performed as described in laboratory trial.

Data analyses

Lesion length measurements of all shoots showing necrosis were subjected to Kruskal–Wallis test (P=0.05) and Mann-Whitney (P=0.05) using PAST (Hammer *et al*., 2001) to assess statistically significant differences between treatments for each host species, as the data did not follow normal distributions.

Results

Morphological identification of fungi

One hundred and seventy-six isolates showed typical *Diaporthe* spp. cultural characteristics, including white, creamy or grayish aerial mycelia with dark pycnidia and the presence of alpha and/or beta conidia. Alpha conidia were each one-celled, hyaline, fusiform to globose and aseptate, the beta conidia were one-celled, hyaline, aseptate and filiform with straight or curved ends, while no gamma conidia were observed. Among these isolates eight morphotypes were described (Table 1).

Molecular characterization and phylogeny of isolated fungi

The phylogenetic analysis of ITS sequences included 19 isolates of *Diaporthe* representing the eight morphotypes from this study, and 19 obtained sequences from GenBank (Table 2). Of 536 total characters, 405 were constant, 99 were parsimony informative and 32 were variable non-informative. After the heuristic search, one hundred most parsimonious trees were retained (TL = 253, CI = 0.6759 RI = 0.8869). One most parsimonious tree showed six well-supported groups (Figure 2).

The first group was formed by *D. ampelina* (STE-U2660) together with sequences of isolates obtained from *Vitis* in Uruguay from a previous study. None of the sequences generated in the present study fell in this clade. The second group included Fi2341 and Fi2342 that formed a highly supported clade (99.9% bootstrap) next to the *D. foeniculina* cluster (97.9% bootstrap), but without any ex-type sequences from GenBank. Therefore these could not be assigned to any known species. Isolate Fi2340 formed the third clade with *D. foeniculina* ex-type (98.2% bootstrap), while the fourth clade comprised isolate Fi2333 and *D. eres* ex-type sequence (99.8% bootstrap). The fifth group comprised several sub-clades formed by sequences such as Fi2334 which clustered with *D. phaseolorum* (92.1% bootstrap support), Fi2335 which clustered with *D. infecunda* ex-type sequence (97.9% bootstrap support), Fi2336 which clustered with *D.*

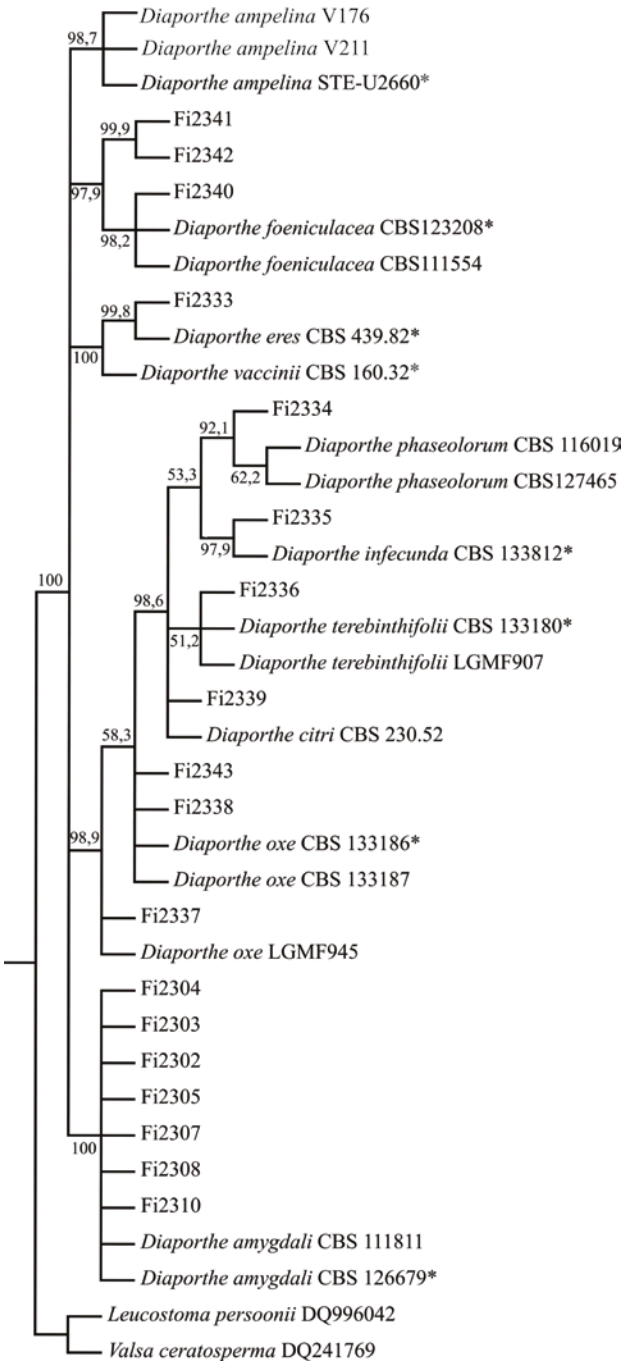


Figure 2. One of 100 most parsimonious trees resulting from the analysis of the internal transcribed spacer (ITS) sequences from fungal isolates. Maximum-parsimony bootstrap support values based on 1,000 replicates are shown in the nodes. Ex-type isolates are highlighted with an asterisk. The tree is rooted with *Leucostoma persooni* (DQ996042) and *Valsa ceratosperma* (DQ241769) isolates.

terebinthifolii (51.2% bootstrap support), and Fi2343, Fi2338 and Fi2337 which clustered with *D. oxe* sequences including the ex-type, with 58.3% bootstrap support. Due to the low support values of some of these clades, the identity of some isolates could not be unequivocally determined. The last group comprised seven isolates that formed a clade with sequences of *D. amygdali* including the ex-type (100% bootstrap support).

A phylogenetic analysis was performed using the concatenated sequences of ITS and EF1-α sequences of the *Diaporthe* isolates of the fifth group that could not be fully resolved in the ITS phylogeny. Sequences of several isolates already identified in the ITS phylogeny were also added for species confirmation. Of 814 total characters, 548 were constant and 228 were parsimony informative. After a heuristic search, four most parsimonious trees were retained (TL = 542, CI = 0.7177, RI = 0.8090). One of the most parsimonious trees is shown (Figure 3). Isolate Fi2336 clustered with *D. terebinthifolii* (100% bootstrap support), isolate Fi2335 with *D. infecunda* (100% bootstrap support) and isolate Fi2334 with *D. phaseolorum* (98.2% bootstrap support). Isolates Fi2337 and Fi2338 clustered with *D. oxe* with 100% bootstrap support. Isolates Fi2339 (*Diaporthe* sp. 4) and Fi2341 (*Diaporthe* sp. 1) did not cluster with any sequence retrieved from GenBank.

Isolate Fi2340 grouped with the ex-type sequence of *D. foeniculina* (100% bootstrap support), Fi2301 grouped with an ex-type CBS126679 sequence of *D. amygdali* (100% bootstrap support) and isolate Fi2333 clustered with a sequence of *D. eres* (99.9% bootstrap support), confirming the species identifications.

Fungi and host symptoms

Symptoms observed in the field survey included reddish cankers on twigs and branches, cankers with gummosis, dead shoots and twigs, internal wood necrosis and shoot blights (Figure 1). Eleven percent of the *Diaporthe* isolates (19 isolates) were obtained from apple, 40% (71 isolates) from pear and 49% (86 isolates) were obtained from peach.

Diaporthe amygdali came exclusively from peach twigs showing shoot blight symptoms, sometimes with gummosis (Figures 1c, 1d and 1e). This species represented 95% of the *Diaporthe* isolates from peach. *Diaporthe oxe* was also found in peach shoots associated with internal necrosis (Figure 1f) and *Diaporthe*

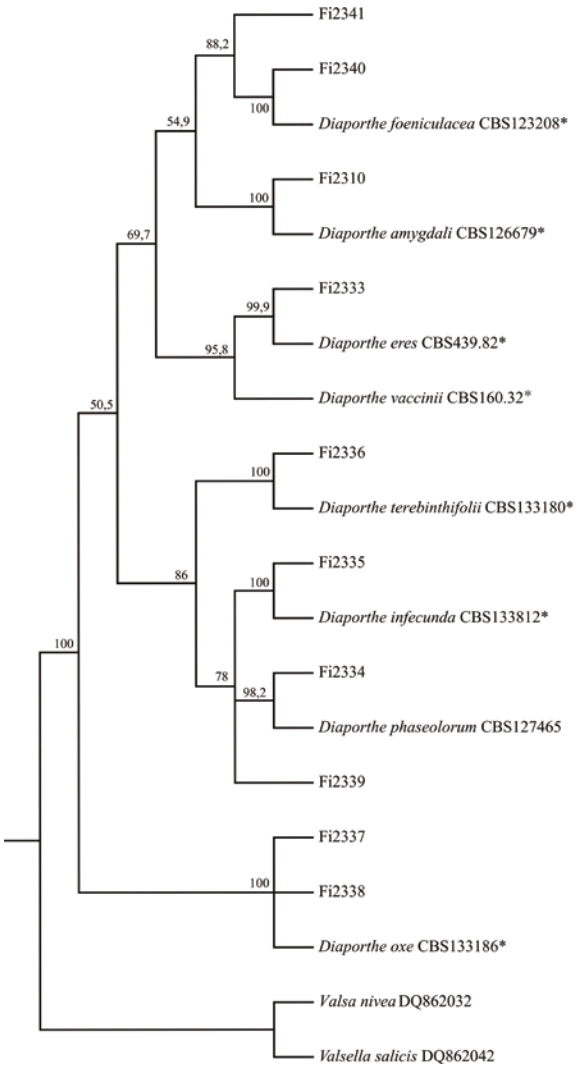


Figure 3. One of four most parsimonious trees resulting from the analysis of sequences of combined ITS and partial elongation factor 1 α (EF1-α) from fungal isolates. Maximum-parsimony bootstrap support values based on 1,000 replicates are shown in the nodes. Ex-type isolates are highlighted with an asterisk. The tree is rooted with *Valsa nivea* (DQ862032) and *Valsella salicis* (DQ862042) isolates.

sp. 1 was also found in peach shoots associated with reddish necrosis (Figure 1h).

Diaporthe phaseolorum and *D. terebinthifolii* were isolated from wood samples of pear branches associated with apical dead shoots (Figure 1a). *Diaporthe infecunda* and *D. eres* were found associated with wedge-shaped necrosis on pear branches (Figure 1b). *Diaporthe eres* was isolated from apple branches showing reddish sunken cankers, representing 53% of the *Diaporthe* spp. obtained in this fruit species, and *D. foeniculina* represented 18% of the isolates from apple tree lesions (Figure 1i).

Pathogenicity trials

The laboratory test showed that all inoculated isolates produced lesions in at least two hosts which were larger ($P \leq 0.05$) than those from the controls. Only *D. eres*, *D. oxe and D. infecunda* produced lesions that differed from the controls in all three hosts. *Diaporthe eres* produced the largest lesions observed on any host, followed by *D. phaseolorum* and *D. infecunda,* both of which produced equally extensive lesions in peach and apple shoots. Although *D. oxe* was also pathogenic on the three hosts, the necroses from this fungus in apple and pear shoots were significantly shorter than those from *D. eres* (Table 3).

Diaporthe infecunda, *D. phaseolorum and Diaporthe* sp. 1 were not selected for the field inoculation on pear shoots, since they failed to produce consistent symptoms on this host in the favorable conditions of the laboratory trial. In addition, *Diaporthe* sp. 1 was excluded for the field inoculation of peach shoots for the same reason.

In the field inoculation, only *D. eres* and *D. phaseolorum* inoculated in apple and peach shoots produced lesions that were larger ($P \leq 0.05$) than the lesions in the respective control shoots. Notably, *D. eres* and *D. oxe*, the only isolates selected in the preliminary laboratory trial to be inoculated on the three hosts, failed to produce symptoms in pear shoots in the field (Table 4).

Necroses produced in peach shoots were larger than the necroses produced by the same isolates in apple and pear shoots (Table 4, Figure 4). The four species inoculated in peach induced dark-brown to reddish cankers and gummosis, as shown in Figures 5A and 5B. On apple, the symptoms produced were brown depressed cankers cracked at the margins, sometimes showing pycnidia (Figures 5c and 5d).

The number of shoots with positive re-isolation of inoculated fungi was erratic in the laboratory experiment, except for *D. eres* which was re-isolated from all shoots from the three hosts. In the field inoculation, all five *Diaporthe* spp. were re-isolated from the margins of necrotic tissues of the inoculated hosts, while no fungi were isolated from control shoots (Tables 3 and 4).

Table 3. Numbers of shoots with lesions after artificial inoculation of five *Diaporthe* spp. on detached shoots of peach, apple or pear trees, mean lesion length and numbers of shoots with positive re-isolation of inoculated isolates.

Fungal species	Isolate	Number of shoots with lesions			Mean of lesion length (mm)[a]			Number of shoots with positive re-isolation		
		Peach	Apple	Pear	Peach	Apple	Pear	Peach	Apple	Pear
Diaporthe infecunda	Fi2335	5	5	3	14.8 ± 10.31 ab	10.9 ± 1.52 ab	7.3 ± 4.10 c	1	1	2
D. oxe	Fi2338	5	5	5	14.1 ± 2.39 a	9.0 ± 1.17 b	8.4 ± 1.14 b	2	4	3
D. phaseolorum	Fi2334	5	5	0	14.6 ± 9.76 ab	15.8 ± 2.17 a	0 c	3	2	0
D. eres	Fi2333	5	5	5	30.8 ± 15.04 a	15.2 ± 2.66 a	27.2 ± 13.70 a	5	5	5
Diaporthe sp. 1	Fi2341	5	5	0	9.6 ± 0.55 b	24.2 ± 7.86 a	0 c	2	5	0
Control		0	0	0	0 c	0 c	0 c	0	0	0

[a] Values are the mean of five replicates ± SD. Different letters in each column indicate significantly different ($P \leq 0.05$) according to Mann-Whitney tests.

Table 4. Numbers of shoots developing lesions after artificial inoculation of five *Diaporthe* spp. inoculated on branches of peach, apple or pear trees, mean lesion lengths and numbers of shoots with positive re-isolation of inoculated isolates.

Fungal species	Isolate	Number of shoots with lesion			Mean of lesion length (mm)[a]			Number of shoots with positive re-isolation		
		Peach	Apple	Pear	Peach	Apple	Pear	Peach	Apple	Pear
Diaporthe infecunda	Fi2335	10	10	ND	15.1 ± 3.98 ab	11.0 ± 3.54 abc	ND	9	8	ND
D. oxe	Fi2338	10	10	10	15.0 ± 3.77 ab	8.3 ± 0.98 bc	8.2 ± 1.86 a	6	10	5
D. phaseolorum	Fi2334	10	10	ND	17.2 ± 2.04 a	11.5 ± 1.66 b	ND	10	10	ND
D. eres	Fi2333	10	10	10	26.5 ± 9.34 a	31.0 ± 5.44 a	9.6 ± 1.64 a	8	9	7
Diaporthe sp. 1	Fi2341	ND	10	ND	ND	8.4 ± 0.91 bc	ND	ND	10	ND
Control		6	1	0	8.8 ± 2.04 b	6.4 ± 0.97 c	8.2 ± 1.03 a	0	0	0

[a] Values are the mean of ten replicates ± SD. Different letter in the same column are significantly different ($P \leq 0.05$) according to Mann-Whitney.
ND, not determined.

Discussion

This study is the first comprehensive attempt to identify and assess the pathogenicity of *Diaporthe* spp. associated with wood disease symptoms in pear, apple and peach trees, and to evaluate the risks of reciprocal host infections by these fungi.

In agreement with previous reports, precise identification of *Diaporthe* spp. required multigene phylogenetic analyses, using ITS and TEF-1α gene datasets (Baumgarthner *et al*., 2013; Urbez-Torres *et al*., 2013; van Niekerk *et al*., 2005). This approach revealed the presence of nine *Diaporthe* spp., including two unknown species (*Diaporthe* sp. 1 and sp. 4), associated with wood disease symptoms of apple, pear and peach trees. These fungi included *D. amygdali*, *D. foeniculina*, *D. infecunda*, *D. eres*, *D. terebinthifolii*, *D. oxe* and *D. phaseolorum.*

Diaporthe infecunda, D. eres, D. terebinthifolii, D. phaseolorum and D. oxe on *Py. communis*, and *D. foeni-*

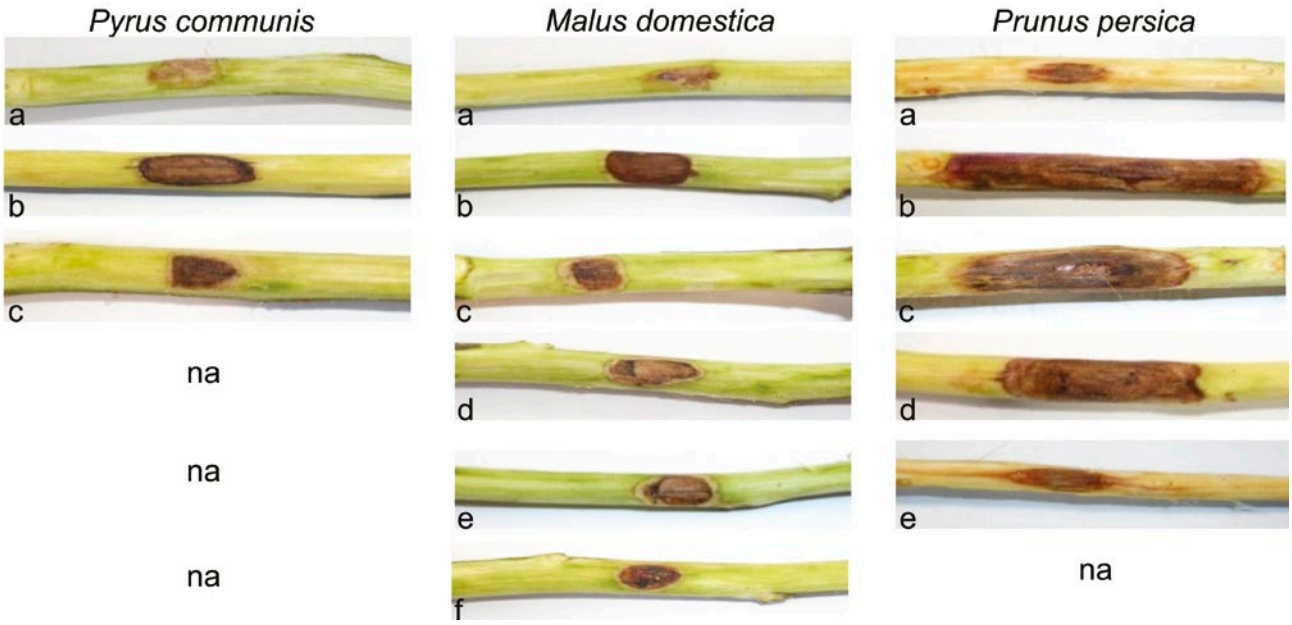


Figure 4. Lesions observed in the xylem of pear, apple or peach branches after the field inoculation trial caused by (a) Control; (b) *Diaporthe eres*; (c) *D. oxe*; (d) *D. infecunda*; (e) *D. phaseolorum*; (f) *Diaporthe* sp. 1; na: not available.

culina on *Malus domestica* represent new records in these hosts in Uruguay, while *D. oxe* isolated from *Pr. persica* is a new record for this species.

The genus *Diaporthe* comprises species that are host-specific, and species with broad host ranges. Pathogenic species are often reported as host-specific, whereas species with wide host range are mostly opportunistic pathogens or saprobes (Udayanga *et al*., 2014). None of the species recorded in the present study were isolated from symptomatic tissue in the three hosts, although some species occurred in more than one host, as was the case for *D. eres* and *D. oxe*. While *D. eres* and *D. oxe* are considered as generalists, infecting several hosts, these fungi had different life strategies on the same host trees.

Diaporthe eres isolate Fi2333 obtained from apple cankers showed the greatest virulence in all three inoculated hosts in the laboratory trial, and its virulence was reproduced in the field trial on apple and peach trees. Whereas its pathogenicity in apple had already been demonstrated (Abreo *et al*., 2012), it is now evident that this species can also be ranked among the most virulent in peach trees. Although in the field trial lesions from *D. eres* did not differ from control shoots in pear trees, this species was isolated both from apple and pear diseased trees. Bai *et al*. (2015) isolated *D. eres* from *Py. communis* and from *Malus* sp. in China, and showed that one isolate obtained from *Malus* was pathogenic when inoculated into *Py. communis*. Altogether, these findings suggest that, despite differences among isolates, *D. eres* is pathogenic to these three species, and there is risk of reciprocal infections by this fungus.

Similarly to *D. eres*, *D. oxe* was also isolated in this study from two hosts. *Diaporthe oxe* is a recently described species, which was reported as an endophyte from medicinal plants in Brazil (Gomes *et al*., 2013), so its pathogenicity was unknown. From the pathogenicity trials conducted in the present study, *D. oxe* isolate Fi2338 produced lesions in the three hosts, with intermediate virulence on detached shoots. However, lesions observed on the three hosts in the field experiment were not significantly larger than lesions in the controls, so this fungus could be considered as a saprobe.

Some species were often associated with specific host species and specific symptoms, as was the case for *D. amygdali*. This species has been previously reported worldwide as the causal agent of peach shoot blight (Farr *et al*., 1999), and has also

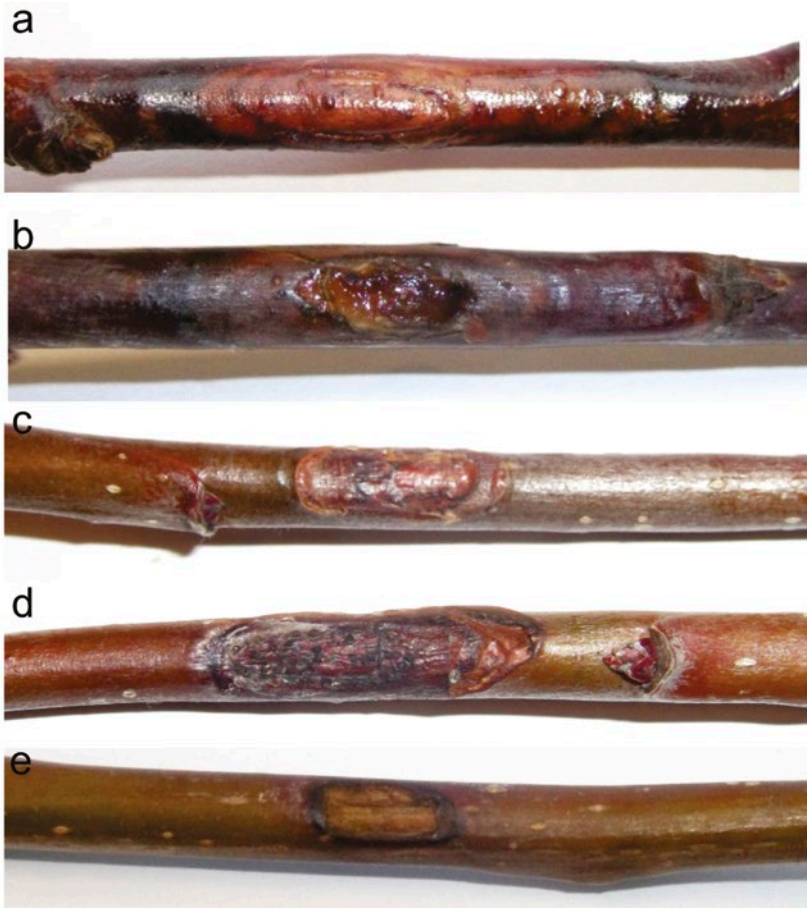


Figure 5. Symptoms observed on the cortices of branches of peach (a-b), apple (c-d) and pear (e) after the field inoculation trial. (a) reddish canker; (b) dark-brown canker with gummosis; (c) brown depressed canker cracked at the margins; (d) pycnidia on brown depressed canker cracked at the margins; (e) necrotic lesion.

been reported in Uruguay (Alvarez *et al*., 2014). The present study confirms its association with this typical symptom. Similarly in this study, *D. phaseolorum* was isolated together with *D. terebinthifolii* only from pear twigs. In the laboratory pathogenicity trial, *D. phaseolorum* isolate Fi2334 was non-pathogenic on its original host, which could indicate that this fungus was present as an opportunistic saprobe. Nonetheless, this isolate of *D. phaseolorum* was pathogenic on apple and peach twigs, both in the laboratory and field pathogenicity trials, so infected pear trees could be considered as sources for this pathogenic species. *Diaporthe infecunda* has long been known as the causal agent of pod and stem blight and stem canker in soybean, and it can also cause seed rot (Pioli *et al*., 2003). In the present study it was obtained from pear branches showing wedge-shaped necroses, and was of high to moderate virulence on apple, peach and pear shoots when inoculated in detached shoots. However, since its pathogenicity on peach and apple was not confirmed in the field trial, *D. infecunda* can also be considered a saprobe. *Diaporthe foeniculina* was found associated with reddish sunken cankers on apple trees. This species is known to have a wide host range, including *Citrus, Malus, Prunus* and *Pyrus* (Udayanga *et al*., 2014), and causes shoot blight and canker diseases of kiwifruit in Greece (Thomidis *et al*., 2013). Since isolates of *D. foeniculina* were not included in the pathogenicity trials, its virulence has not been verified.

Pyrus communis harboured the greatest diversity of *Diaporthe* spp., being associated with seven spe-

cies. Nonetheless, the pathogenicity trials showed that this host was the least susceptible to disease caused by these fungi. On the other hand, *Malus domestica* was the host with the least diverse *Diaporthe* population, as it was associated with only two species, one of which had already been reported in Uruguay (Abreo *et al*., 2012). Nevertheless, *M. domestica* was susceptible to *D. eres* and *D. phaseolorum* in the field pathogenicity trial. This apparent contradiction suggests that pear trees are more tolerant to *Diaporthe*. As no protective or cultural measures would be taken against these potential pathogens, pear trees could act as reservoirs of the majority of the identified fungal species. On the contrary, as apple trees are more susceptible to *Diaporthe* spp., they are probably subjected to control measures that reduce pathogen diversity. Furthermore, the removal of dead branches may prevent their sampling in surveys that concentrate on symptomatic host tissues.

In the case of peach trees, the number of *Diaporthe* spp. isolated was intermediate to those isolated from pear or apple, while the species that caused necroses in the field trial were the same as in apple. However, the most notorious feature was that length of lesions was much greater in peach shoots than in pear or apple. Therefore, peach trees should be considered the most susceptible host to pathogenic *Diaporthe* spp.. Of the *Diaporthe* spp. tested in the field experiment, the isolates of *D. eres* and *D. phaseolorum* were capable of producing symptoms in both apple and peach shoots. This suggests that these fungi have the potential to cause disease in these alternative host species as long as host jumps occur.

In conclusion, several wood disease symptoms in apple, pear and peach trees were described, and nine species of *Diaporthe* were found associated with these symptoms in orchards in close proximity to one another. *Diaporthe eres* and *D. phaseolorum* can be considered the species posing the greatest risks due to their distribution and pathogenicity in at least two fruit tree hosts. Orchards of the three hosts examined can be considered potential reciprocal sources of these pathogenic *Diaporthe* spp.. While peach and apple trees were more susceptible, pear trees should be considered as reservoirs of several of the identified species of *Diaporthe*, including *D. eres* and *D. phaseolorum*. Disease management strategies should therefore be deployed to address the threats that these pathogens pose to fruit production.

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