Brown spot of pear, an emerging disease in Argentina: identification and pathogenicity characterization of Argentinean *Stemphylium vesicarium* isolates

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Abstract Recently, a new plant disease was detected in the rural area of the High Valley of Río Negro, in Northern Patagonia, Argentina, called "Brown Spot of Pear" (BSP), caused by *Stemphylium vesicarium* (teleomorph: *Pleospora herbarum*, syn. *Pantoea allii*) in its sexual and asexual stages which produces lesions on leaves and fruits. BSP has been reported previously in pear growing areas of Europe and Japan where research studies have assessed the production of two host-specific toxins (HSTs) in culture filtrates of *S. vesicarium* (SV-I and SV-II toxin). The purpose of the present study was to characterize *Stemphylium* isolates from air and from brown spot lesions on pear leaves and fruits grown in this rural area, at a morphological, molecular and pathogenic level. The pathogen's identity was determined as *S. vesicarium* and its aggressiveness and toxicity were demonstrated through conidia and culture filtrate bioassays showing different degrees of pathogenicity. D'Anjou and Abate Fetel were the most susceptible pear cultivars to *S. vesicarium* isolates.

Keywords Brown spot of pear · *Stemphylium vesicarium* · SV-toxins · Emerging disease · Climate change

Introduction

The High Valley of Rio Negro is a rural zone located in Northern Patagonia, Argentina, and it is the main production region of pome fruits in the country. The most

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important pear cultivars grown in this valley are, in order of abundance, Williams Bon Chrétien, Packham's Triumph, Beurré D'Anjou and Abate Fetel. In the last few years, changes in climatic conditions have been observed in this area such as a considerable increase in rainfall and humidity (Nördenstrom, 2006; Rodriguez, 2013a, 2013b; Rodriguez, 2014a, 2014b). Several authors report that changes like increased temperatures and the quantity and pattern of precipitation, are expected to affect pathogen development with a resulting impact on crop diseases (Coakley et al., 1999; Elad & Pertot, 2014; Moragrega et al., 2018). In January 2013, a new pathology was detected in this rural valley, called "Brown Spot of Pear" (BSP), caused by *Stemphylium vesicarium* (Wallr.) E.G. Simmons (teleomorph: *Pleospora herbarum* [Pers.] Rabenh, syn. *Pantoea allii* [Rabenh.] Ces. & De Not.). Symptoms were observed on leaves and fruits of pear trees of cv. Beurré D'Anjou (Dobra & Garcia, 2015). The disease was detected again during the fruit growing season in 2016 and after the fruit harvest period in 2017 on cultivars Beurré D'Anjou, Abate Fetel and, on a few occasions, on Williams Bon Chrétien and Packham's Triumph cultivars. The presence of the sexual and asexual stages of the pathogen was also confirmed (Di Masi, 2017).

This economically important disease in pear production occurs from spring to harvest and it has been previously reported in several pear-growing areas in Europe and Japan (Llorente et al., 2012; Llorente & Montesinos, 2006; Tanahashi et al., 2008). To study the pathogenicity of the causal agent and the host susceptibility, tests included some of the cultivars grown in this valley such as Abate Fetel, which was one of the most susceptible cultivars, and Williams Bon Chrétien and Packham's cultivars which have shown moderate or no susceptibility to the disease (Cavanni & Ponti, 1994; Llorente et al., 2012; Llorente & Montesinos, 2006).

It was also reported that *S. vesicarium* produces two host-specific toxins (HSTs) in culture filtrates (SV-I and SV-II toxin). HSTs play an extremely important role in the virulence or pathogenicity of the fungus and they are responsible for the vein necrosis with the specific "V-shaped" on pear leaves. The severity of the necrosis may be related to the toxin concentration and varietal susceptibility (Köhl et al., 2009; Singh et al., 1999) and the toxin production in these cultivars could be induced by

host factors (Otani et al., 1998; Singh et al., 1999). Singh et al. (2000) showed that these toxins exert their action on the plasma membrane by generating a cellular dysfunction that finally leads to the necrosis. SV-toxin production was demonstrated for European (Italy) and Japanese isolates (Singh et al., 1999; Tanahashi et al., 2017).

Although at present the incidence of this disease is incipient, it is extremely important to study this pathogen in the area. In several Mediterranean regions of Europe, a few years after the detection of the disease it progressively extended, becoming an important economic problem. In Europe, this disease causes significant losses in pear-producing areas of Italy (where it was first detected in 1975), The Netherlands and Belgium (Köhl et al., 2009; Llorente & Montesinos, 2006; Polfliet, 2002), France and Spain (Llorente et al., 2012).

The purpose of the present study was to characterize *S. vesicarium* isolates obtained from air and from brown spot lesions on leaves and fruits at a morphological, molecular and pathogenic level in order to evaluate the behavior of Argentinean isolates and their potential production of SV-toxins.

Materials and methods

Sampling area and isolation method: Isolates recovered from plant material were isolated from Beurré D'Anjou pear cultivars from rural settings located in the central and eastern zone of the High Valley of Río Negro during the harvest and post-harvest seasons of 2016–2018 (Online Resource 1). Symptomatic fruits and leaves were superficially disinfected with 0.5% sodium hypochlorite and rinsed in distilled water (DW). Then, small pieces of tissue were placed in Petri dishes containing potato dextrose agar (PDA) and were incubated at 25 °C for 5–7 days (Belisario et al., 2002; Pitt & Hocking, 2009; Pose, 2007). Airborne spores were collected on a previous work from eight rural settings located in the central and eastern zone of the High Valley of Río Negro between autumn 2014 and summer 2017 with a microflow air sampler (Microflow α 90 Aquaria version 3.0.0 cod. G.1015) using Petri dishes containing PDA supplemented with chloramphenicol (0.1 g/L) and dichloran (2 mg/L) which were incubated for 5 days at 25 °C (Temperini et al., 2019) (Online Resource 1). The annual mean count of airborne *Stemphylium* spores in

samples collected was 2 CFU/m3 (CFU: colony forming unit) and its relative frequency among other 27 fungal genera isolated was 0.05% (Temperini et al., 2019).

Morphological identification: After the incubation period, differential count of fungal genera was carried out after microscopic observation and macroscopic analysis of colonies in PDA according to Samson et al. (2000) and Pitt and Hocking (2009) and *Stemphylium* isolates were identified by macroscopic and microscopic observation according to Simmons (1969, 1985). Eleven *Stemphylium* isolates recovered from air (SE1, SE2, SE3, SE4, SE5, SE6, SE7, SE8, SE9, SE10, SE11) and twenty-one *Stemphylium* isolates recovered from plant material were randomly selected to carry out this study. Ten isolates were recovered from brown spot lesions of pear fruits (SF4, SF5, SF8, SF9, SF13, SF19, SF20, SF21, SF23, SF24) and eleven were recovered from brown spot lesions on leaves (SL22, SL35, SL37, SL39, SL40, SL61, SL62, SL63, SL65, SL68, SL70), both from Beurré D'Anjou cultivars. To carry out further tests, all 32 *Stemphylium* isolates were obtained from a single spore culture maintained on PDA. For species identification, all the isolates were inoculated on Petri dishes with V8 agar medium and potato carrot agar (PCA) medium (decoction of 20 g potato and 20 g carrot, and 20 g agar/L) and incubated for 30 days at 15–25 °C under black-blue light to induce sporulation according to the method used by Kurose et al. (2015). The lengths and widths of 50 conidia from PCA agar of each isolate were measured with the calibrated ocular micrometer of an optic microscope (Motic BA210) using a total magnification of 400X.

Molecular methods

Mycelia harvest: Pieces of mycelium from each of the 32 isolates were aseptically taken and placed into a Penicillin vial containing 5 mL of yeast extract with supplements medium (sucrose 15% and yeast extract 2%). Vials were incubated at 25 °C for 48 to 96 h until observation of a mycelium layer free of spores. Then mycelia were dried with Whatman grade 1 paper filter, weighed and stored in Eppendorf tubes at −80 °C until use.

DNA extraction: The deep-frozen mycelia were immersed in liquid nitrogen for one minute and then ground to a fine powder using sterile plastic pylons.

DNA Extraction was performed with the DNeasy Plant Mini Kit following manufacturer's instructions (Qiagen, Intl). Total DNA was quantified with the fluorimeter Qubit 2.0 (Life Technologies, Intl.).

PCR amplifications and sequencing: To obtain resolution at species level, partial gene sequences of the translation elongation factor 1-α gene (EF-1α) were obtained through PCR amplification and sequenced to identify the isolates. PCR amplifications for EF-1α gene were performed using primers EF1-728F: CATCGAGA AGTTCGAGAAGG and EF1-986R: TACTTGAA GGAACCCTTACC (Carbone & Kohn, 1999). Reactions contained a total volume of 40 μl with 20 ng of DNA template, 1X PCR buffer, 0.5 μM of each primer (GBT Oligos, Buenos Aires), 0.2 μM of each dNTP, 2 mM MgCl2, 1%, DMSO, and 0.5 U of *Taq* DNA polymerase (Thermo Fischer Scientific, Invitrogen Argentina S.A.). Reaction tubes were placed in a Multigene Thermal Cycler (Labnet International Cycler) with the following running conditions: initial denaturation step at 95 °C for 8 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 30 s and extension at 72 °C for 1 min, with a final extension step at 72 °C for 5 min. Water was used as negative control. Amplicons were visualized under UV light on a 1% agarose gel with Gel Red. Sequencing of the fragments was performed with EF1-728F primer by Macrogen Inc. (Seoul, Korea).

Phylogenetic analysis: The resulting sequences were compared with other fungal DNA sequences from NCBI's GenBank sequence database using a BLAST search to identify species. Phylogenetic analyses were run using the MEGA 10 program package (Kumar et al., 2018) and *Stemphylium* strain reference sequences used were obtained from GenBank (http://www.ncbi.nlm. nih.gov/genbank/). The species strain numbers, and the EF-1α sequences with their respective GenBank accession numbers are indicated in Table 1. The sequence of *Cercospora beticola*, strain CPC 18813, was used as outgroup to root the tree. The isolates nucleotide sequences were aligned all together with the reference sequences using the Muscle algorithm (Edgar, 2004), and when necessary they were adjusted manually. The phylogenetic study was performed using the Maximum Likelihood analyses with Kimura 2 parameters using Gamma distribution model and treating gaps, and missing data as partial deletion (Greco et al., 2015). The bootstrap values were generated with 1000 replicates, and they were considered significant when they were

higher than 85%. The EF-1α alignment consisted of approximately 257 nucleotide positions.

Aggressiveness and toxicity tests

Plant material and bioassays: Pear cultivars Beurré D'Anjou, Williams Bon Chrétien, Packham's Triumph, and Abate Fetel were used as host plants and the apple cultivars Red Delicious and Cripp's Pink were used as non-host plants in two bioassay experiments. All selected plants belong to the experimental orchard of The National Institute of Agricultural Technology (INTA). Nine isolates, three from air (5SE, 6SE, 10SE), three from leaves (22SL, 62SL, 70SL) and three from fruits (8SF, 20SF, 23SF) were randomly chosen to carry out the bioassays. The aggressiveness of the isolates on host and non-host plants was evaluated by inoculating conidial suspensions on leaves and the host selectivity of toxins on host and non-host plants was determined by inoculating culture filtrates (CF) on leaves (Tanahashi et al., 2017). In both bioassays, results are described as percentage of foliar area affected by necrosis and are organized in levels of aggressiveness or toxicity based on the scale from Tanahashi et al. (2017) as follows: (−): no necrosis, 0%; (±): a small amount of necrosis around the inoculation point; 0% < necrosis≤5%; (+); 5% < necrosis≤25%; (++); 25% < necrosis≤50%; (+++); 50% < necrosis≤100%. According to Moragrega et al. (2018), levels of aggressiveness or toxicity (−) and (±) represent non-pathogenic isolates. Isolates included in level (+) were considered of low aggressiveness/toxicity, those included in level (++) were considered of moderate aggressiveness/toxicity and those included in level (+++) were considered of high aggressiveness/toxicity (Moragrega et al., 2018). Aggressiveness and toxicity were confirmed according to the Koch's postulates.

Conidial suspensions: Isolates were cultured on water agar medium (agar 20 g/L) in Petri dishes (90 mm) and incubated for 3 weeks at 25 °C and 16-h photoperiod under black-blue light until full sporulation. The surface of the colonies was flooded with DW, and conidia were collected by gently rubbing the surface with a paintbrush. The conidial suspensions were filtered through a layer of cheese cloth to remove mycelial debris. Conidial concentration was adjusted to 5×10^5 conidia/mL with DW using a Neubauer chamber (Tanahashi et al., 2017).

Table 1 EF-1α sequences used in the phylogenetic analyses

Species	Isolate	GenBank nucleotide accession number
Cercospora beticola	CPC 18813	JX143310
Stemphylium botryosum	CBS 714.68	KC584729
Stemphylium eturmiunum	EGS29–099	AY324712
Stemphylium lancipes	CBS 333.73	KC113194
Stemphylium loti	NO 1364	AY324776
Stemphylium majusculum	EGS16–068	AY324710
Stemphylium paludiscirpi	EGS31–016	AY324769
Stemphylium solani	EGS42–027	AY324760
Stemphylium trifolii	EGS12–142	AY324744
Stemphylium triglochinicola	EGS36–118	AY324753
Stemphylium xanthosomatis	EGS17–137	AY324758
Stemphylium vesicarium	EGS37–067	AY324708
	BRIP:65181	KY009905
	CBS 191.86	KC584731
	SE1	MK419915
	SE2	MK419916
	SE3	MK419917
	SE4	MK419918
	SE5	MK419919
	SE6	MK419920
	SE7	MK419921
	SE8	MK419922
	SE9	MK419923
	SE10	MK419924
	SE11	MK419925
	SF4	MK424777
	SF5	MK424778
	SF8	MK424779
	SF9	MK424780
	SF13	MK424781
	SF19	MK424782
	SF20	MK424783
	SF21	MK424784
	SF23	MK424786
	SF24	MK424787
	SL22	MK424785
	SL35	MW032465
	SL37	MW032466
	SL39	MW032467
	SL40	MW032468
	SL61	MW032469
	SL62	MW032470
	SL63	MW032471
	SL65	MW032472

Table 1 (continued)

Species	Isolate	GenBank nucleotide accession number
	SL68	MW032473
	SL70	MW032474

Aggressiveness bioassay: Three young fresh detached leaves of each cultivar were washed with DW and placed individually on Petri dishes representing three replicates. Their undersides were inoculated by spraying 3 mL of conidial suspensions ($1{,}5 \times 10^3$ conidia/mL) with an atomizer. Negative controls were performed by spraying the same volume of DW. Afterwards, leaves were incubated at 25 °C under darkness in a moist chamber (Tanahashi et al., 2017) composed of the Petri dish sealed inside a polyethylene bag. Humidity conditions were maintained using sterile pieces of cotton of equal size soaked in 5 mL of distilled sterile water. After 48 h of incubation, photographs of the injured leaves were taken using a 16 megapixel resolution camera and the images obtained were processed with the ImageJ software (Schneider et al., 2012). The program determined the total area and the necrotic area of each leaf, and with these data the percentage of foliar area affected by necrosis was then calculated in order to obtain the level of aggressiveness. This assay was carried out three times.

Culture filtrates: Small pieces of mycelia from a 7-day-old PDA colony of each of the thirty two isolates were inoculated in a 500-mL flask containing 200 mL of modified Richard's liquid medium (KNO_3 10 g/L, KH_2PO_4 5 g/L, $MgSO_4$/7H2O 2.5 g/L, $FeCl_3$ 0.02 g/L, glucose 25 g/L, yeast extract 1 g/L) and were incubated at 27 °C under continuous diffuse light for 20 days. The culture medium was then filtered through a piece of Whatman grade 50 filter paper assisted with a vacuum pump and then re-filtered through a 0.2 μm syringe filter (Tanahashi et al., 2017).

Toxicity bioassay: Three young fresh detached leaves of each cultivar were washed with DW and placed individually on Petri dishes representing three replicates. The underside of the leaves was slightly injured in their central portion with a pair of tweezers and a 40-μl drop of each culture filtrate was placed on a wound site. Negative controls were performed with a drop of DW (Tanahashi et al., 2017). Incubation of the leaves was performed in the same way as in the aggressiveness bioassay as well as the determination of the percentage of foliar area affected by necrosis. Then, the correspondent level of toxicity was obtained. This assay was carried out three times.

Statistical analysis of data: For aggressiveness and toxicity tests, a completely randomized design with factorial structure was used, with isolate and cultivar as factors. Data were analyzed with R software version 4.0.3 (R Core Team, 2021, and the packages emmeans (Lenth et al., 2020), ggplot2 (Wickham et al., 2016) and nlme (Pinheiro et al., 2021) were used. For both analyses, the percentage of foliar area affected by necrosis was used as the response variable and isolate, cultivar, and their interaction were used as predictive variables. Since data of the three independent experiments for each bioassay were analysed pooled, an analysis to determine the effect of the experiment factor was previously performed and, as a result, it was not significant neither for the aggressiveness test ($p = 0.33$) nor for the toxicity test ($p = 0.69$). Planned comparisons were carried out between airborne isolates and plant material isolates (leaf and fruit) and between the pear cultivars. Comparison of aggressiveness and toxicity between airborne isolates and plant material isolates per cultivar were also performed in order to determine if the isolation source was a significant factor. All comparisons were verified to be orthogonal and the degrees of freedom were calculated by Satterthwaite method. Linear models, to which the variance-covariance matrixes were adjusted due to lack of homoscedasticity between the cultivars tested, were selected to perform the statistical analysis of the data, presenting an adequate estimation. A varident function was used to model heteroscedasticity according to Pinheiro & Bates, 2000. The assumptions were verified graphically and through the likelihood ratio test. An Analysis of Variance (ANOVA) was carried out ($p < 0.05$). In the means comparison test the Tukey method was used. Pearson correlation coefficient was applied to correlate aggressiveness and toxicity for each isolate and also per cultivar.

Results

Relative frequencies of isolation

Ninety-one *Stemphylium* isolates and 12 *Alternaria* isolates were obtained from plant material during the

sampling period, 21 from pear fruits and 46 from pear leaves. The pathogen was collected from 98% of the symptomatic fruits, from 95% of leaves with the specific "V-shaped" symptom and from 65% of leaves with circular necrotic spots. 14 *Stemphylium* isolates were obtained from air of agricultural environments during the sampling period.

Morphological characterization

Growth on V8 and PCA media of all 32 airborne and plant-material isolates selected showed morphological characteristics consistent with *S. vesicarium* (Simmons, 1969, 1985). Colonies on V8 consisted of a pale brown to grayish mycelium with abundant white aerial mycelium showing a floccose or cottony texture. Occasionally, they presented a radiating appearance with colorless and regular margins. On PCA medium, colonies were whitish or colorless with

scarce aerial mycelium and, only in the center of the colony, and showed similar characteristics to growth on V8 medium. On both media, the colonies covered the entire diameter of a 90 mm Petri dish after three weeks of incubation. On both media, 9 of the 11 airborne isolates and all plant-material isolates developed pseudothecia in abundance at the end of 3 weeks which matured 6–9 months later to produce ascospores (Fig. 1). On both media, conidia were ovoid to oblong, rounded at the apex, brown with verrucose wall ornamentation, developed singly and not in chains, with 1–5 transverse septa and 0–3 longitudinal septa (Table 2). Pseudothecia were usually rounded or oval shaped with a diameter of 1–2 mm, easily observable with the naked eye and dark brown to blackish in colour. Asci were hyaline and contained 8 oblong or ellipsoidal dark brown ascospores, each 32–35 μm long by 10–12 μm wide, up to 6 transverse septa and multiple longitudinal septa (Fig. 2).
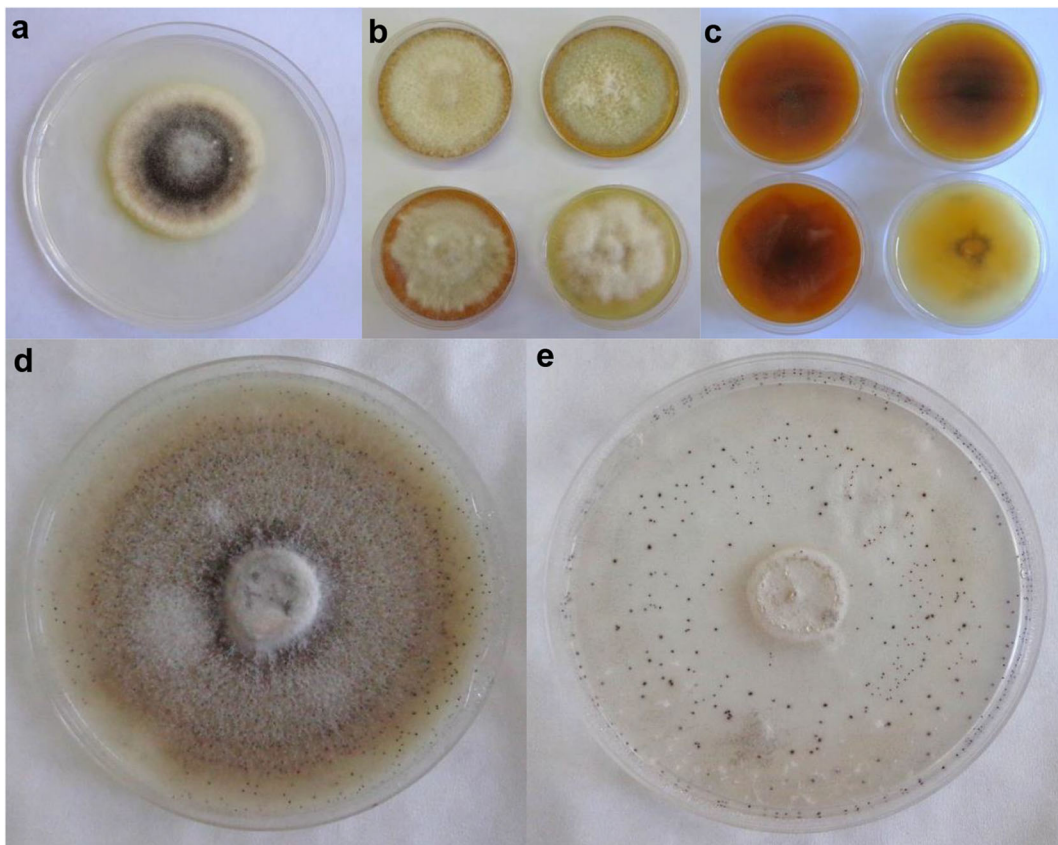
Fig. 1 Macroscopic characteristics of *Stemphylium vesicarium*. Anamorph isolates of *S. vesicarium* after 14 days of incubation under 16 h photoperiod with black-blue light in PDA (**a-c**).

Teleomorph isolates of *S. vesicarium* after 21 days of incubation on V8 (**d**) and PCA (**e**) medium exhibiting pseudothecia

Table 2 Morphological characteristics of *Stemphylium vesicarium* conidia on PCA agar corresponding to 32 isolates

Variable	Mean and Standard deviation	Minimum	Maximum
Length (μm)	23.45±4.10	12.5	40
Width (μm)	13.49±2.15	7.5	21
Ratio L/W	1.76±0.30	1.08	2.95
N° of transverse septa	2.64±0.84	1	6
N° of longitudinal septa	1.00±0.39	0	3

Molecular characterization and phylogenetic analysis

Although, in this work the molecular characterization of the *Stempyhlium* isolates was based on the EF-1α gene, many authors suggest carrying it out through a multilocus approach. To this end, sequences of the internal transcribed spacers (ITS) of the nuclear ribosomal DNA and protein coding genes are important molecular markers to perform phylogenetic analyses of fungi (Begerow et al., 2010; Graf et al., 2016; Schoch et al., 2012; White et al., 1990). Many authors have chosen ITS and EF-1α among the genes used to verify taxonomic relations between isolates of the *Stemphylium* genus (Inderbitzin et al., 2009; Köhl et al., 2009; Kurose et al., 2015; Moslemi et al., 2017). Thus, we carried out a preliminary phylogenetic approach, characterizing a subgroup of *Stemphylium* isolates (airborne) using sequences of EF-1α gene combined with ITS genes. The phylogenetic trees obtained for each locus separately as well as both loci combined showed that EF-1α gene provided the best taxonomic resolution of the isolates with the highest bootstrap values for each clade obtained as shown in Online Resource 2. Even more, this gene has
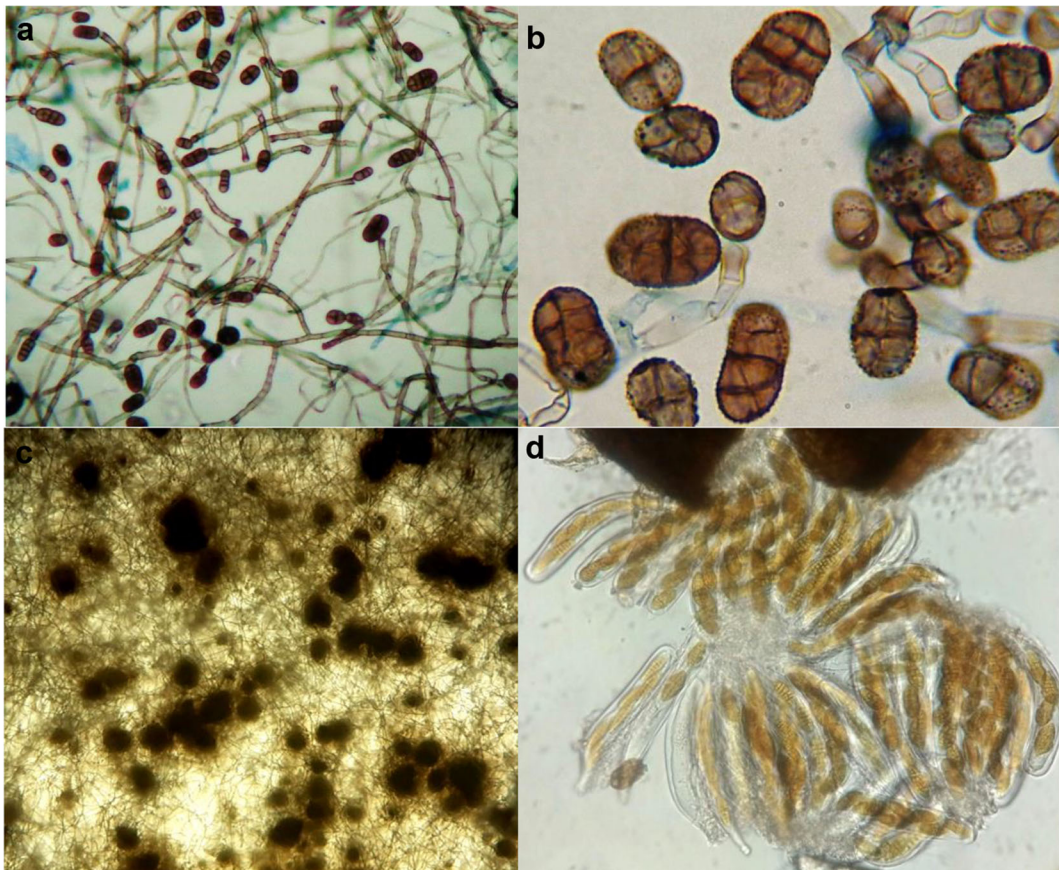

Fig. 2 Microscopic characteristics of *Stemphylium vesicarium*. Conidia of *S. vesicarium* anamorphs after 14 days of incubation on PDA medium (**a**: 100X, **b**: 1000X); pseudothecia of *S. vesicarium* teleomorphs after 14 days of incubation on V8 medium (**c**: 20X); ascospores of *S. vesicarium* teleomorphs emerging from a mature pseudothecium after 9 months of incubation on PDA medium (**d**: 400X)

demonstrated its ability to differentiate close species such as *S. vesicarium* and *S. botryosum*, for which some authors recommend sequencing of the cytochrome b gene (Graf et al., 2016). Therefore, EF-1α gene was chosen to characterize the 32 isolates included in this study. Their respective sequences were clustered together in one terminal clade strongly supported by a high bootstrap value containing the strain references of *S. vesicarium* with greater homology including the ex-type strain deposited by Simmons (1967) and sequenced

by Inderbitzin et al. (2005) (EGS 37–067) and the isolates deposited by Moslemi et al. (2017) (BRIP:65181) and by Woudenberg et al. (2017) (CBS 191.86). The phylogenetic analysis based on the EF-1α gene fragment resulted, therefore, in the molecular confirmation of all the isolates as *S. vesicarium* (Fig. 3). Due to the one-fungus one name concept, the accepted name for this fungus is named after the anamorph state (McNeill et al., 2012). Therefore, all the isolates under study on this work are named as *S. vesicarium.*
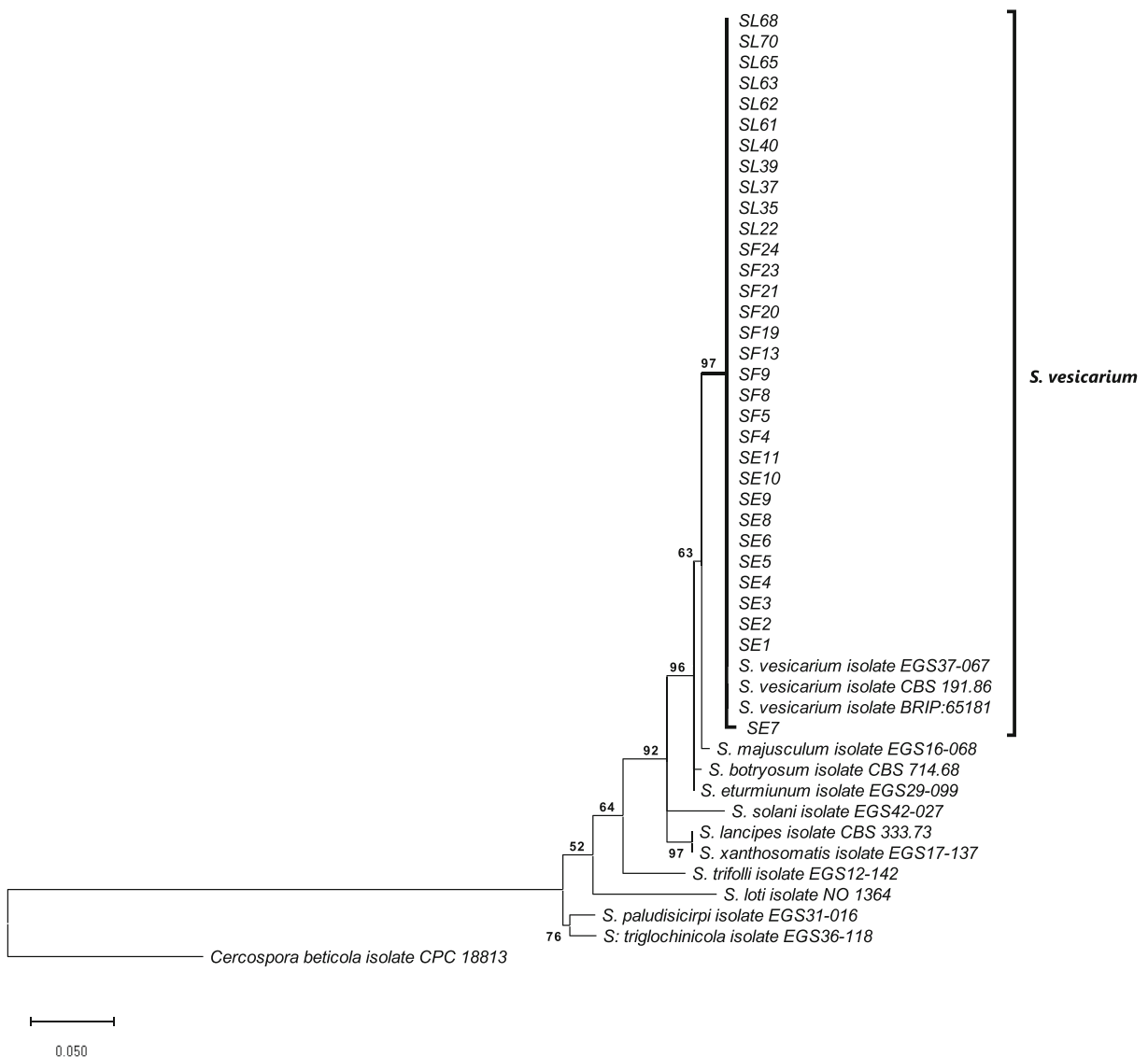
Fig. 3 Maximum likelihood phylogenetic tree based on the translation elongation factor 1-α gene displaying the analysis of the 32 *Stemphylium* isolates obtained from air samples and pear leaves and fruits with the type strain reference sequences (CBS 191.86, BRIP:65181, and EGS 37–067). *Cercospora beticola*, strain CPC 18813 was used as an outgroup to root the tree

Aggressiveness of *S. vesicarium* conidia and host-selective toxicity of culture filtrates

The conidial suspensions and the culture filtrates were able to reproduce the characteristic symptoms of the disease on pear leaves (host plant), but not on apple leaves (non-host plant). Lesions obtained on leaves of the different pear cultivars assayed are shown in Fig. 4.

According to the means obtained for each isolate on each cultivar, results from the aggressiveness test showed that 33.3% of the isolates (n = 3) were pathogenic on D'Anjou leaves, 22.2% were pathogenic on Abate Fetel (n = 2) and the highest level of pathogenicity they reached was low necrosis (+). On Williams and Packham's, none of the isolates was pathogenic. On D'Anjou, only plant isolates, two from leaf and one from fruit, caused low necrosis (+). All airborne isolates produced necrosis around the inoculation point (±) as well as two isolates from leaf and one from fruit. On Abate Fetel, one airborne isolate and one from fruit caused low necrosis (+). All leaf isolates originated necrosis around the inoculation point (±) as well as two isolates from fruit and two from air. On Williams, all plant isolates produced necrosis around the inoculation point (±) as well as one from air. The remaining two airborne isolates did not cause injury (−). Finally, on Packham's, most of plant isolates (all from fruit and two from leaf) caused necrosis around the inoculation point (±) as well as one from air. One leaf isolate and two airborne ones did not cause injuries (−). The mean percentage of affected area for each isolate and cultivar combination are presented in Fig. 5 and the levels of aggressiveness corresponding to each isolate on pear leaves are described in Table 3. The frequency of isolates within each level of aggressiveness on each pear cultivar sorted by their source of origin is shown in Fig. 6.

According to the means obtained for each isolate on each cultivar, in the toxicity analysis, all isolates were pathogenic on D'Anjou (n = 9), 77.8% of the isolates were pathogenic on Abate Fetel leaves (n = 7), 11.1% on Packham's (n = 1) and no isolates were pathogenic on Williams. As observed in the aggressiveness bioassay, the highest level of pathogenicity they reached was low necrosis (+). On D'Anjou, all CFs presented low necrosis (+). On Abate Fetel, all CFs from plant isolates caused low necrosis (+) as well as one from air, and the remaining two airborne isolates caused necrosis around the inoculation point (±). On Williams, all CFs presented produced necrosis around the inoculation point (±). On Packham's cultivar, all CFs caused some type of injury: one CF from a leaf isolate caused low necrosis (+) and all CFs from fruit and from air as well as the remaining two from leaf produced necrosis around the inoculation point (±). The mean values of percentage affected area are shown in Fig. 7 and the levels of toxicity corresponding to each isolate on each pear cultivar are described in Table 4. The frequency of isolates within each level of toxicity sorted by source of origin is shown in Fig. 8.

Pearson correlation coefficient indicated a low correlation (0,45) between aggressiveness and toxicity for each isolate on each cultivar (p value <2.2e-16). ANOVA test showed significant differences between aggressiveness of the isolates within each pear cultivar. The same effect was observed for toxicity variable.

With respect to the impact of the source of origin, in aggressiveness bioassays, air isolates were less aggressive than those extracted from plant material (p < 0.001). Likewise, Abate Fetel and D'Anjou cultivars
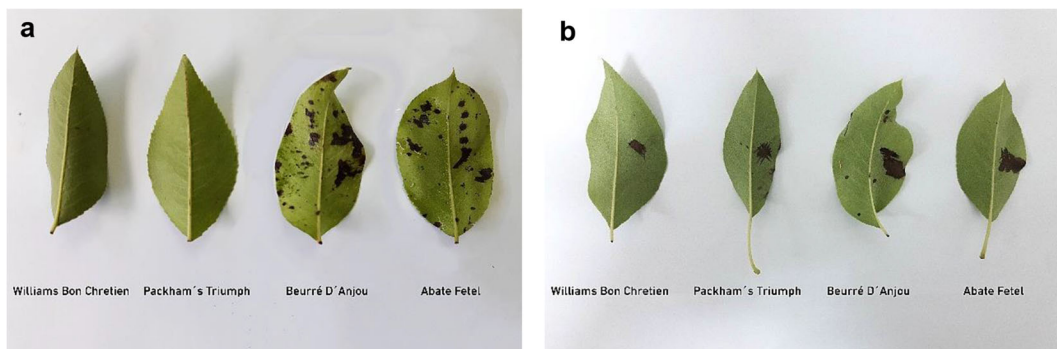
Fig. 4 Lesions caused by conidial suspensions of *Stemphylium vesicarium* (isolate SF23) on leaves of pear cultivars (**a**). Lesions caused by culture filtrates of *S. vesicarium* on leaves of pear cultivars (**b**)
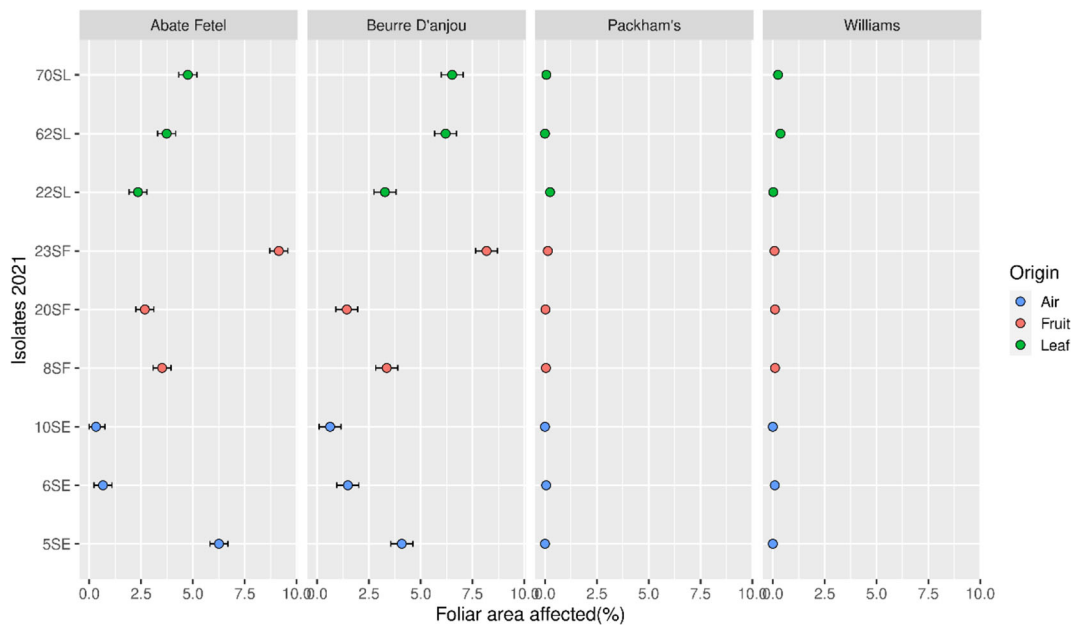
Fig. 5 The effect of inoculation with *Stemphylium vesicarium* isolates (1×10^5 conidia/mL) on leaves of pear cultivars Abate Fetel, D'Anjou, Packham's and Williams varieties

were more sensitive to damage than Williams and Packham's (p < 0.001). In toxicity bioassays, air isolates showed less toxicity than those obtained from plant material (p = 0.001). As in aggressiveness bioassays, Abate Fetel and D'Anjou were also more sensitive to damage than Williams and Packham's (p = 0.002), and Packham's was more sensitive than Williams (p < 0.0001).

Raw statistical data including results of Tukey test from both bioassays are presented in Online Resource 3.

Discussion

Brown spot of pear has a large economic impact in the Mediterranean region in several pear producing areas

Table 3 Aggressiveness of *Stemphylium vesicarium* conidia on host pear leaves

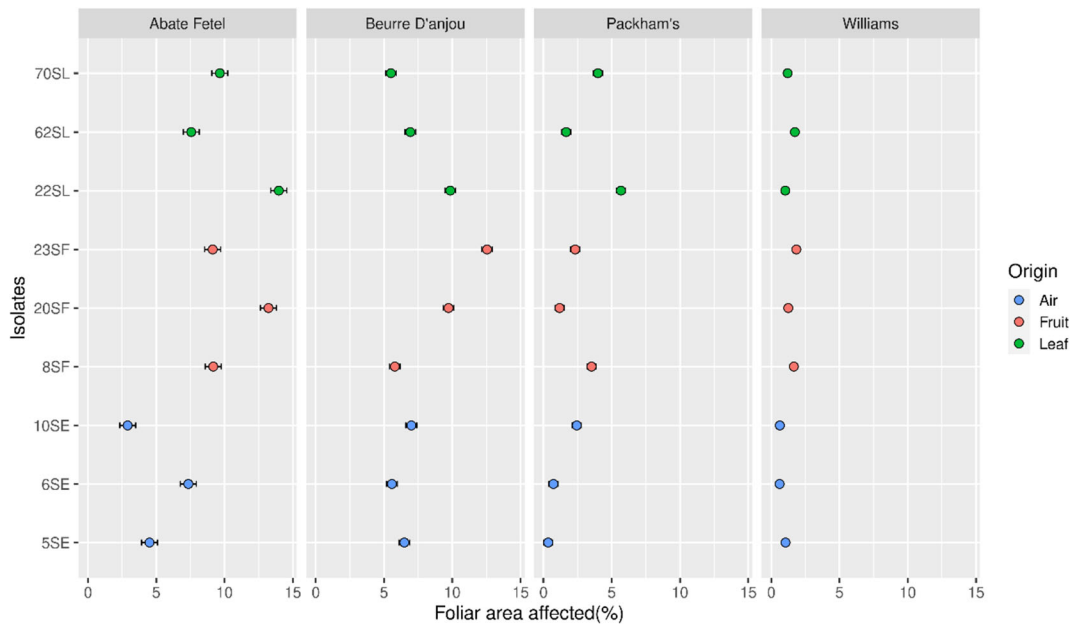| Isolate	Cultivars							
	Williams		Packham's		D'Anjou		Abate Fetel	
	Mean+SD (%)	Index	Mean+SD (%)	Index	Mean+SD (%)	Index	Mean+SD (%)	Index
SE5	0.00	(−)	0.00	(−)	4.08±0.75	(±)	6.26±2.29	(+)
SE6	0.09±0.28	(±)	0.06±0.17	(±)	1.48±3.34	(±)	0.66±0.58	(±)
SE10	0.00	(−)	0.00	(−)	0.63±0.20	(±)	0.33±0.27	(±)
SF8	0.11±0.32	(±)	0.05±0.14	(±)	3.36±2.88	(±)	3.51±1.55	(±)
SF20	0.11±0.22	(±)	0.03±0.08	(±)	1.43±1.32	(±)	2.68±0.89	(±)
SF23	0.08±0.24	(±)	0.14±0.32	(±)	8.17±3.42	(+)	9.14±5.23	(+)
SL22	0.02±0.06	(±)	0.25±0.38	(±)	3.28±1.97	(±)	2.35±0.97	(±)
SL62	0.37±0.35	(±)	0.00	(−)	6.20±3.90	(+)	3.74±1.84	(±)
SL70	0.25±0.30	(±)	0.07±0.20	(±)	6.51±6.23	(+)	4.75±4.56	(±)

[a] Aggressiveness classification: (−): no necrosis, 0%; (±): a small amount of necrosis around the inoculation point; 0% < necrosis≤5%; (+); 5% < necrosis≤25%; (++); 25% < necrosis≤50%; (+++); 50% < necrosis≤100%
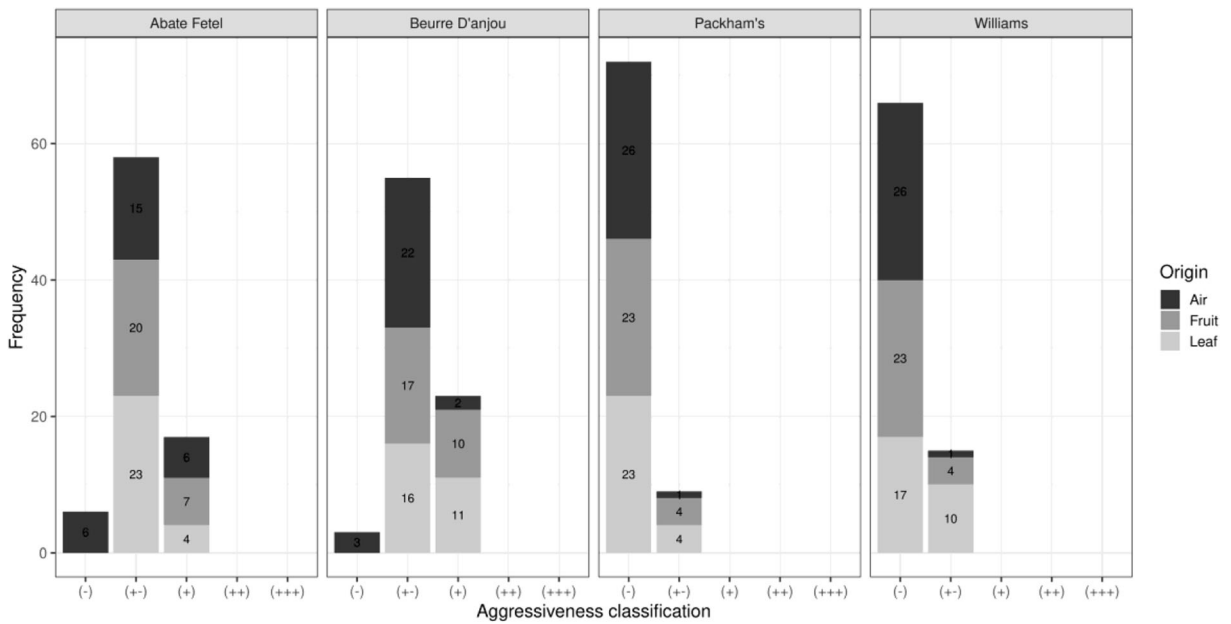
Fig. 6 Frequency of *Stemphylium vesicarium* isolates within each level of aggressiveness discriminating their source of origin on leaves of pear cultivars Abate Fetel, D'Anjou, Packham's and Williams

located in Italy, Spain, and France, and has been lately described also in the Netherlands and Portugal (Llorente et al., 2012; Llorente & Montesinos, 2006; Rossi et al., 2005). It was also reported in Niigata Prefecture, Japan (Tanahashi et al., 2008). The disease has an estimated incidence of 1–10% and its impact was compared in

some areas to that of apple scab caused by the fungal pathogen *Venturia inaequalis* in apple orchards (Heijne & Van Mourik, 2001; Llorente et al., 2012; Llorente & Montesinos, 2006; Rossi et al., 2005). In Argentina, BSP was detected for the first time in the High Valley of Río Negro and symptoms were described, but no

Fig. 7 The effect of culture filtrates of *Stemphylium vesicarium* on leaves of pear cultivars of Abate Fetel, D'Anjou, Packham's and Williams cultivars

Table 4 Toxicity of *Stemphylium vesicarium* culture filtrates on host pear leaves
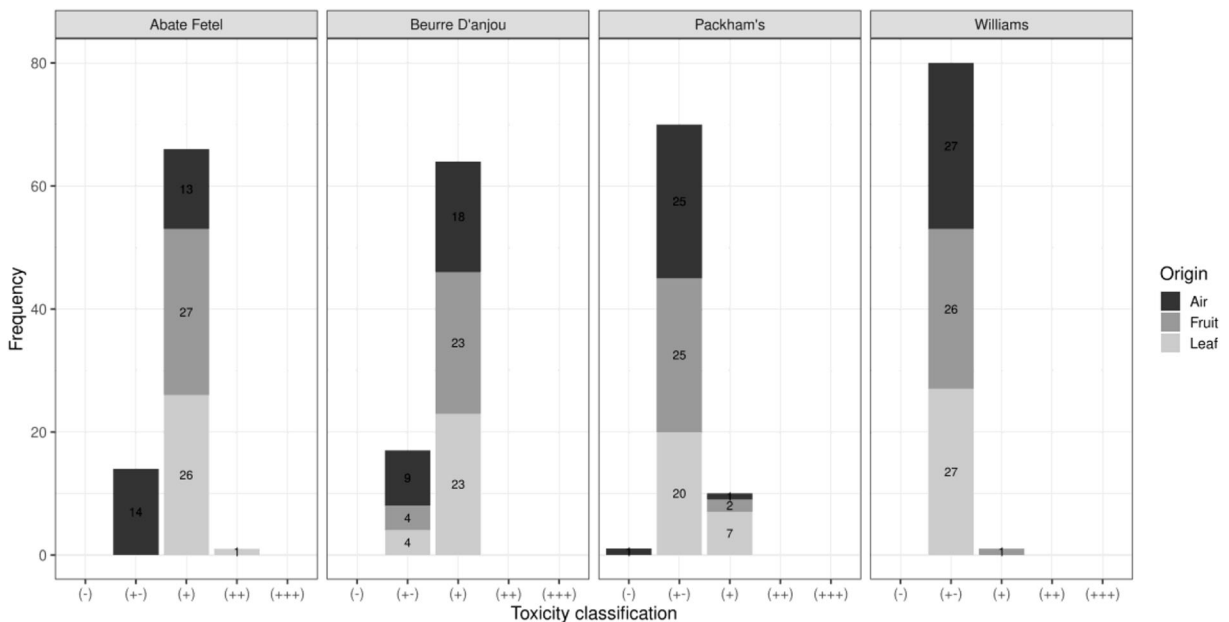
Isolate	Cultivars							
	Williams		Packham's		D'Anjou		Abate Fetel	
	Mean+SD (%)	Index	Mean+SD (%)	Index	Mean+SD (%)	Index	Mean+SD (%)	Index
SE5	1.05±0.57	(±)	0.35±0.18	(±)	6.49±4.23	(+)	4.51±2.32	(±)
SE6	0.62±0.27	(±)	0.73±0.17	(±)	5.58±0.92	(+)	7.35±1.21	(+)
SE10	0.63±0.29	(±)	2.44±1.49	(±)	7.00±2.09	(+)	2.90±1.06	(±)
SF8	1.66±1.00	(±)	3.52±3.21	(±)	5.79±1.54	(+)	9.18±2.49	(+)
SF20	1.25±1.70	(±)	1.18±0.44	(±)	9.72±1.27	(+)	13.22±3.92	(+)
SF23	1.84±1.30	(±)	2.32±1.04	(±)	12.55±3.66	(+)	9.13±1.54	(+)
SL22	1.03±0.46	(±)	5.67±4.12	(+)	9.85±2.04	(+)	13.97±7.66	(+)
SL62	1.73±1.11	(±)	1.66±0.56	(±)	6.93±1.72	(+)	7.56±0.93	(+)
SL70	1.20±0.36	(±)	3.99±1.78	(±)	5.51±1.35	(+)	9.66±4.39	(+)

[a] Toxicity classification: (−): no necrosis, 0%; (±): a small amount of necrosis around the inoculation point; 0% < necrosis≤5%; (+); 5% < necrosis≤25%; (++); 25% < necrosis≤50%; (+++); 50% < necrosis≤100%

analysis or description of the causal agent or pear cultivars susceptibility were conducted (Dobra & Garcia, 2015). Thus, this is the first study focused on characterizing *S. vesicarium* isolates outside Europe and Japan and the susceptibility of pear cultivar D'Anjou to this pathogen.

The High Valley of Río Negro, the most important production and exporting region in the country, presents a cold, dry desert climate with warm summers according to the Köppen climatic classification (BWk type) (Köppen, 1931). In this region, the concentration and relative frequency of *S. vesicarium* airborne spores with respect to other fungal genera in rural environments is low (Temperini et al., 2019). However, in recent years, climatic conditions seemed to have favored its development and pathogenic capacity, allowing for the presence

Fig. 8 Frequency of *Stemphylium vesicarium* isolates within each level of toxicity discriminating their source of origin on leaves of pear cultivars Abate Fetel, D'Anjou, Packham's and Williams

of a new pathology in the main pear cultivars grown in this region (Di Masi, 2017; Dobra & Garcia, 2015). The symptoms of the disease observed in the field matched with those described for BSP in Europe (Llorente & Montesinos, 2006; Rossi et al., 2005). The morphological characteristics of Argentinean fungal isolates, such as colony characteristics and spore morphology, were similar to those described for isolates of the morphological group which contains the *S. vesicarium* EGS 37–067 (Type) strain (Simmons, 1969) and isolates obtained from lesions on pear fruits and leaves and from air samples from pear orchards in Spain, France, and the Netherlands (Puig et al., 2015). Moreover, these characteristics also matched with those described for *S. vesicarium* isolates EGS 37–067, which was used in the taxonomic re-examination of Japanese isolates (Kurose et al., 2015), strain BRIP:65181 (Moslemi et al., 2017), and strain CBS 191.86 (Woudenberg et al., 2017). All isolates in this study were grouped in the same clade that contains the *S. vesicarium* isolates EGS 37–067 (Type), BRIP:65181, and CBS 191.86 with a high bootstrap support (97). Therefore, morphological and molecular analyses identified the isolates in this study as *S. vesicarium* (Wallr.) E.G. Simmons (teleomorph: *P. herbarum* [Pers.] Rabenh, syn. *P. allii* [Rabenh.] Ces. & De Not.).

With respect to the aggressiveness and toxicity studies, the results obtained show that *S. vesicarium* isolates obtained from air and plant material were pathogenic to Argentinean pear cultivars and presented different degrees of aggressiveness and/or phytotoxicity on them. The *S. vesicarium* isolates were not pathogenic to apple leaves, which is a non-host crop. The non-pathogenicity of some of the isolates recovered from lesions of pear leaves and fruit might be explained by the existence of only the saprophytic phase of *S. vesicarium* (Köhl et al., 2009; Llorente et al., 2010; Llorente et al., 2012; Rossi et al., 2005). The pathogenic behavior of the isolates exhibited variability between them and the results suggest that D'Anjou and Abate Fetel cultivars were the most affected based on both assays. This is consistent with the observations in the field, since severe infections were detected on leaves and fruits of Abate Fetel and D'Anjou, while in Williams and Packham's, symptoms were only observed on fruits and in a very low proportion. Similar results were obtained from cultivar susceptibility tests using European *S. vesicarium* isolates and pear cultivars, including Abate Fetel, Williams and Packham's, in which Abate Fetel was characterized as

highly susceptible while Williams and Packham's were characterized as slightly or not susceptible (Llorente et al., 2012; Montesinos et al., 1995; Pattori et al., 2006; Rossi et al., 2005). This host specificity demonstrated by Argentinean *S. vesicarium* isolates suggests the existence of pathotypes, which other researchers have also mentioned in their studies (Llorente & Montesinos, 2006; Singh et al., 1999). Other research works have also reported different degrees of aggressiveness and toxicity on a variety of host plants (Foster et al., 2019; Köhl et al., 2009; Singh et al., 1999). Italian isolates of *S. vesicarium* showed that 5 of 9 isolates were pathogenic on 7 European pear cultivars whereas an isolate from asparagous and an isolate that was not pathogenic on European pears were both pathogenic on Japanese pear (cvs. Chojuro, Nijisseiki and Osa Nijisseiki). Regarding phytotoxicity, one isolate (IT37) showed higher toxicity compared with the other isolates tested, and the most susceptible European pear cultivar was Alexandrine Douillard (Singh et al., 1999). Japanese studies showed similar results: European pear cultivar Le lectier was the most susceptible to *S. vesicarium* infection whereas European cultivar Bartlett and Japanese cultivar Nijisseiki were not affected by some isolates (Tanahashi et al., 2017). It was proposed and shown that Japanese and European isolates could produce host-specific SV toxins (Singh et al., 1999; Tanahashi et al., 2017). Although the system used to determine the aggressiveness and toxicity of the isolates in this study was based on a quantitative estimate of the lesions extension instead of the qualitative one that Singh et al. (1999) and Tanahashi et al. (2017) used, it can be concluded that, as in their works, the range of host sensitivity to the toxicity of CFs was comparable to the susceptibility of cultivars to *S. vesicarium* conidia. As a result, Abate Fetel and D'Anjou were more sensitive than Williams and Packham's. On the other hand, no significant correlation between aggressiveness and toxicity was found within any pear cultivar. This in contrast to what Singh et al. (1999) observed in their study where they affirmed that the sensitivity of European pear cultivars to the SV-toxins was directly correlated to the susceptibility to the causal agent (Singh et al., 1999).

In reference to the source of origin of pathogenic isolates, the airborne isolates seemed to be less aggressive ($p < 0.001$) and less toxic ($p = 0.001$) than those collected from plant material. This is in line with the results obtained by other authors where the highest

proportion of non-aggressive isolates corresponded to air samples, and most of the isolates recovered from plant lesions were aggressive to pear (Moragrega et al., 2018; Puig et al., 2015). Authors suggest that the reason why a high proportion of airborne conidia are not aggressive is due to the fact that an important amount of the air inoculum may be produced by populations of *S. vesicarium* that have only the saprophytic phase (Moragrega et al., 2018) and that may come from a wide variety of plant hosts including weed and grass species surrounding pear cultivars which are affected mainly by aggressive isolates that re-infect the host (Foster et al., 2019; Rossi et al., 2005). The existence of a saprophytic phase may also explain their lower toxicity due to their minor capacity o inability to produce SV-toxins during this phase.

This work describes the finding of *S. vesicarium* pathogenic isolates on Argentinean pear cultivars and their behavior in terms of their aggressiveness and toxicity on host plants revealing a different range of host sensitivity introducing new cultivars under study such as D'Anjou. This knowledge will eventually allow for the design of better preventive or treatment strategies to manage BSP.

Conclusion

This is the first study conducted in South America about the characterization of autochthonous *S. vesicarium* isolates and their pathogenicity on commercial pear cultivars including D'Anjou, whose susceptibility was never tested before. Increased rainfall in the High Valley of Río Negro, the main pear production and exporting region of Argentina, seems to have been one of the predisposing factors for the development of the disease, although further studies should be conducted. The symptoms observed and the identity of the pathogen after morphological and molecular analysis matched with those described for BSP in European and Japanese cultivars. The D'Anjou and Abate Fetel cultivars were the most susceptible to both *S. vesicarium* infection and culture filtrates. The information obtained in this work is a valuable tool for future studies to develop novel disease management strategies.

Declarations

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