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Pathogenicity of *Stemphylium vesicarium* from different hosts causing brown spot in pear

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Abstract *Stemphylium vesicarium* (teleomorph: *Pleospora herbarum*) is the causal agent of brown spot disease in pear. The species is also able to cause disease in asparagus, onion and other crops. Saprophytic growth of the fungus on plant debris is common. The objective of this study was to investigate whether isolates of *S. vesicarium* from different hosts can be pathogenic to pear. More than hundred isolates of *Stemphylium* spp. were obtained from infected pear fruits, dead pear leaves, dead grass leaves present in pear orchard lawns as well as from necrotic leaf parts of asparagus and onion. Only isolates originating from pear orchards, including isolates from dead grass leaves, were pathogenic on pear leaves or fruits in bioassays. Non-pathogenic isolates were also present in pear orchards. *Stemphylium vesicarium* from asparagus or onion, with one exception, were not pathogenic to pear. Analysis of the genetic variation between isolates using Amplified Fragment Length Polymorphism (AFLP) showed significant concordance with host plants. Isolates from asparagus or onion belonged to clusters separate from the cluster with isolates from pear or grass leaves collected in pear orchards. Multilocus sequencing of a subset of isolates showed that such isolates were similar to *S. vesicarium*.

Keywords AFLP · Asparagus · Grass · Onion · *Pleospora herbarum*

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Introduction

Stemphylium vesicarium (teleomorph, according to current taxonomy, *Pleospora allii*) is a filamentous fungus able to cause disease on a range of different hosts, such as asparagus (Falloon et al. 1987), garlic (Prados-Ligero et al. 1998), and onion (Aveling and Snyman 1993). The morphologically-related species *S. botryosum* (teleomorph: *P. tarda*) has been found as a pathogen on several crops including asparagus (Leuprecht 1990), alfalfa (Cowling et al. 1981) and spinach (Koike et al. 2001). A third species, *S. alfalfae* (teleomorph: *P. alfalfae*) has been reported as a pathogen on alfalfa (Chaisrisook et al. 1995).

Since the late 1970s, *S. vesicarium* has been observed in the Po Valley in Italy causing brown spot on leaves and fruits of pear (Ponti et al. 1982). This disease was subsequently found in Spain and France in the late 1980s and first observations of brown spot in The Netherlands and Belgium were reported in the early 1990s (Llorente and Montesinos 2006; Polfliet 2002).

Stemphylium species are typically colonisers of dead plant tissues (Hudson 1971). Limited information is available on the relationship between saprophytic and pathogenic populations of *Stemphylium* spp. as well as on the possible host-specificity of pathogenic isolates. Most species of the closely related genus *Alternaria* are saprophytic but some are opportunistic pathogens on a large variety of crops. Characteristically, pathogenic *Alternaria* isolates produce host-specific toxins (Thomma 2003). Host-specific toxins, SV-toxin I and SV-toxin II, were found in culture filtrates of isolates of *S. vesicarium* pathogenic to pear (Singh et al. 1999). It could be demonstrated that severity of necrosis increased with toxin concentration and susceptibility of pear varieties. The typical v-shaped symptom of brown spot on pear leaves could be explained by vein necrosis caused by the toxins (Singh et al. 1999).

The question whether isolates are host-specific is a major issue for understanding routes of transmission and modes of resistance. As yet limited information is available on the pathogenicity to pear of isolates pathogenic on other crops (Singh et al. 1999; Pattori et al. 2006) or found as saprophytes in the environment. Ascospores are found on fallen pear leaves on orchard floors only during early spring, but epidemics usually start in summer (Llorente and Montesinos 2006). Since only airborne conidia are detected in spore traps during summer (Rossi et al. 2005a), conidia may be the prevalent primary inoculum of the disease. Conidia are rarely observed in lesions on affected pear leaves during summer. Thus, sources of conidia other than infected pear tissue, e.g. tissue of other living hosts or of saprophytically colonised necrotic material of other plant species may play a role in brown spot epidemiology. Rossi et al. (2005b, 2008) suggested that dead tissues of plants present in the orchard lawn may act as sources of the disease. Onion and asparagus, potential host crops of *S. vesicarium* frequently grown in The Netherlands, may possibly also act as sources.

The objectives of our study were to investigate whether isolates of *S. vesicarium* pathogenic to pear have a reservoir on necrotic plant tissues present in pear orchards or their close vicinity. Over one hundred isolates were obtained from pear fruits showing symptoms of brown spot, dead leaves of pear and various grasses collected from pear orchard floors and from necrotic leaf tissues of onion and asparagus crops. Isolates were tested for pathogenicity on pear. The genetic variation between isolates was assessed for a sub-set of isolates of different origin using Amplified Fragment Length Polymorphism (AFLP) as a full genome approach (Vos et al. 1995).

Materials and methods

Isolates of *Stemphylium*

Stemphylium spp. were isolated from diseased pear fruits collected in summer 2003 in thirteen orchards, necrotic pear leaves collected in spring 2003 from the floor of five pear orchards, from necrotic leaves of grasses collected in two pear orchards in spring 2003, from necrotic parts of onion leaves collected in autumn 2003 from ten commercial crops or necrotic parts of asparagus leaves collected in autumn 2003 from ten different commercial crops. A few isolates of *Stemphylium* spp. were obtained from surface-sterilised green apple leaves from one orchard. All crops were grown in different parts of The Netherlands. Brown spot has been observed in 2003 or previous years in all sampled pear orchards.

For isolation of *Stemphylium* spp., segments of leaf or fruit surfaces, approx. 1×1 mm, were placed on *Alternaria radicina* selective agar (ARSA; Pryor et al. 1994) and incubated for 10 days at 22°C. On this semi-selective medium the majority of observed fungi was found to belong to the Dematiaceae such as *Alternaria*, *Stemphylium* and *Ulocladium*. Plates were observed at 100 × magnification and spores reminiscent of *Stemphylium* spp. were transferred with a sterile needle to MEA plates (malt extract 1 g l^{-1}, agar 15 g l^{-1}). For identification based on morphology, cultures were grown on MEA for 14 days at 25°C with 12 h black light (350 nm) per day (Simmons, 1969), and the shape and size of conidia were used as the main criteria to distinguish *S. vesicarium* from *S. botryosum* and intermediary types (Irwin et al. 1986). Strains were stored at −80°C in vials containing sterile cryopreservative fluid on beads (Technical Service Consultants Limited, Lancashire, UK). Four isolates from diseased pear fruits obtained from Dr. P. Creemers, PCF-Royal Research Station of Gorsem, Belgium, and several isolates obtained from various hosts from the culture collection of Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, were also included in the study.

Pathogenicity assays

Bioassay on leaves Four experiments were performed during the period between May and September 2004. Symptomless pear leaves of cv. Conference were collected from an orchard not treated with fungicides. Only young fully expanded leaves were used, not more than five leaves were taken per tree and leaves from different trees were pooled. Leaves were placed into bags immediately after sampling and kept at approximate 5°C and high humidity during transportation. On the same day, 12 leaves were placed upper sides up on a plastic grid in plastic trays (size 50×30×6 cm), containing two layers of sterile filter paper moistened with tap water and covered by a polyethylene bag. The trays were stored overnight at 22°C in the dark and used for experiments at the following day. Four leaves within each tray served as one replicate of a treatment, so that each tray contained three different treatments. Each experiment consisted of a non-inoculated control treatment and two reference treatments, i.e. application of conidial suspensions of isolates *S. vesicarium* PRI 867 and *S. vesicarium* PRI 891, both pathogenic on pear. The first experiment consisted of 23 other treatments: applications of conidial suspensions of isolates of *S. vesicarium*. All 26 treatments were replicated in four blocks, where a block consisted of nine trays. The different treatments were completely randomised between and within trays within a block. In the other three experiments different isolates (and a different number of isolates) were tested. In total 116 isolates were tested, which were randomly allocated to one of the four experiments.

To obtain conidial suspensions of *S. vesicarium* isolates for inoculations, cultures stored at −80°C were sub-cultured in Petri dishes containing PDA for 28 days at 18°C with 12 h black light (350 nm) per day. Cultures were flooded with sterile tap water and, after gently rubbing with a rubber spatula, the resulting suspensions were filtered through sterile nylon gauze with a mesh of 200 µm. Concentrations of the conidial suspensions were determined with a haemocytometer and adjusted to 2.5×10^4 conidia ml^{-1} with sterile tap water. Flasks containing the conidial suspensions were kept in ice-water until use during the same day. Each leaf was inoculated with six droplets of 10 µl of a conidial suspension on the upper leaf surface, with three droplets on each side of the middle vein at a distance of approximately 2 cm between droplets. Inoculated leaves were incubated for 10 days at 22°C in the dark.

For classification of lesions occurring on inoculation sites the following rating was used: 0: no brown lesion; 1: brown lesion with diameter <2 mm; 2: brown lesions with diameter >2 mm. The mean disease incidence and severity was calculated per replicate. Disease incidence was established as the number of inoculation sites with brown lesions per replicate (maximum is 24). Disease severity (DS) was calculated from the numbers of lesions with a rating of 0, 1 or 2 (n_0, n_1 and n_2, respectively) as follows: $DS=(n_0\times0+n_1\times1+n_2\times2)/(n_0+n_1+n_2)\times100$. Symptoms that developed outside the inoculated sites were not recorded. In the few cases that such symptoms expanded and affected the correct assessment of the inoculation site, the leaf was disregarded.

Bioassay on fruits Healthy pear fruits of cv. Conference were harvested from an orchard not treated with fungicides. Fruits were stored for 17–21 weeks at −0.5°C (with 21% O_2 and 0% CO_2 during the first 6 weeks, followed by 3% O_2 and 0.5% CO_2). Three experiments were performed, each with 42 to 45 treatments. Treatments consisted of application of conidial suspensions from cultures of *S. vesicarium* which had been tested on leaves except three isolates which produced insufficient numbers of conidia. In addition, isolates *S. vesicarium* CBS 668.80 from *Lycopersicum esculentum*, and *P. herbarum* isolates CBS 322.49 from *Lathyrus odoratus* and CBS 205.82 from *Lunaria annua* were tested. Isolates were distributed randomly amongst the three experiments. Isolates PRI 867 and PRI 891 served as standards in each of the experiment. In the control treatment, water was applied to the pears.

For each treatment, four replicate plastic trays (size 36.5×23×5 cm) containing two layers of sterile filter paper moistened with tap water and a plastic grid separating fruits from the water layer were used. Each tray contained four fruits (kept at 22°C overnight before use to avoid condensation of atmospheric water on the surface during inoculation) which were inoculated with six droplets of 10 µl of a conidial suspension (1×10^5 conidia ml^{-1}) at a distance of approximately 2 cm on the fruit. Since droplets easily rolled off the pear surface when trays were handled, trays with inoculated pear fruits were kept open for

approximately 2 h at 20°C to allow drying of the droplets. Each tray was enclosed in a plastic bag and trays were put in a climate chamber in a randomised block design for 10 days at 22°C in the dark. Fruits were assessed as described above for leaves and the mean disease incidence and severity were calculated.

AFLP analysis

A sub-set of 65 isolates of *S. vesicarium*, representative of the different sampling locations and hosts, and some reference isolates (Table 1) was grown for four weeks on PDA at 18°C and 12 h black-light (350 nm) per day. Mycelium and spores were scraped from the agar surface and stored in sterile vials at −20°C. DNA was extracted using the Mo-bio Ultrapure soil DNA isolation kit (Biozym, Landgraaf, The Netherlands). The mechanical lysis step was modified by using bead-beating for 30 s at 4500 rpm in a ribolyser (Hybaid, UK). Two mixes were made per sample: 0.1 µl T4 DNA ligasebuffer (New England Biolabs, UK), 0.1 µl NaCl (0.5 M), 0.2 µl Mse*I* endonuclease (10 U μl^{-1}), 0.5 µl Eco*RI* endonuclease (20 U μl^{-1}), 0.08 µl T4 DNA ligase (400 U ml^{-1}) and 0.5 µl sterile water. The other mix contained 1 µl T4 DNA ligasebuffer, 1 µl NaCl (0.5 M), 0.5 µl BSA (1 mg ml^{-1}), 1 µl Mse*I*-adaptor, and 1 µl Eco*RI*-adaptor. Ten to 100 ng of genomic DNA was added to a total of 11 µl enzyme–buffermix and incubated at 37°C for 2 h. A 10 µl reaction mixture was used containing 2 µl of the diluted amplicons with ligated adaptors, 8 µl PCR-mix (0.25 µl Eco*RI* CoreSeq, 0.25 µl Mse*I* CoreSeq; Applied Biosystems), and 7.5 µl AFLP amplification CoreMix (Applied Biosystems). The pre-selective PCR programme started with a initial incubation at 72°C for 120 s, followed by 25 cycles: 94°C for 30 s, 56°C for 40 s, 72°C for 70 s, and cooled to 4°C; 10 µl of water was added to every pre-amplification sample. A diluted pre-amplification sample (1.5 µl) was mixed with 8.5 µl selective PCR-mix containing 0.5 µl NED-labelled Eco*RI*-primer+[C] (Applied Biosystems) or JOE-labelled Eco*RI* primer+[AG], 0.5 µl Mse*I*-primer+[A] (Applied Biosystems) and 7.5 µl AFLP amplification CoreMix. The selective PCR-programme used: initial denaturation at 94°C for 120 s, followed by ten stepdown-cycles (94°C for 20 s, 66°C–56°C for 30 s decreasing temperature by 1°C every cycle, 72°C for 120 s), 20 cycles 94°C for 20 s, 56°C for 30 s, 72°C for 120 s, followed by one cycle of 60°C for 30 min and cooled to 4°C. To analyse the selective PCR samples, 2 µl was mixed with 24.7 µl deionised formamide (Sigma, USA) and 0.25 µl GeneScan 500 ROX standard (Applied Biosystems). The mixture was denatured at 96°C for 5 min and snap-cooled on ice-water. The denatured samples were analysed on a ABI Prism 310 Genetic Analyser using performance optimised polymer and running the samples at 60°C, 15 kV running voltage for 30 min. Results were imported in Gelcompar II software (Applied Biomaths, Kortrijk, Belgium). The ROX internal size standard in each pattern was used for normalisation of the fingerprint database according to the software instruction manual.

Multilocus sequencing

For multilocus sequence analysis, fragments of three different genes were sequenced. For PCR and sequencing of elongation factor 1α (EF1) primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) were used. The ITS1-5.8S-ITS2 region was amplified with primers V9G and LS266 (Gerrits van den Ende and de Hoog, 1999) and sequenced using ITS1 and ITS4 (White et al. 1990). Part of the glutaraldehyde-3-phosphate-dehydrogenase gene (GPD) was amplified with gpd-f (5′-GCACCGACCACAAAATC-3′) and gpd-r (5′-GGGCCGTCAACGACCTTC-3′) followed by sequencing the amplicon with gpd-if (5′-CACGGCCAGTTCAAG-3′) and gpd-ir (5′-GGCGGGGTCCTTCTCC-3′; Câmara et al. 2002). The sequencing results were assembled using SeqMan of the Lasergene software packages (DNAStar Inc., Madison, U.S.A.) and analysed with BioNumerics 4.6 (Applied Maths, Kortrijk, Belgium).

Statistics

Disease incidences for leaves were analysed over all experiments using a generalised linear model (GenStat for Windows release 10.2; Genstat Committee, Algorithm Group Inc.). Experiment number, block within experiment and isolate are the explanatory variables. Since the incidences are percentages, a binomial error distribution was assumed with a logit link function and binomial totals of 24 (four leaves × six droplets). In case of overdispersion, the dispersion parameter was estimated and used to calculate the standard errors.

Table 1 Origin of fungal isolates analysed by AFLP

Isolate	Origin			
	Host	Location	Latitude	Longitude
Stemphylium vesicarium PCF-KOG 312	Pear fruit	Roeselare, Belgium	50°56′	3°07′
Stemphylium vesicarium PRI 850	Pear fruit	Leerbroek, The Netherlands	51°54′	5°03′
Stemphylium vesicarium PRI 859	Necrotic grass leaf	Randwijk, The Netherlands	51°57′	5°43′
Stemphylium vesicarium PRI 930	Pear fruit	Schoonrewoerd, The Netherlands	51°55′	5°07′
Stemphylium vesicarium PRI 931	Pear fruit	Schoonrewoerd, The Netherlands	51°55′	5°07′
Stemphylium vesicarium PRI 932	Pear fruit	Kapelle, The Netherlands	51°29′	3°58′
Stemphylium vesicarium PRI 933	Pear fruit	Dinteloord, The Netherlands	51°38′	4°22′
Stemphylium vesicarium PRI 917	Pear fruit	Lexmond, The Netherlands	51°58′	5°02
Stemphylium vesicarium PRI 872	Fallen pear leaf	Rossum, The Netherlands	51°48′	5°20′
Stemphylium vesicarium PRI 912	Pear fruit	Dinteloord, The Netherlands	51°38′	4°22′
Stemphylium vesicarium PRI 920	Pear fruit	Numansdorp, The Netherlands	51°38′	4°22′
Stemphylium vesicarium PRI 922	Pear fruit	Zuidland, The Netherlands	51°49′	4°16′
Stemphylium vesicarium PRI 921	Pear fruit	Schoonrewoerd, The Netherlands	51°55′	5°07
Stemphylium vesicarium PRI 894	Fallen pear leaf	Randwijk, The Netherlands	51°57′	5°43′
Stemphylium vesicarium PRI 926	Onion leaf	Nagele, The Netherlands	52°39′	5°43′
Stemphylium vesicarium PRI 916	Pear fruit	s' Gravenpolder, The Netherlands	51°27′	3°54′
Pleospora herbarum CBS 205.82	Lunaria	The Netherlands	–[a]	–
Stemphylium vesicarium PRI 924	Onion leaf	Slootdorp, The Netherlands	52°51′	4°58′
Stemphylium vesicarium PRI 895	Fallen pear leaf	Randwijk, The Netherlands	51°57′	5°43′
Stemphylium vesicarium PRI 915	Pear fruit	s' Gravenpolder, The Netherlands	51°27′	3°54′
Stemphylium vesicarium PCF-KOG 316	Pear fruit	Beveren, Belgium	51°13′	4°15′
Stemphylium vesicarium PRI 888	Fallen pear leaf	Randwijk, The Netherlands	51°57′	5°43′
Stemphylium vesicarium PCF-KOG 315	Pear fruit	Limburg, The Netherlands	51°16′	5°54′
Stemphylium vesicarium PRI 873	Fallen pear leaf	Velddriel, The Netherlands	51°46′	5°18′
Stemphylium vesicarium PRI 875	Necrotic grass leaf	Randwijk, The Netherlands	51°57′	5°43′
Stemphylium vesicarium PRI 852	Pear fruit	Krabbendijke, Netherlands	51°26′	4°07′
Pleospora tarda CBS 714.68 (T)	Medicago sativa	Australia	–	–
Stemphylium vesicarium PRI 913	Pear fruit	Kapelle, The Netherlands	51°29′	3°58′
Stemphylium vesicarium PCF-KOG 314	Pear fruit	Spalbeek, Belgium	50°57′	5°14′
Pleospora herbarum CBS 322.49	Lathyrus	The Netherlands	–	–
Pleospora herbarum CBS 156.45	Dianthus	The Netherlands	–	–
Stemphylium vesicarium PRI 870	Fallen pear leaf	Velddriel, The Netherlands	51°46′	5°18′
Stemphylium vesicarium PRI 865	Fallen pear leaf	Rossum, The Netherlands	51°48′	5°20′
Stemphylium vesicarium PRI 861	Necrotic grass leaf	Rossum, The Netherlands	51°48′	5°20′
Pleospora herbarum CBS 155.24	Allium	Netherlands	–	–
Stemphylium vesicarium PRI 871	Fallen pear leaf	Velddriel, The Netherlands	51°46′	5°18′
Stemphylium vesicarium PRI 911	Pear fruit	Vianen, The Netherlands	51°59′	5°06′
Stemphylium vesicarium PRI 855	Onion leaf	Randwijk, The Netherlands	51°57′	5°43′
Stemphylium vesicarium PRI 896	Onion leaf	Rhenen, The Netherlands	51°57′	5°34′
Stemphylium vesicarium PRI 897	Onion leaf	Rhenen, The Netherlands	51°57′	5°34′
Stemphylium vesicarium PRI 883	Fallen pear leaf	Kerkdriel, The Netherlands	51°46′	5°20′
Stemphylium vesicarium PRI 906	Onion leaf	Slootdorp, The Netherlands	52°51′	4°58′
Stemphylium vesicarium PRI 961	Asparagus leaf	Maasbree, The Netherlands	51°22′	6°03′
Stemphylium vesicarium PRI 946	Asparagus leaf	Roggel, The Netherlands	51°16′	5°55′
Stemphylium vesicarium PRI 963	Asparagus leaf	Roggel, The Netherlands	51°16′	5°55′
Stemphylium vesicarium PRI 964	Asparagus leaf	Ell, The Netherlands	51°13′	5°48′
Stemphylium vesicarium PRI 960	Asparagus leaf	Maasbree, The Netherlands	51°22′	6°03′
Stemphylium vesicarium PRI 956	Asparagus leaf	South-East Netherlands	–	–
Stemphylium vesicarium PRI 951	Asparagus leaf	Neeritter, The Netherlands	51°10′	5°48′
Stemphylium vesicarium PRI 952	Asparagus leaf	Neeritter, The Netherlands	51°10′	5°48′

Table 1 (continued)

Isolate	Origin			
	Host	Location	Latitude	Longitude
Stemphylium vesicarium PRI 941	Asparagus leaf	Maasbree, The Netherlands	51°22′	6°03′
Stemphylium vesicarium PRI 936	Asparagus leaf	Maasbree, The Netherlands	51°22′	6°03′
Stemphylium vesicarium PRI 943	Asparagus leaf	Maasbree, The Netherlands	51°22′	6°03′
Stemphylium vesicarium PRI 953	Asparagus leaf	Ell, The Netherlands	51°13′	5°48′
Stemphylium vesicarium PRI 945	Asparagus leaf	Roggel, The Netherlands	51°16′	5°55′
Stemphylium vesicarium PRI 948	Asparagus leaf	Horst, The Netherlands	51°27′	6°03′
Stemphylium vesicarium PRI 949	Asparagus leaf	Horst, The Netherlands	51°27′	6°03′
Stemphylium vesicarium PRI 889	Fallen pear leaf	Randwijk, The Netherlands	51°57′	5°43′
Stemphylium vesicarium PRI 902	Onion leaf	Westmaas, The Netherlands	51°47′	4°28′
Stemphylium vesicarium PRI 898	Onion leaf	Rhenen, The Netherlands	51°57′	5°34′
Stemphylium vesicarium PRI 904	Onion leaf	Westmaas, The Netherlands	51°47′	4°28′
Stemphylium vesicarium PRI 923	Onion leaf	Zeeland, The Netherlands	51°29′	3°47′
Stemphylium vesicarium PRI 885	Fallen pear leaf	Randwijk, The Netherlands	51°57′	5°43′
Stemphylium vesicarium PRI 908	Onion leaf	Munnekezijl, The Netherlands	53°18′	6°16′
Stemphylium vesicarium PRI 929	Onion leaf	Lelystad, The Netherlands	52°31′	5°29′
Stemphylium vesicarium PRI 899	Onion leaf	Marknesse, The Netherlands	52°43′	5°52′
Stemphylium vesicarium PRI 910	Onion leaf	Nagele, The Netherlands	52°39′	5°43′
Stemphylium vesicarium PRI 856	Onion leaf	Randwijk, The Netherlands	51°57′	5°43′
Stemphylium vesicarium PRI 925	Onion leaf	Nagele, The Netherlands	52°39′	5°43′
Stemphylium vesicarium PRI 927	Onion leaf	Nagele, The Netherlands	52°39′	5°43′
Stemphylium vesicarium CBS 668.80	Lycopersicon	Crete	–	–
Embellisia annulata CBS 302.84 (T)	Shell Cancer	North Sea Denmark	–	–

Isolates are presented in the same order as in Fig. 1

[a] Not recorded

Estimates of isolate effects, predictions of isolate means and standard errors were obtained. With the RPAIR procedure the isolates were classified in groups of which the predicted means did not differ significantly at $P=0.05$. Isolates, which were not part of the group that included the water control, were considered to be pathogenic. The same analysis was performed for disease incidence on fruits.

AFLP data were analysed using the cluster analysis tool in the Gelcompar II software package and a single linkage tree was constructed using the Pearson correlation. Robustness of the tree topology was assessed by bootstrap analysis using the programme standard of 100 replicates.

Results

Stemphylium spp. isolates were obtained from most samples of brown spot disease on pear fruits or from necrotic parts of leaves of pear, grasses, asparagus and onion after plating on ARSA. Isolates obtained differed in morphology, especially with respect to conidial shape and size. Strains were classified preliminarily according to these criteria and identified with the morphological key provided by Simmons (1969) as *S. vesicarium* (65 isolates) or *S. botryosum* (41 isolates), while ten *Stemphylium* isolates could not

Fig. 1 Composite dendrogram of AFLP primer AG-A with AFLP primer C-A. Isolates of *Stemphylium vesicarium* were obtained from brown spots of pear fruits collected in orchards in summer, necrotic pear leaves or grass leaves sampled from pear orchard floors in spring, or from necrotic leaf parts of asparagus or onion crops. *A–F* Clusters formed at 68% similarity cut-off. I and II are indicated clusters at a 56% similarity cut-off. Only bootstrap values >50 are given at the tree nodes of clusters >68% similarity. [1]Preliminary classification of isolates of *S. vesicarium* as morphological groups indicated as *S.v.* ('*S. vesicarium*'), *S.b.* ('*S. botryosum*') or *S.sp.* ('*Stemphylium* sp.'). Isolates of *Pleospora herbarum* (*P.h.*), *P. tarda* (*P.t.*) and *Embellisia annulata* (*E.a.*) are from the CBS collection. Isolates *PCF-KOG 312*, *314*, *315* and *316* were provided by P. Creemers, Gorsem, Belgium. [2]Locations: see Table 1. [3]–Disease incidence on pear leaves or fruits not significantly different from control treatment, +disease incidence significantly different from control treatment, *n.d.* not determined ▶

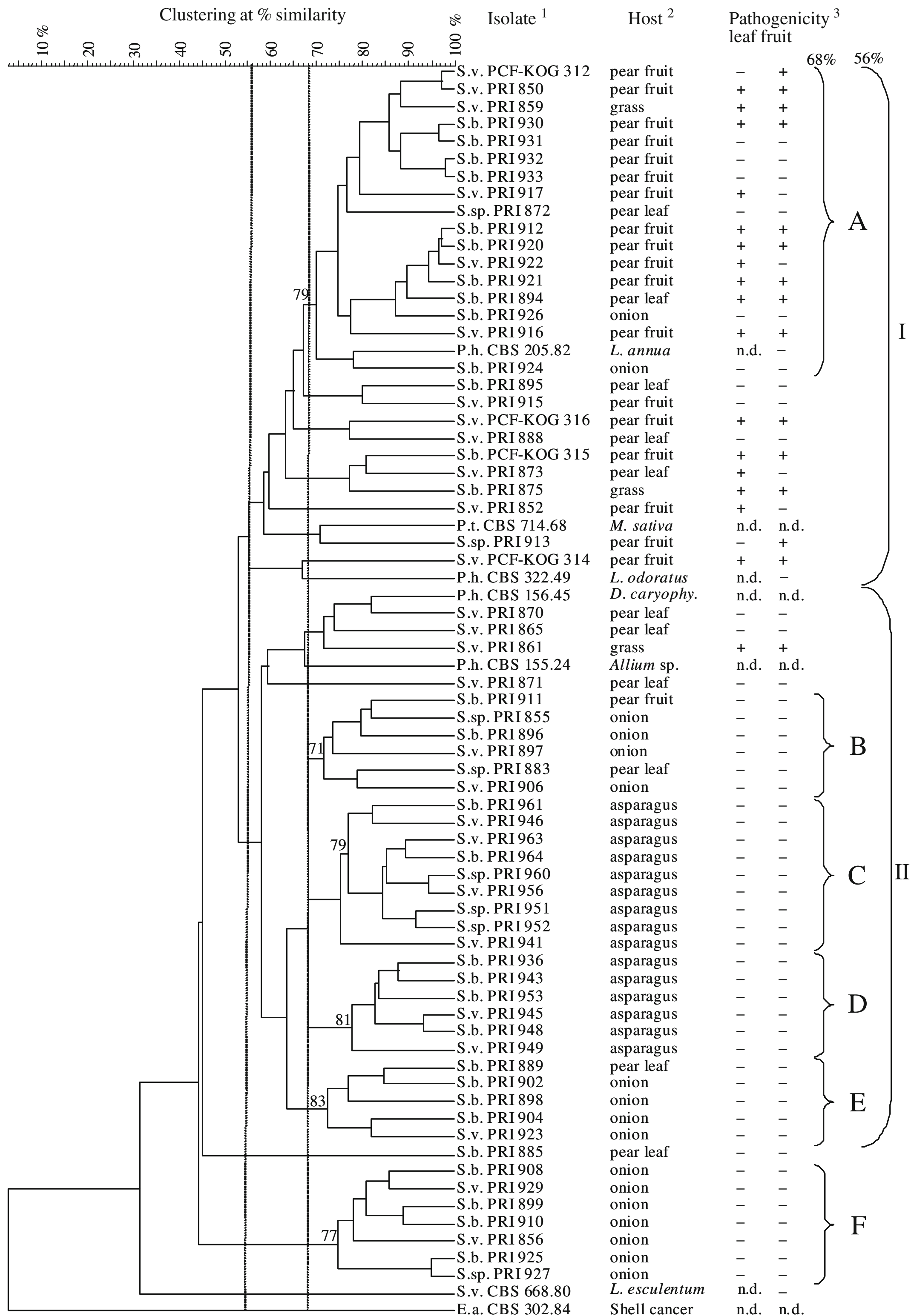
Clustering at % similarity
10 %
20
30
40
50
60
70
80
90
100 %
Isolate 1
Host 2
Pathogenicity 3
leaf fruit
68%
56%
S.v. PCF-KOG 312 pear fruit – +
S.v. PRI 850 pear fruit + +
S.v. PRI 859 grass + +
S.b. PRI 930 pear fruit + +
S.b. PRI 931 pear fruit – –
S.b. PRI 932 pear fruit – –
S.b. PRI 933 pear fruit – –
S.v. PRI 917 pear fruit + –
S.sp. PRI 872 pear leaf – –
S.b. PRI 912 pear fruit + +
S.b. PRI 920 pear fruit + +
S.v. PRI 922 pear fruit + –
S.b. PRI 921 pear fruit + +
S.b. PRI 894 pear leaf + +
S.b. PRI 926 onion – –
S.v. PRI 916 pear fruit + +
P.h. CBS 205.82 L. annua n.d. –
S.b. PRI 924 onion – –
S.b. PRI 895 pear leaf – –
S.v. PRI 915 pear fruit – –
S.v. PCF-KOG 316 pear fruit + +
S.v. PRI 888 pear leaf – –
S.b. PCF-KOG 315 pear fruit + +
S.v. PRI 873 pear leaf + –
S.b. PRI 875 grass + +
S.v. PRI 852 pear fruit + –
P.t. CBS 714.68 M. sativa n.d. n.d.
S.sp. PRI 913 pear fruit – +
S.v. PCF-KOG 314 pear fruit + +
P.h. CBS 322.49 L. odoratus n.d. –
P.h. CBS 156.45 D. caryophy. n.d. n.d.
S.v. PRI 870 pear leaf – –
S.v. PRI 865 pear leaf – –
S.v. PRI 861 grass + +
P.h. CBS 155.24 Allium sp. n.d. n.d.
S.v. PRI 871 pear leaf – –
S.b. PRI 911 pear fruit – –
S.sp. PRI 855 onion – –
S.b. PRI 896 onion – –
S.v. PRI 897 onion – –
S.sp. PRI 883 pear leaf – –
S.v. PRI 906 onion – –
S.b. PRI 961 asparagus – –
S.v. PRI 946 asparagus – –
S.v. PRI 963 asparagus – –
S.b. PRI 964 asparagus – –
S.sp. PRI 960 asparagus – –
S.v. PRI 956 asparagus – –
S.sp. PRI 951 asparagus – –
S.sp. PRI 952 asparagus – –
S.v. PRI 941 asparagus – –
S.b. PRI 936 asparagus – –
S.b. PRI 943 asparagus – –
S.b. PRI 953 asparagus – –
S.v. PRI 945 asparagus – –
S.b. PRI 948 asparagus – –
S.v. PRI 949 asparagus – –
S.b. PRI 889 pear leaf – –
S.b. PRI 902 onion – –
S.b. PRI 898 onion – –
S.b. PRI 904 onion – –
S.v. PRI 923 onion – –
S.b. PRI 885 pear leaf – –
S.b. PRI 908 onion – –
S.v. PRI 929 onion – –
S.b. PRI 899 onion – –
S.b. PRI 910 onion – –
S.v. PRI 856 onion – –
S.b. PRI 925 onion – –
S.sp. PRI 927 onion – –
S.v. CBS 668.80 L. esculentum n.d. –
E.a. CBS 302.84 Shell cancer n.d. n.d.
79
71
79
81
83
77
A
B
C
D
E
F
I
II

Table 2 Selected reference strains analysed with sequencing for calibration of identifications

Number	Name	Substrate
Camara 537	*S. botryosum / P. tarda*	*Lupinus angustifolia*
AF071345 = EGS 08-069=CBS 116596	*S. botryosum / P. tarda*	*Medicago sativa*
CBS 714.68 (T) = EGS 04-118C	*S. botryosum / P. tarda*	*Medicago*
CBS 192.86=EGS 36-088 (T)	*S. alfalfae / P. alfalfae*	*Medicago sativa*
CBS 482.90=EGS 37-073 (T)	*S. gracilariae / P. gracilariae*	*Gracilaria*
CBS 191.86=IMI 276975 (T)	*S. vesicarium / P. herbarum*	*Medicago sativa*
CBS 311.92	*S. vesicarium / P. herbarum*	*Allium cepa*
CBS 205.82	*S. vesicarium / P. herbarum*	*Lunaria annua*
CBS 407.54 (T)	*S. loti*	*Lotus*

unambiguously be attributed to one of these species and were maintained as 'intermediary type'. Naming was corrected on the basis of sequence data, as specified below. No preferences of morphological types were noted for particular host tissues (Fig. 1).

For identification at species level, 21 strains representing each of the AFLP clusters (Fig. 1) and either of the sampled host plant fragments of EF1-, ITS- and GPD-genes were sequenced and compared to reference strains (Table 2) maintained in the CBS collection. All strains except the ex-type strain of *S. botryosum* were found to be identical, and no difference was found either with the ex-type strains of *Stemphylium herbarum* (teleomorph: *Pleospora herbarum*), *S. alfalfae* (teleomorph: *P. alfalfae*) and *S. gracilariae* (teleomorph: *P. gracilariae*). Of *S. vesicarium* no type strain is available, but a strain deposited by Simmons (1967) under this name, EGS37-067, was also found to be identical. Identical sequences were found in strains originating from divergent host plants including mono- and dicots (*Allium, Asparagus, Dianthus, Cucumis, Gracilaria, Lycopersicon, Lunaria, Malus, Medicago*), and originated from different continents (The Netherlands, Israel, India, Australia). The ex-type strain of *S. botryosum* (teleomorph: *Pleospora tarda*) differed from *S. vesicarium / P. herbarum* in two ITS positions and significant deviations in EF1 and GPD.

Thirty-six of 116 isolates tested caused disease incidences on pear leaves that were statistically significantly different from the disease incidence found in water-treated control leaves (Table 3). The pathogenic isolates all originated from pear orchards, with the exception of a single isolate from onion that caused low disease incidence. Pathogenic isolates originated either from brown spots of fruits or from necrotic pear leaves collected from orchard floors in spring. In addition, seven out of ten isolates from necrotic leaves of orchard lawn grasses were pathogenic to pear leaves. Isolates from asparagus or onion caused no symptoms on pear leaves with the exception of the aforementioned isolate. Data on disease severity gave similar results (data not shown). When the pear leaf experiments were repeated on pear fruits, the majority of the tested isolates showed the same pathogenicity pattern (Table 4; Fig. 2). No isolates from asparagus or onion caused brown spot on pear fruit, but the majority of isolates from pear orchards induced brown spots regardless of their origin, i.e., from pear fruits or leaves, or from grasses.

Disease incidences for pathogenic isolates ranged from approximately 10% to 70% on both leaves and fruits. No difference in the incidence (Fig. 2) or

Table 3 Pathogenicity on detached pear leaves of *Stemphylium vesicarium* isolates originating from different hosts and tissues under controlled conditions

Origin	Number of isolates (number of locations)		
	Total	Pathogenic[a]	Non pathogenic[a]
Pear orchards			
Infected fruit (summer)	29 (18)	20 (16)	9 (8)
Dead pear leaf (spring)	22 (5)	8 (4)	14 (5)
Dead grass leaf (spring)	10 (3)	7 (3)	3 (1)
Other crops			
Onion leaf	22 (10)	1 (1)	21 (10)
Asparagus leaf	30 (10)	0 (0)	30 (10)
Green apple leaf	3 (1)	0 (0)	3 (1)

[a] For pathogenic isolates, incidence of leaf symptoms was significantly higher than in the water control (P=0.05)

Table 4 Pathogenicity on detached pear fruits of *Stemphylium vesicarium* isolates originating from different hosts and tissues under controlled conditions

Origin	Number of isolates (number of locations)		
	Total	Pathogenic[a]	Non-pathogenic[a]
Pear orchard			
Infected fruit (summer)	29 (18)	18 (16)	11 (11)
Dead pear leaf (spring)	22 (5)	5 (4)	17 (5)
Dead grass leaf (spring)	9 (3)	7 (3)	2 (1)
Other crops			
Lathyrus odoratus	1 (1)	0 (0)	1 (1)
Lunaria annua	1 (1)	0 (0)	1 (1)
Lycopersicon esculentum	1 (1)	0 (0)	1 (1)
Onion leaf	21 (10)	0 (0)	21 (10)
Asparagus leaf	29 (10)	0 (0)	29 (10)
Green apple leaf	3 (1)	0 (0)	3 (1)

[a] For pathogenic isolates, incidence of leaf symptoms was significantly higher than in the water control ($P=0.05$)

severity (data not shown) was found for pathogenic isolates originating from fruits or leaves of pear or from orchard lawn grasses. Also, no difference in disease incidence (Fig. 3) or severity (data not shown) was found between isolates of *S. vesicarium* preliminary classified as morphological groups '*S. vesicarium*' or '*S. botryosum*' or isolates with intermediary conidial characteristics.

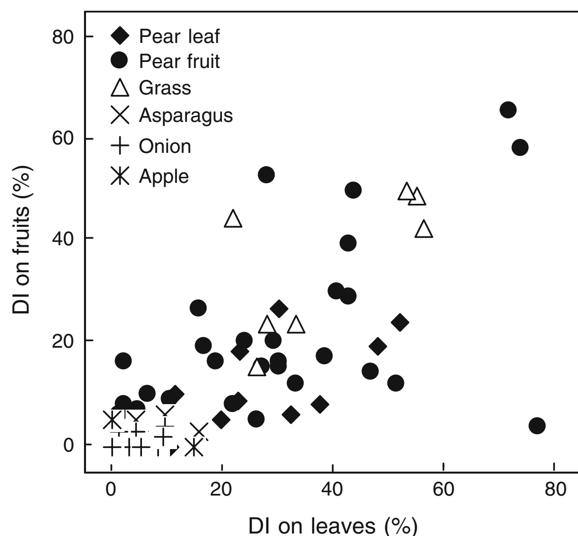

Fig. 2 Disease incidence (%) (DI) in pear leaves and fruits under controlled conditions caused by *Stemphylium vesicarium* isolates from different hosts and tissues

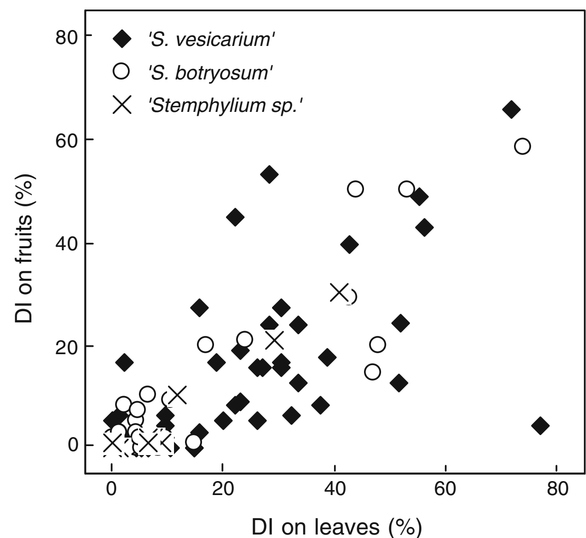

Fig. 3 Disease incidence (%) (DI) in pear leaves and fruits under controlled conditions caused by *Stemphylium vesicarium* isolates preliminarily classified as morphological groups '*S. vesicarium*', '*S. botryosum*' or '*Stemphylium* sp.'

Cluster analysis of the AFLP patterns (Fig. 1) of *Stemphylium* strains showed three main clusters, I, II and F at a level of 56% similarity. Cluster I contained 30 isolates, 25 of which were from pear orchards and 19 were found to be pathogenic on pear leaves or fruits. Cluster II contained 32 isolates including all strains from asparagus and the majority of the isolates from onion. Within this group, only six isolates were from pear orchards, of which only a single isolate proved to be pathogenic to pear. Isolates of *S. vesicarium* preliminary classified as morphological groups '*S. vesicarium*', '*S. botryosum*' or '*Stemphylium* sp.' (Fig. 1) were found in all clusters without any tendency to cluster according to conidial characteristics.

Above a level of 68% similarity, various clusters could be distinguished with a bootstrap value above >70. Cluster A (70% similarity, bootstrap 79) contained 18 isolates, 15 of which originated from pear orchards including an isolate from a dead grass leaf. Eleven of these isolates proved to be pathogenic to pear leaves or fruits. Cluster C (80% similarity, bootstrap 79) with nine isolates and Cluster D (80% similarity, bootstrap 81) with six isolates exclusively contained isolates from asparagus which were not pathogenic to pear. Three remaining clusters (B, E and F) in total contained 18 isolates of which 14 originated from onion, while three were from pear tissues. None of these 18 isolates were

pathogenic to pear. The remaining 21 isolates of *Stemphylium* or *Pleospora* species (from various hosts) belonged to smaller distinct clusters.

Discussion

Identification of isolates of *Stemphylium* proved to be problematic. Initial identifications were established on the basis of conidial dimensions as described by Simmons (1967), distinguishing *S. botryosum* (*P. herbarum*) and *S. vesicarium* as the main species. In a later publication, Simmons (1985) segregated *S. herbarum* (*P. herbarum s. stricto*) from *S. botryosum*, for which the teleomorph *P. tarda* was introduced. In addition, *S. alfalfae* (*P. alfalfae*) was erected. Câmara et al. (2002) analysed the phylogenetic relationships between various *Stemphylium* species on the basis of rDNA ITS and glyceraldehyde-3-phosphate dehydrogenase (GPD) sequence data. *Stemphylium herbarum*, *S. vesicarium* and *S. alfalfae* could not be distinguished. This is confirmed by our morphological and molecular data; the species should be regarded as synonymous. In contrast, molecular data of Câmara et al. (2002) and the present authors supported the separation of *S. botryosum* (*P. tarda*), as advocated by Simmons (1985). The oldest teleomorph name available for the taxon presently known under the anamorph name *S. herbarum* is *P. herbarum* (based on *Sphaeria herbarum*), and the oldest anamorph name for the same taxon is *S. vesicarium*. The name *S. vesicarium* is used in the present paper.

Initial identifications on the basis of conidial dimensions alone were incorrect, as molecular data indicated that our entire set of strains belonged to *S. vesicarium* (*P. herbarum*), despite the frequent occurrence of strains microscopically resembling *S. botryosum*. We conclude that distinguishing these species based on conidial characteristics is impossible. *Pleospora herbarum* teleomorphs of *S. vesicarium* are characterised by thin-walled, rapidly maturating ascomata, whereas the *P. tarda* teleomorphs of *S. botryosum* develop slowly from hard sclerotial bodies (Simmons 1985). Reliable identification is possible with the aid of rDNA ITS data, showing two base differences with *S. vesicarium* (Câmara et al. 2002)

Host and pathogenicity traits showed a reasonable degree of homogeneity with the AFLP data analysis of strains from The Netherlands. AFLP patterns of *S. vesicarium* strains that were geographically remote, such as Crete, U.S.A., Germany, Spain or Australia (not shown), patterns were not clearly clustering. The structuring of our dataset suggests that *S. vesicarium* populations disperse slowly, suggesting inbreeding and leading to low degrees of polymorphism.

The clustering based on AFLP fingerprinting showed concordance with host plants in case of isolates originating from asparagus, which are represented in clusters C and D. Isolates from onion are found in clusters B, E and F with two exceptions (PRI 924 and PRI 926). Non-pathogenic haplotypes originating from pear appear in clusters I and II. All pathogenic isolates to pear with one exception are found within cluster I, but AFLP analysis was not able to separate pathogenic from non-pathogenic isolates within this cluster. *Stemphylium* isolates originating from onion and asparagus crops in The Netherlands at some geographic distance from pear orchards proved to be not-pathogenic to pear. AFLP typing showed that such isolates belong to separate clusters from the majority of isolates obtained from pear. Some of the isolates originating from pear orchards were found to be pathogenic to pear. These isolates originated from diseased fruits, also from necrotic leaves of pear or orchard lawn grasses. These plant tissues also harboured non-pathogenic isolates.

These findings indicate that haplotype variants within a population of *Stemphylium* may occur beside each other in the same crop. Within a single pear orchard, pathogenic isolates have the potential to invade healthy pear tissue, but are also able to grow saprophytically in necrotic pear tissue as well as on necrotic tissues from other plants. In addition to these pathotypes to pear, non-pathogenic haplotypes are present in the orchard. These findings are in line with reports on host-specificity of *S. vesicarium* in pear (Singh et al. 1999) and on the saprophytic potential of *S. vesicarium* (Rossi et al. 2005b).

Singh et al. (1999) investigated the role of host-specific toxins in *S. vesicarium*. Sterile culture filtrates of pear-pathogenic isolates induced necrosis on pear leaves whereas culture filtrates of non-pathogenic isolates did not induce necrosis (Singh et al. 1999). Neither the isolates nor their sterile culture filtrates caused symptoms on other hosts such as apple, tomato or rose. Two host-specific toxins, SV-toxin 1I and SV-toxin II, were found in culture filtrates of pathogenic isolates and it could be demonstrated that

severity of necrosis correlated with concentration of the toxins and on the susceptibility of pear varieties. The typical v-shaped symptom of brown spot on pear leaves could be explained by transportation of the toxins from infected sites through veins followed by vein necrosis (Singh et al. 1999).

Rossi et al. (2005b) demonstrated that dead leaves of different grasses as well as white clover are a potential growth substrate for *Stemphylium vesicarium* isolates pathogenic to pear. They inoculated autoclaved leaves of *Poa pratensis*, *Festuca rubra*, *F. ovina*, *Lolium perenne*, *Digitaria sanguinalis, Setaria glauca* and *Trifolium pratensis* with *S. vesicarium* isolates pathogenic to pear and incubated them under controlled conditions. *Stemphylium vesicarium* was able to grow on the autoclaved substrates, and formed conidia and pseudothecia on the dead leaves. The fungus was re-isolated and it was proven that isolates were still pathogenic to pear. Rossi et al. (2005b) also observed formation of pseudothecia with ascospores on autoclaved and artificially inoculated leaves of lawn grasses exposed to orchard conditions. Green leaves of lawn grasses artificially inoculated with *S. vesicarium* were not colonised by *S. vesicarium*.

In our study we demonstrated that the majority of *Stemphylium* isolates from necrotic leaves of grasses collected in pear orchards with a brown spot history were pathogenic to pear. From the combined results of both studies it can be concluded that *S. vesicarium* pathogenic to pear has the potential to colonise dead lawn leaves saprophytically under natural conditions, and to reproduce on such plant residues present on the orchard floor; the propagules subsequently may become airborne and eventually reinfect pear.

Epidemics of brown spot are initiated by primary inocula in spring. In the similar epidemiological situation of apple scab (*Venturia inaequalis*), sanitation measures have been developed directed against overwintering of the scab fungus in apple leaves as the main primary inoculum source. These measures include leaf removal, leaf shredding, stimulation of leaf decomposition and pathogen control by application of urea or antagonistic fungi (MacHardy et al. 2001). Similar attempts have been directed against *S. vesicarium* overwintering in pear leaves (Llorente et al. 2006). However, since pathogenic *S. vesicarium* isolates also have the potential to develop saprophytically on residues of plants different from pear, the epidemiological situation is more complex than for apple scab. Besides pear leaves, the residues of the lawn vegetation may also be sources of primary inoculum. Furthermore, residues of lawn vegetation are present during the entire year and may thus also play a role during summer epidemics as a continuous source of conidia. In this situation, sanitation measures focusing on fallen pear leaves can lead only to partial success.

Many necrotrophic pathogens have a life-cycle with a pathogenic stage alternating with a saprophytic stage. Pathogens colonise host tissue after induction of necrosis, and subsequently spread within the plant during senescence. Based on this competitive advantage, crop residues are often colonised by necrotrophic pathogens of the respective crop and are considered as inoculum sources for future epidemics. Necrotrophic pathogens can also colonise non-host tissue in competition with other naturally occurring saprophytic micro-organisms. Such a development on non-host residues may play an underestimated role in the epidemiology of fungal diseases. More quantitative studies on pear brown spot epidemiology are needed to understand the importance of a saprophytic stage on non-host tissues compared to host tissue for overwintering but also during summer epidemics. For such studies, *Stemphylium* populations non-pathogenic on pear must be distinguished from pear-pathogenic isolates. Testing for pathogenicity in laborious bio-assays as we did in our study is not suitable for such quantitative studies. We are currently developing quantitative molecular techniques to discriminate pear-pathogenic *S. vesicarium* populations from *Stemphylium* populations non-pathogenic on pear. Quantification of these populations may contribute to the development of sanitation measures and epidemiological studies. Furthermore, it may become possible to forecast risks of brown spot epidemics using such tools.

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