Brown spot disease in ‘Rocha’ pear Portuguese orchards

P. Reis[1], C. Rego[1], M. Mota[1], T. Comporta[2] and C.M. Oliveira[1]

[1]Linking Landscape, Environment, Agriculture and Food (LEAF), Instituto Superior de Agronomia (ISA), Universidade de Lisboa, Lisboa, Portugal; [2]CPF - Centro de Produção e Comercialização Hortofruticola, Lda., Estrada Nacional 8, Km 69 - Sobreiral, 2540-170 Bombarral, Portugal.

Abstract

Brown spot, caused by *Stemphylium vesicarium*, is the main fungal disease of pear causing severe crop losses. Disease incidence was evaluated in flowers and fruits throughout the growing season in two orchards with moderate (MI) and low (LI) disease incidence history, both of them with two different weed control systems, mechanical cultivation (M) and natural cover (C). In both orchards. vegetation in the tree strip was controlled with herbicide. Selected isolates were used to fulfill pathogenicity tests. These tests were performed in a commercial orchard, without the disease. Fruits without lesions were inoculated with mycelial discs of a total of 15 isolates. Controls were inoculated with PDA discs. Flowers’ disease incidence on May 16 (BBCH 65) in LI-C orchard was lowest (31.7%) differing significantly from the other treatments. Sepals, styles and stigma were the most infected organs, however, the pathogen was also isolated from ovaries and stamens. Regarding fruits’ disease incidence, on the May 30 (BBCH 72), LI-M showed the lowest incidence (20.0%) and MI-C, the highest (56.7%) meaning that infection in the initial stages of fruit growth is strongly influenced by natural cover (inoculum source). However, on July 15, orchard history disease incidence was dominant with 51.7% of the fruits in MI C and M average was symptomatic whereas in LI-C and M only 17.5% average showed symptoms. Fruit disease prevalence was also lower in the orchards LI history. Results from pathogenicity tests revealed that 53.4% of the isolates were pathogenic. Among them 25% of the isolates obtained from flowers and 75% of the isolates obtained from fruits produced typical brown spot symptoms. The most virulent isolates were obtained from fruits, but there was no relation with orchard treatments. These results show that cultural control measures based on inoculum reduction are of utmost importance and that an isolate’s pathogenicity is a genetic feature independent from the orchards.

Keywords: flower and fruits infections, Koch postulates, *Stemphylium vesicarium*

INTRODUCTION

The disease caused by *Stemphylium vesicarium* (Wallr.) E.G. Simmons has been identified in the most relevant areas of pear production in Europe, mainly in Italy, Spain, the Netherlands, France, Belgium and Portugal (Llorente and Montesinos, 2006). Also referred to as brown spot of pear (BSP), this disease has gained importance causing damages whose severity varies from year to year according to factors such as weather conditions and the orchard disease history. In the main production region of ‘Rocha’ pear, there are favourable conditions for the disease development, becoming the key disease leading to orchard losses over 50%. ‘Rocha’ pear is moderately susceptible to the pathogenic fungus (Sousa et al., 2004) and the high incidence of the disease can arise from several factors, such as the opportunity and effectiveness of the application of plant protection products, the implementation of cultural practices aimed at reducing the inoculum (Llorente et al., 2010, 2012) such as the removal of affected fruits and potentially infected leaves, pruning wood, weed control, mobilizations, nutrition, plant growth regulators and other problems (e.g., *Phylloxera*), which may influence the incidence of the disease. Despite the development of new strategies to control the disease that combine good sanitation practices and the application of fungicides based on forecast models, the saprophytic behaviour of this pathogen has hampered the management of this disease (Moragrega et al., 2018). Parasitic and saprophytic phases in the life cycle of *S. vesicarium* and *Pleospora allii* on pear orchards were described by Llorente et al. (2012).

Puig et al. (2015) showed that several species of *Stemphylium* coexist in pear orchards with *S. vesicarium*, the causal agent of BSP, and only isolates identified as *S. vesicarium* were pathogenic on pear and consequently, direct measurements of the airborne inoculum using volumetric spore traps may overestimate the actual pathogen population. On the other hand, within the species *S. vesicarium*, pathogenic (with different degrees of virulence) and non-pathogenic isolates have been reported (Köhl et al., 2009) and recently, a specific TaqMan-PCR technique has been developed to detect and quantify pear-pathogenic inoculum of *S. vesicarium* in pear orchards (Köhl et al., 2013). Moreover, the relationship between the source of inoculum (air samples, leaf debris, and infected host and non-host tissues) and pathogenic/aggressiveness profile was confirmed by Moragrega et al. (2018).

A trial was conducted in 'Rocha' commercial orchards in order to answer the following questions: are all the floral parts infected from the start of flowering? What is the influence of the soil orchard management strategies in the incidence of the disease? Are the *Stemphylium* isolates obtained from flowers and fruits at different developmental stages all pathogenic? The present results will influence orchard practices and may help to implement a program of strategies to reduce BSP.

MATERIAL AND METHODS

Isolates of *Stemphylium*

Stemphylium spp. were isolated from pear flowers and fruits collected along the spring and summer of 2016 in two 'Rocha' pear grafted on quince EMA orchards, trained as central axis and located in Bombarral, Roliça 39°19'21.6"N; 9°11'34.9"W. One orchard had a low brown spot incidence (LI) history and other had a moderate incidence (MI) history of the disease. The LI orchard was planted in 1999 and trees spaced 4×1.5 m, while the MI orchard was planted in 2008 with a spacing of 3.5×1 m. In each orchard weed control systems were established, alleyways with mechanical cultivation (M) and with natural cover (C). In both orchards tree strip vegetation was controlled with herbicide.

In each orchard, three rows with 10 trees were selected per each treatment. Two flowers were collected from each tree from opposite sides of the canopy on the May 9 and 16 – BBCH 62 and 65, respectively. Two asymptomatic or symptomatic fruits were collected from each tree form opposite sides of the canopy on May 30 (15 mm diameter, BBCH 72) and on July 15 (45 mm diameter, BBCH 77), respectively. Twenty flowers and fruits were collected from each treatment. For isolation of *Stemphylium* spp. from pear flowers, styles, stigmas, ovaries, stamens and sepals were surface-washed while for the pear fruits, asymptomatic or symptomatic fragments were surface disinfected with a 1% hypochlorite solution. All tissues were then rinsed in sterilized distilled water and four pieces of tissues were plated onto Petri dishes containing PDA (PDA, Difco, BD, Sparks, MD, USA) amended with chloramphenicol (250 mg L^{-1}), and incubated for a period of 10 days, at 24°C, in darkness. Plates were observed at 100×magnification and mycelial discs of *Stemphylium* spp. were transferred to MEA plates (malt extract 1 g L^{-1}, agar 15 g L^{-1}). After 10 days, the colony characters were noted. Colony colours were rated according to Rayner (1970) and for morphological identification cultures were grown on MEA for 14 days at 25°C with 12 h black light day^{-1} (Simmons 1967, 1969). Conidia were measured with the Leica Suite v3.16 program, for each isolate under study. DNA extraction was performed using DNeasy™ Plant Mini Kit (QiaGen) according to the manufacturer's instructions. The ITS regions were amplified using primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al. 1990). Sequences of gpd gene were amplified using GPD1 and GPD2. Amplifications were performed according to Puig et al. (2015). Both strands of the PCR products were sent for sequencing at STABVIDA (Lisbon, Portugal). Sequences obtained were edited and aligned using MEGA X to find a consensus sequence. These sequences were then compared with sequences from GenBank in BLAST searches, and species identification was obtained when at least 99% of similarity was found.

Incidence and prevalence were evaluated by the percentage of flower and fruits infected and by the percentage of colonies of *S. vesicarium* obtained from infected flower and fruits, respectively.

Pathogenicity tests

Pathogenicity tests were carried out in one 'Rocha' pear orchard without brown spot incidence history, grafted on quince EMA orchards, trained as central axis and located in Freiria, Torres Vedras. Ten randomly chosen fruits in different tree canopies were inoculated with each isolate (treatment). A total of 15 isolates of *S. vesicarium* were selected among the isolates previously obtained from flowers and fruits. Six isolates were obtained from asymptomatic flowers (FL7, FL8, FL9, FL10, FL11, FL17) and nine isolates from asymptomatic/symptomatic fruits (FR2, FR8, FR10, FR11, FR12, FR15, FR13, FR16, FR17).

Inocula were produced on PDA Petri dishes incubated for a period of 10 days, at 24°C, in darkness. Fruits were surface disinfected with 70% ethanol and inoculated with 3-mm mycelial plugs taken from the actively growing margin of the colonies. Each inoculation point was covered with moist cotton wool and sealed with Parafilm. Control fruits were inoculated with PDA plugs and following the above procedure. Ten replicates were made for each of isolate. After one month, fruits showing symptoms were collected for reisolations. Firstly, they were washed for 15 min under running tap water and left to dry. Isolations were then performed by cutting small pieces from the margin of the lesions, which were surface disinfected with sodium hypochlorite (1%) for 1 min rinsed in sterile water and dried under a sterile air flow. Four pieces were placed in Petri dishes with PDA amended with chloramphenicol (250 mg L^{-1}) and incubated at 24°C, in darkness. Colonies of *S. vesicarium* were identified (Simmons, 1967, 1969) and their frequency of isolation determined.

Data analysis

When assumptions for variance analysis were accomplished, data were analysed by ANOVA, and the means compared by Tukey test ($\alpha=0.05$) using Statistix 9 (Analytical Software, Tallahassee, Florida). In other cases, a non-parametric analysis using the ranks-based Kruskal-Wallis test was used ($\alpha=0.05$).

RESULTS

Cultural and morphological characters of the 15 isolates under study could be assigned to *S. vesicarium* (Simmons, 1967, 1969). Colonies texture of isolates was cottony and colour varied from pale or dark brown. Conidia dimensions ranged from 24.4 to 33.8 µm (length) × 15.0 to 22.1 µm (width) and ratio l/w from 1.7 to 2.2. Moreover, the species identification was confirmed through the sequences obtained from the amplification of ITS and gpd regions. Analyses of consensus sequences indicated that all the isolates were in fact *S. vesicarium* in accordance to phenotypic characters.

Infection percentage of *S. vesicarium* assessed on flowers and fruits infected are shown in Table 1. The mean percentage of infection at the beginning of flowering May 9 (BBCH 62) was 58.5% and the mean values between orchards and soil maintenance systems were not statistically different, however a week later (BBCH 65) flower percentage of infection was slightly higher (67.8%) except in the low incidence orchard where the alleyways had grass cover (31.7%) ($P<0.05$).

Regarding fruits with 15 mm diameter (May 30) the level of infection ranged from 20% in the low incidence orchard with alleyways with mechanical cultivation to 56.7% in the moderate incidence orchard where the alleyways were mechanical cultivated ($P<0.05$). Overall incidence of infection was lower when the management soil system was mechanical cultivation compared with alleyways with natural cover. On July 15 (fruits with 45 mm diameter, BBCH 77) the difference in incidence was related with the orchard incidence history, 51.7 and 29.2% of incidence in the moderate incidence and low incidence orchard, respectively ($P<0.05$).

Table 1. Incidence of infection of *S. vesicarium* on flowers and fruits.

Treatments		Infection (%)			
		on flowers (%)		on fruits (%)	
		May 9	May 16	May 30	July 15
MI	C	61.6	70.0 a	56.7 a	51.7 a
MI	M	60.0	65.0 a	35.0 bc	51.7 a
LI	C	58.3	31.7 b	38.3 b	23.3 b
LI	M	53.3	68.3 a	20.0 c	11.7 b
Prob		N.S.	<0.001	<0.001	<0.001

Significance level P or N.S. not significant, according to the analysis of variance (ANOVA). Means in the same column followed by different letters differ according to the Tukey test α=0.05.

MI – moderate incidence orchard; LI – low incidence orchard; C – alleyways with natural cover; M – alleyways with mechanical cultivation.

All flower organs were infected as shown in Figure 1. Overall, there were differences between the two flowering stages and regarding the distribution between them, gynoecium (ovary, styles and stigma) account for 51.6%, stamens 30.7% and sepals 28.7%.

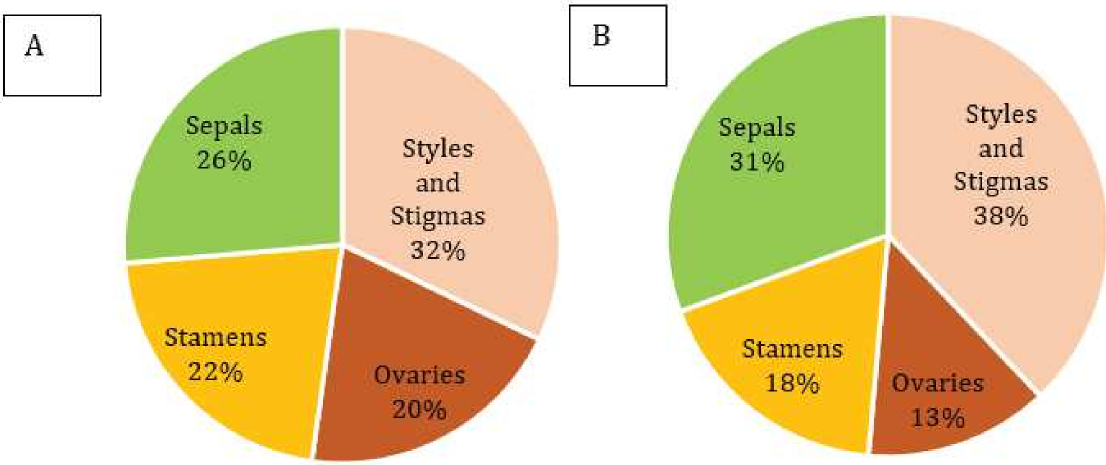

Figure 1. Percentage of flower organs infected with *S. vesicarium*: A – beginning of flowering (BBCH 62) and B – full flowering (BBCH 65).

S. vesicarium prevalence, assessed by the percentage of colonies of *S. vesicarium* isolated from infected flower and fruits, is presented in Table 2. The mean *S. vesicarium* prevalence at the beginning of flowering, May 9 (BBCH 62) ranges from 20.8 to 26.3%. Although not statistically different, the lowest values were obtained in the LI history. A week later (BBCH 65), flower infection of LI-C was the lowest and differed statistically from other treatments (12.0%) (P<0.05). Regarding fruitlets with 15 mm diameter (May 30), the value of prevalence ranged from 6.8% in the LI-M treatment to 18.0% in the MI-C treatment (P<0.05). No significantly differences occurred among treatments. On July 15 (fruits with 45 mm diameter, BBCH 77), the lowest values of prevalence were achieved in the LI orchard history. In general, BSP incidence orchards history seemed to be a crucial feature regarding the prevalence of infection in flowers and fruits.

Table 2. Prevalence of *S. vesicarium* on flowers and fruits.

Treatments		On flower		On fruits	
		May 9	May 16	May 30	July 15
MI	C	25.0	31.8 a	18.0 a	13.3 A
MI	M	26.3	28.3 a	8.8 b	13.8 A
LI	C	21.3	12.0 b	9.5 b	5.8 B
LI	M	20.8	28.3 a	6.8 b	3.0 B
Prob		N.S.	<0.001	<0.001	<0.001

Significance level P or N.S. not significant. according to the analysis of variance (ANOVA). Means in the same column followed by different letters differ according to the Tukey test α=0.05.
MI – moderate incidence orchard; LI – low incidence orchard; C – alleyways with natural cover; M – alleyways with mechanical cultivation.

Pathogenicity of *S. vesicarium* isolates

Seven isolates, corresponding to 47% of the total evaluated, did not produce lesions and were non-pathogenic on cultivar 'Rocha' pear fruit. This group included four isolates obtained from flowers and three isolates from fruits (Table 3). Among the non-pathogenic isolates obtained from flowers, three were obtained in the first sample collected on May 9 and one in the second sample collected on May 16. Concerning isolates obtained from fruits, three were obtained on May 30, June 21, and July 15 showing that non-pathogenic isolates can be isolated all over the growing season. Also, there were no differences among non-pathogenic isolates and orchards' brown spot incidence history and mechanical cultivation versus natural cover (Table 3).

Table 3. Pathogenicity results obtained under orchard conditions: isolates reference, collecting date, orchard isolates origin, reisolations percentage and necrosis area. Isolates obtained from flowers (FL), from fruits (FR), control – medium, disks; numbers are the isolate reference.

Isolate reference	Collecting date	Orchard isolate origin	Reisolation (%)	Necrosis area (mm^2)[a]
FL11	May 9	MI-M	0 d	3.5 b
FL17	May 16	LI-M	0 cd	3.5 b
FL8	May 9	LI-M	0 d	3.7 b
FL9	May 9	MI-C	0 d	3.5 b
FR11	May 30	MI-M	0 d	3.5 b
FR12	June 21	MI-C	0 d	3.9 b
FR16	July 15	LI-C	0 d	3.8 b
Control	-	Control	0 d	3.9 b
FL7	May 16	LI-M	16.6 d	14.7 ab
FR13	June 21	MI-M	16.6 cd	29.6 a
FR8	June 21	MI-M	16.6 cd	29.6 a
FL10	May 16	LI-C	33.3 cd	39.4 a
FR10	May 30	LI-M	50 bc	44.4 a
FR17	July 15	LI-C	50 bc	39.4 a
FR15	July 15	MI-M	83.8 ab	44.4 a
FR2	July 15	MI-M	100 a	49.3 a

Letters in column correspond to differences in Kruskal Wallis ranks test.
Significance level P or n.s. not significant, (α=0.05).
MI – moderate incidence orchard; LI – low incidence orchard; M – alleyways with mechanical cultivation; C – alleyways with natural cover.
[a]Circle area ($A = \pi . r^2$) of the necrosis was evaluated.

Aggressiveness of pathogenic isolates ranged from approximately 16 to 100% on fruits. Significant differences were found among pathogenic isolates originated from flowers or fruits (Table 3). Isolates FL7, FR13, FR8 and FL10 differ significantly from isolates FR15, FR2. Regarding the colleting date, results showed that isolates collected early in May 16 (FL7, FL10) do not differ significantly from isolated collected latter in June 15 (FR13, FR8). Also, no differences in aggressiveness of isolates were found between orchards' brown spot incidence history and mechanical cultivation versus natural cover (Table 3).

Regarding the colleting date, results showed that isolates collected early on May 16 (FL7, FL10) do not differ significantly from isolated collected latter in June 15 (FR13, FR8). Also, no differences in aggressiveness of isolates were found between orchards' brown spot incidence history and mechanical cultivation versus natural cover (Table 3).

Necrosis area caused by isolates under study ranged from approximately 49.3 to 3.5 mm^2 on infected fruits. Significant differences were found among pathogenic isolates originated from flowers or fruits (Table 3). Necroses caused by isolates FR2, FR15, FR17, FR10, FL10, FR8 FR13 differ significantly from necroses caused by control and isolates FR16 FR12 FR11 FL9 FL8 FL17 FL11 (Table 3).

DISCUSSION

As Rossi et al. (2005) showed not only dead pear leaves and fruits host fungi that can produce spores but also dead grasses in the orchard, and in Portuguese growing conditions, isolates from *Rumex crispus* L., *Picris echioides* L., *Oxalis pes-caprae* L., *Epilobium* sp. and *Sonchus* sp., common weeds in 'Rocha' orchards, caused lesions in 'Rocha' fruits 15 days after inoculations (Isidoro and Azevedo, 2005). More recently Köhl et al. (2013) showed that DNA of pear-pathogenic *S. vesicarium* was detected at high concentration not only in fallen pear leaves and fruit, but in residues of weeds and grass leaves, confirming that these residues can be considered as major inoculum sources of pear-pathogenic *S. vesicarium*. In consequence, fruit producers questioned their orchard floor management practices in relation to the spread of the pathogen population. According to our results, overall incidence and prevelance of infection were lower when the management soil system was mechanical cultivation compared with alleyways with natural cover, according to the observations that disease incidence is higher in pear orchards managed on grassy soils than on bare soils (Cavanni and Ponti, 1994). However, the major differences were between orchards with different BSP incidence history, that is, with different inoculum pressure. In agreement with Köhl et al. (2009) the incidence levels were also different over time and local because the progression of brown spot epidemics depends on the inoculum produced on the orchard floor during the growing season (Llorente et al., 2012). BSP incidence in all flower organs was detected since the beginning of flowering, stressing the need of early chemical control and also the importance of timing and frequency of mowing.

The majority of pathogenicity tests with *S. vesicarium* isolates was conducted in bioassay on detached leaves or detached fruits (Köhl et al., 2009; Puig et al. 2015; Tanahashi et al., 2017; Moragrega et al., 2018) or in potted plant in orchard (Puig et al., 2015). As far as we are aware, our results obtained with inoculations of not detached fruits in a commercial pear orchard are therefore a starting point for giving insights on several aspects regarding disease infection process and plant defenses responses in orchard conditions. Also, it was proved that the pathogen's infection occurs through the epidermis of the fruits with no lesion.

Monitoring of airborne inoculum of BSP has been useful to know fluctuations in the inoculum and periods in which the inoculum is low or high (Köhl et al., 2009; Llorente et al., 2012). Puig et al. (2015) in a study performed in Spain concluded that some *S. vesicarium* isolates revealed different patterns of disease progress and even could show saprophytic ability depending on type of source (air, pear lesions, pear leaf debris non host). Recent studies with DNA molecular techniques focused on pathogenicity/aggressiveness profiles of *S. vesicarium* revealed that populations might have been overestimated and that saprophytic and pathogenic isolates may occur in the orchard during growing season (Puig et al., 2015). Our findings are in accordance with this evidence and pointed out the coexistence of different profiles of pathogenicity/aggressiveness among *S. vesicarium* isolates obtained from different pear organs over the growing season.

CONCLUSIONS

This work showed that all floral parts are infected since the beginning of flowering. Soil orchard management strategies (mechanical cultivation or natural cover) can influence the incidence and prevelance of *S. vesicarium* infection. Mechanical cultivation showed to be more useful in inoculum reduction than natural grass cover. However, the major differences were recorded between orchards with different BSP incidence history. Inoculations in vivo in a commercial orchard showed that the infection of fruits can occur without lesions present on the fruit epidermis. *Stemphylium* isolates demonstrated to have different virulence degrees and no clear relation between the organ origin and the pathogenicity could be established.

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