Trop. plant pathol. (2016) 41:78–83
DOI 10.1007/s40858-016-0070-z

ORIGINAL ARTICLE

Optimal conditions for conidial germination and infection of European pear leaves by *Diplocarpon mespili*

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Received: 6 August 2015 / Accepted: 4 January 2016 / Published online: 14 January 2016
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Abstract The epidemiology of Entomosporium leaf spot (ELS) affecting European pear is poorly understood, which limits the development of an effective management strategy. In vitro assays were conducted to study the effect of temperature levels (5, 10, 15, 20, 25, and 30 °C) on *Diplocarpon mespili* conidial germination evaluated at different incubation times (0, 2, 4, 6, 8, 12, 24, and 48 h). Inoculation experiments were conducted to assess the effect of leaf wetness duration (0, 6, 12, 24, and 48 h) under constant temperature (20 °C) on ELS disease severity on leaves of cultivar 'Rocha'. The temperature × time interaction significantly affected conidial germination in both experiments and a response surface model was fitted to percent conidial germination data. The optimal temperature for conidial germination was estimated at 20 °C. The incubation period was estimated at 4 days for all leaf wetness durations, excepting the 'zero' duration for which no infection occurred. A minimum of 6 h of leaf wetness duration was required for *D. mespili* infection. Severity reached maximum values after 24 h of leaf wetness duration. A linear regression model described ELS severity increase over time in the absence of reinfection conditions and a monomolecular model described the increase of disease severity influenced by leaf wetness duration in both experiments.

Keywords *Pyrus communis* · Fabraea leaf spot · Epidemiology · Monocyclic component

Section Editor: Harald Scherm

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Introduction

Pear (*Pyrus communis* L.) crops are often grown in temperate climatic zones but are not yet fully acclimated to southern Brazil (Faoro and Orth 2010). Brazilian annual pear production in 2013/14 was approximately 22,000 t (IBGE 2015). However, the annual domestic demand for fresh pear fruit is around 230,000 t, making Brazil the third largest pear importer worldwide (FAO 2014; IBRAF 2014). The pear crop offers an expansive market opportunity in southern Brazil because of the suitable climate and available infrastructure, but limiting factors affect the economic production of the crop.

Entomosporium leaf spot (ELS), also known as Fabraea leaf spot, is the main foliar disease of pear in southern Brazil (Bogo et al. 2013). The disease is caused by the fungus *Diplocarpon mespili* (Sorauer) B. Sutton (anamorph: *Entomosporium mespili* (DC.) Sacc.) (Van der Zwet 1990). Symptoms on young leaves are comprised by lesions that first appear as reddish to purple, pinpoint spots of 1–3 mm in diameter on either leaf surface. The spot enlarges, turns dark brown, and sometimes develops a chlorotic halo that may coalesce. Asexual fruiting bodies (acervuli) bearing conidia are formed on the lesions. Severely affected leaves become necrotic, turn yellow, and abscise. Defoliation is more pronounced at the lower half of the tree canopy and, by the time of harvest, only the top leaves may remain attached to the tree (Van der Zwet 1990; Fioravanço 2007). ELS can also cause cankers in branches and twigs (Bell and Van Der Zwet 2005). In the absence of control, severe early defoliation may occur during summer, leading to reduced photosynthetic capacity and weakening of the trees by reduction of vigor and yield (Van der Zwet 1990).

Most cultivars of European pear are susceptible to ELS (Van der Zwet 1990; Bell and Van Der Zwet 2005; Bogo et al. 2013) and little is known about the epidemiology of the disease such as environmental requirements for spore germination and infection (Fioravanço 2007). Thus, this study aimed to i) model the effect of temperature and incubation time on *D. mespili* conidial germination and ii) model the effect of leaf wetness duration on the *D. mespili* disease severity.

Material and methods

Inoculum production

Fully developed leaves of European pear cultivar 'Rocha' exhibiting ELS symptoms were collected from plants in an orchard and washed with sterile distillated water. The ELS lesions were gently scraped with a scalpel to remove the acervuli, which were then macerated in a depression microscope slide. The conidia were agitated in sterile distilled water with Tween 80 (1:50) and counted with the aid of a Neubauer chamber. A spore suspension with a fixed concentration was further adjusted for each experiment.

Effects of temperature and time on conidial germination

A 50-μL aliquot of 10^5 conidia/mL concentration was added to water agar (2 %) medium in Petri dishes and incubated in a biochemical oxygen demand chamber (BOD) in the dark at temperatures of 5, 10, 15, 20, 25, and 30 °C (±0,5 °C).

A 100-μL aliquot of lactoglycerol was added to the Petri dishes after 0, 2, 4, 6, 8, 12, 24, and 48 h to interrupt conidial germination. Germination was estimated under an optical microscope at 100× magnification by counting 100 radially distributed conidia and classifying them as germinated or non-germinated. Conidia were considered fully germinated when the length of the germ tube was at least as great as the conidial longitudinal diameter. The conidial germination time 'zero' was assessed twice; immediately after lactoglycerol addition, and 48 h thereafter to confirm inhibition of conidial germination.

The experiments followed a completely randomized 6 × 8 factorial design (six temperatures, eight incubation times) with four replicates, using one Petri dish per experimental plot. The experiment was repeated once in time.

The effects of temperature and incubation time on *D. mespili* conidial germination were described separately as a response surface model fitted to the data (Carisse et al. 2000). The equations proposed by Duthie (1997) were used to fit the response surface model for conidial germination. The effect of time on the percent conidial germination (*GER*) was modeled by Eq. 1 as

$$GER_h = A\left(1 - \exp\left\{-[B(h - C)]^D\right\}\right) \quad (1)$$

where *A* is the upper limit of the response (upper asymptote), *B* is the intrinsic rate of increase in the response, *C* is the length of delay in the response, *D* is the portion of the time in which the response decelerates, and *h* is the time in hours.

The relationship of percent conidial germination to temperature (degree Celsius) was described by Eq. 2 as

$$GER_t = E'\{\exp[(T-F)G/(H+1)]\}/\{1+\exp[(T-F)G]\} \quad (2)$$

where GER_t is percent conidial germination at a given temperature (*T*); *E'* is given by $E[(H+1)/H]H^{1/(H+1)}$, where *E* is the maximum response; *F* is a location parameter proportional to the optimum temperature; *G* is the intrinsic rate of decline from the maximum as the temperature deviates from the optimum, and *H* is the degree of asymmetry of the curve.

The parameters *A, B, C,* and *D* (Eq. 1) and *E, F, G,* and *H* (Eq. 2), were estimated using the nonlinear regression (NLIN) procedure in SAS (SAS Institute). This process uses iterative methods to adjust and estimate the parameters by least squares' non-linear statistical method. The asymptotic intervals of 95 % reliability were obtained in order to test such estimates. The quality of adjustment was analyzed by the magnitude of regression residue and by the correlation between predicted values of the methods and the observed data (paired scores).

The combined effects of temperature and incubation time on conidial germination was described by a surface response model (Eq. 3) as

$$GER_{ht} = E'\{\exp[(T-F)G/(H+1)]\} /\{1+\exp[(T-F)G]\}^{*}\left(1-\exp\left\{-[B(h-C)]^D\right\}\right) \quad (3)$$

where GER_{ht} is percent conidial germination, *E'* is equal to $E[(H+1)/H]H^{1/(H+1)}$, and the *B, C, D, E, F, G, H* parameters are the same described above.

Statistical significance of temperature and incubation time, as well as their interaction, on conidial germination was assessed using ANOVA in both factorial experiments in a model without randomization (PROC GLM in SAS).

Effect of leaf wetness duration on disease severity

All leaves of 2-year-old European pear cultivar Rocha plants potted into 5-L containers of organic soil were removed. The plants were sprayed with mancozeb (240 g i.a./100 L) and then with a mixture of 0.5 % Dormex (BASF) and 3 % mineral oil to break dormancy and induce leaf bud break. The plants were kept in a growth room at 20 °C ± 2 °C, 12 h photoperiod, and 65 % relative humidity for 30 days for acclimatization and inducing leaf bud break.

Leaves with approximately 2/3 of maximum development were inoculated with a 200-μL aliquot of a 5×10^4 conidia/mL

suspension onto both leaf surfaces of each plant with a hand-held sprayer until runoff. The inoculated leaves were numbered and ELS severity was assessed after the completion of each leaf wetness duration (LWD). The inoculated plants were covered with a transparent plastic bag containing a moist cotton ball. LWDs of 0 (without plastic bag and cotton ball), 6, 12, 24, and 48 h after inoculation were terminated by removal of the bag and cotton ball and gentle forced ventilation. The growth room was equipped with fans for leaf drying just after removal of the plastic bags.

Inoculum viability was estimated for each experiment by adding 100 μL conidial suspension onto three agar plates at the end of each inoculation. The plates were incubated at 20 °C for 24 h and percent germination was estimated under an optical microscope at 100× magnification by counting 100 radially distributed conidia and classifying them as germinated or ungerminated. Conidia were considered fully germinated when the length of the germ tube was at least as great as the conidial longitudinal diameter.

The inoculated leaves were assessed daily until ELS symptoms appearance to determinate the length of the incubation period. First symptoms were defined as dark circular lesions (black spots) visible on both leaf surfaces. In addition, ELS severity was assessed visually with the aid of a standard area diagram (Nunes and Alves 2012) at 4, 7, 11, 14, 17, and 21 days after inoculation (DAI). The experiment followed a completely randomized design with four replicates, using 20 leaves per plant per experimental plot. The experiment was repeated once in time.

ELS severity data were analyzed by one-way ANOVA and by linear regression, taking into consideration the effect of longitudinal DAI as repeated measure over time. The equation: $Y=y_0+r*DAI$, where Y is the response variable (severity, %), y_0 is the intercept, r is the slope, and DAI is the time (days after inoculation) was fitted.

The ELS severity data at 21 DAI were fitted to the monomolecular model: $Y=B_1*(1-(1-B_2/B_1)\exp(-B_3 LWD))$, where Y is the response variable (severity, %), B_1 is the maximum asymptote, B_2 is a model parameter, B_3 is the growth rate, and LWD is the leaf wetness duration (hours). The GLM, REG and NLIN procedures of SAS were utilized for ANOVA, linear regression analysis, and monomolecular model fit for both experiments, respectively. All model parameters were significantly different from zero, and the quality of the model fitness was assessed as described previously.

Results

Effect of temperature and incubation time on conidial germination

D. mespili conidia germinated at all temperatures evaluated, except at 5 °C, regardless of incubation time. The minimum temperature at which conidial germination occurred was 10 °C. Temperature increases were accompanied by increases in conidial germination from 10 to 20 °C, followed by a gradual decrease in the percentage of germinated conidia at temperatures of 25 and 30 °C. The time of 6 h was the minimum required for *D. mespili* conidial germination at 20 °C. The highest percentage of germinated conidia was obtained at the same temperature and a time of 48 h.

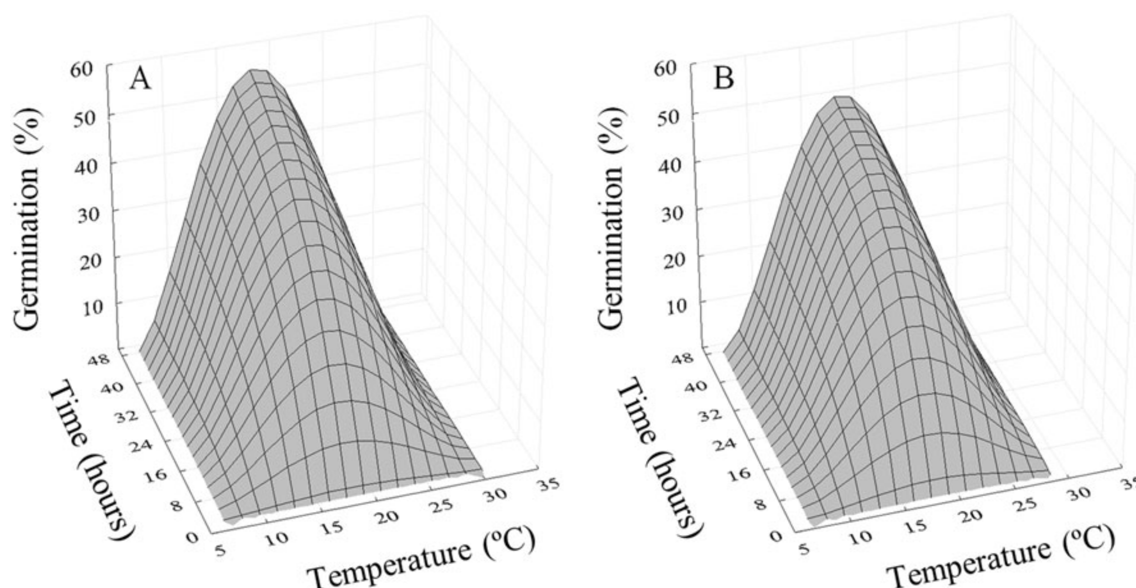

Fig. 1 Three dimensional response of *Diplocarpon mespili* conidial germination to the combined effects of temperature (10, 15, 20, 25, or 30 °C) and incubation times (0, 2, 4, 6, 8, 12, 24, or 48 h). Estimated parameters are given in Table 1. Experiment 1 (**a**); Experiment 2 (**b**). The predicted values were calculated using Eq. 3 (described in text)

Table 1 Parameter estimates for the models that described the response of conidia germination time (GER_h), temperature (GER_t) and combined effects (GER_{ht})

Model[a]	Experiment	Parameter[b]							
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
GER_h at t = 20 °C	1	**61.37**	**0.07**	3.15	1.04	–	–	–	–
	2	**69.13**	0.04	**5.52**	0.48	–	–	–	–
GER_t at h = 48 h	1	–	–	–	–	**58.80**	**20.98**	**0.48**	**2.34**
	2	–	–	–	–	**51.97**	**20.44**	**0.46**	2.02
$GER_{h,t}$	1	–	**0.07**	3.45	**1.07**	**62.55**	**20.43**	**0.51**	**1.98**
	2	–	**0.07**	**5.04**	**0.65**	**62.26**	**19.44**	**0.48**	**1.42**

[a] All regressions were significant ($P < 0.0001$) by F test

[b] Estimated parameters in bold were different from 0 by asymptotic 95 % confidence interval

There was a significant interaction between the effects of temperatures and time on conidial germination in both experiments ($P < 0.001$). Thus, the minimum time required for conidial germination was 6, 6, 8, 8, and 12 h at temperatures of 15, 20, 25, 30 and 10 °C, respectively. The combined model function allowed construction of a response surface for conidial germination (Fig. 1). The estimated model parameters for both experiments were significant ($P < 0.05$), except for the *C* parameter in experiment 1 (Table 1).

Effect of leaf wetness duration on disease severity

The Anova model indicated that LWD significantly affected *D. mespili* infection on pear leaves ($P < 0.001$). Separately for each LWD, the increase in ELS severity was fitted according to a linear model in the absence of reinfection conditions (Fig. 2). The regression slope was significant for all leaf wetness durations, except for 6 h in experiment 1 (Table 2).

There was no *D. mespili* infection on leaves in the absence of wetness. A LWD of 6 h was the minimum required for infection. The incubation period was 4 days for all LWDs evaluated (Fig. 2). Disease severity peaked at 21 DAI for LWDs of 24 and 48 h. At that assessment date, LWD >24 h did not further increase disease severity (Fig. 3).

The effect of LWD on disease severity at 21 DAI was well described by a monomolecular model ($P < 0.001$) for both experiments. However, disease severity was much greater in experiment 1 than in experiment 2 (Fig. 3), although the rate parameter associated with LWD (B_3) did not differ between the experiments (Table 3). The viability of inoculum varied between experiments, being 67 % of germinated in experiment 1 and only 40 % in experiment 2.

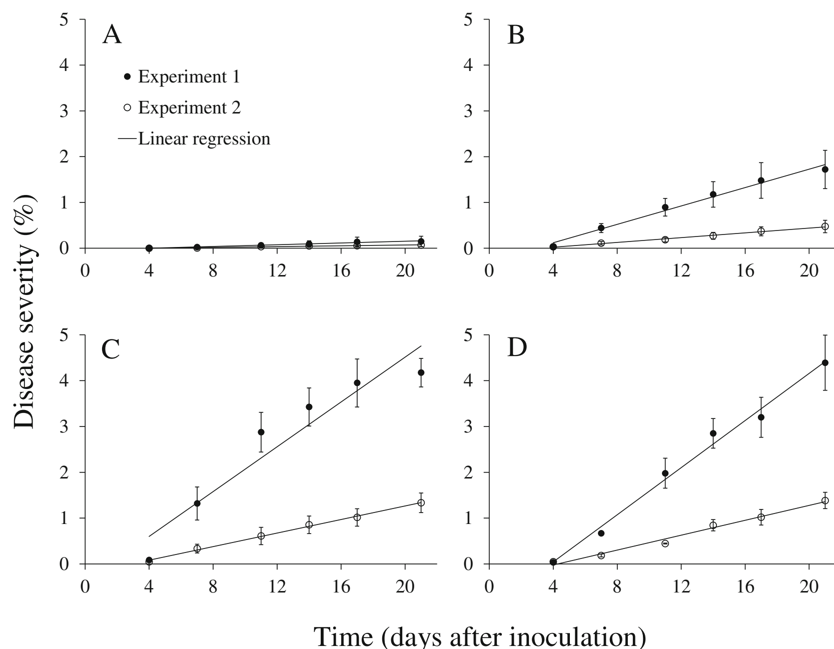

Fig. 2 Entomosporium leaf spot severity progress in leaves of European pear cultivar Rocha after different periods of leaf wetness duration: 6 (**a**), 12 (**b**), 24 (**c**), and 48 h (**d**) at 20 °C. *Each point* represents the mean of four replicates. *Vertical bars* represent standard errors. Estimated parameters are given in Table 2

Table 2 Linear regression parameters of Entomosporium leaf spot severity of European pear cultivar Rocha regressed against incubation time (days after inoculation, *DAI*) under different leaf wetness durations at 20 °C

Leaf wetness duration (hours)	Experiment 1			Experiment 2		
	y_0	r	R^2	y_0	r	R^2
6	NS			−0.018	0.004	0.32
12	−0.283	0.10	0.67	−0.082	0.026	0.60
24	−0.378	0.244	0.74	−0.214	0.074	0.76
48	−0.982	0.257	0.86	−0.346	0.081	0.89

[a] Parameters of equation $Y = y_0 + r*DAI$

[b] NS = not significant at $P < 0.05$

Discussion

Most fungal plant pathogens respond in a distinctive pattern to temperature and leaf wetness (Duthie 1997; Margarey et al. 2005) which can be described by different models. The one proposed by Duthie (1997) was utilized in this work because each parameter of this model has a relatively well-defined biological meaning.

The optimal temperature for *D. mespili* conidial germination was 20 °C. This finding agrees with that of Holstag et al. (2003), who showed that *D. mespili* infection in Saskatoon cherry (*Amelanchier alnifolia*) was reduced at temperatures above 25 °C in Winnipeg, Canada. Baudoin (1986) showed that *D. mespili* conidial germination on Photinia (*Photinia × fraseri*) leaves occurred 6 h after inoculation and required 12 h for leaf penetration at 25 °C. Horie and Kobayashi (1979) and Rosenberger (1981) indicated that the shortest infection time for *D. mespili* in pear was between 20 and 25 °C.

The temperature of 20 °C resulted in the highest percentage of conidial germination in vitro and was used for the in vivo experiments. However, despite this experimental similarity, in vitro and in vivo processes can differ because of biochemical interactions and alterations in the plant–pathogen relationship (Agrios 1997; Pascholati et al. 2008). Different conditions for *D. mespili* infection have been reported for different hosts. Baudoin (1986) and Holstag et al. (2003) suggested that 6 and 12 h were the minimum LWDs for infection in Saskatoon cherry and Photinia leaves at 20 °C, respectively.

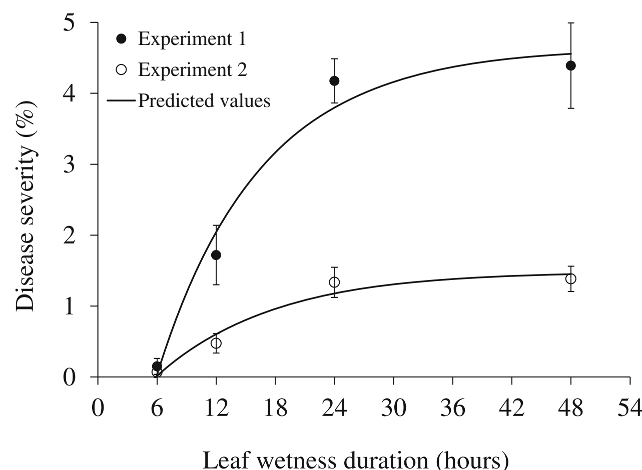

Fig. 3 Entomosporium leaf spot severity progress in leaves of European pear cultivar Rocha under different leaf wetness durations at 20 °C and 21 days after inoculation. *Each point* represents the mean of four replicates. *Vertical bars* represent standard errors. *Lines* show the monomolecular model fitted to the data. Estimated parameters are given in Table 2

The minimum LWD required for *D. mespili* infection in Rocha pear was 6 h at a temperature of 20 °C. In growth chamber experiments, the minimum wetting periods for infection by *D. mespili* were 12, 8, and 8 h at 10, 20, and 25 °C, respectively (Van der Zwet 1990; Bell and Van der Zwet 2005).

ELS symptom appearance in Rocha pear required 4 days of incubation period. This period was similar to that reported by Baudoin (1986) of 5 days for *D. mespili* infection in young leaves of Photinia. However, Rosenberger (1981) and Van der Zwet (1990) reported that at least 7 days were necessary for ELS symptom appearance in pear. ELS lesions are typically very small black spots (Van der Zwet 1990) and many lesions are required for high disease severity. This ELS feature results in low disease severity despite the presence of many lesions, reflecting exactly what was observed in this study. Gonçalves et al. (2014) reported a maximum ELS severity of 6 % in different combinations of European pear cultivars and rootstocks at 56 days after the first evaluation in southern

Table 3 Estimated parameters of a monomolecular model fitted to disease severity data of Entomosporium leaf spot on leaves of European pear cultivar Rocha under different leaf wetness durations (*LWD* = 0, 6, 12, 24 or 48 h) at 20 °C

Experiment	Parameters[a]		
	B_1	B_2	B_3
1	4.6432 (3.6096; 5.6769)	−3.4968 (−7.2567; 0.2631)	0.0948 (0.0261; 0.1635)
2	1.4879 (1.0543; 1.9215)	−0.9962 (−2.3449; 0.3525)	0.0873 (0.00704; 0.1675)

[a] Parameters of equation $Y = B_1 * (1 - (1 - B_2/B_1)\exp(-B_3 LWD))$. Approximate 95 % confidence intervals are show in parentheses

Brazil. However, in orchards with a longer history of the disease and/or poor disease management, ELS can reach much higher severity. Nunes and Alves (2012) reported a maximum ELS severity of 40 % at the end of the pear-growing season before leaf fall in orchards without substantial disease control strategies. The authors indicated that lesions tend to coalesce at disease severities above 11 %. ELS outbreaks are very dependent on the inoculum history and predominant growing season conditions, suggesting that in some years, the epidemic can be very severe and in others years less so (Nunes et al. 2013).

ELS is a polycyclic disease and its development is influenced mainly by disease progress rate. In the present study, ELS progress was evaluated during the monocyclic stage of lesions derived from primary inoculum and described by a linear model. This situation is very different from the data obtained by Gonçalves et al. (2014) who found that the logistic and Gompertz models best described ELS progress in different combinations of European pear cultivars and rootstocks in the edaphoclimatic conditions of southern Brazil. These epidemiological models describe polycyclic diseases in which new infections can occur with inoculum produced during the epidemics. These models indicated that the rate of increase of ELS severity was proportional to the infection rate (Madden 2007). In the present study, the effect of LWD was not evaluated under field conditions because of the difficulties to maintain an orchard without use of chemical control. Nevertheless, we provided knowledge on the weather-based requirements for infection and the development of ELS, a fundamental step for the establishment of disease management strategies for effectively reducing disease impact on pear fruit production.

Acknowledgments This research was financially supported by CNPq, EMBRAPA, CAPES and Universidade Estadual de Santa Catarina (UDESC).

References

Agrios GN (1997) Plant Pathology, 4th edn. Elsevier Academic Press, San Diego

Baudoin ABAM (1986) Infection of photinia leaves by *Entomosporium mespili*. Plant Dis 70:191–194

Bell RL, Van Der Zwet T (2005) Host resistance in *Pyrus* to *Fabraea* leaf spot. Hortscience 40:21–23

Bogo A, Casa RT, Kretzchmar AA, Gonçalves MJ, Correa D (2013) Epidemiologia e manejo das principais doenças da pereira. Revisão Anual de Patologia de Plantas 21:145–161

Carisse O, Bourgeois G, Duthie JA (2000) Influence of temperature and leaf wetness duration on infection of strawberry leaves by *Mycosphaerella fragariae*. Phytopathology 90:1120–1125

Duthie JA (1997) Models of the response of foliar parasites to the combined effects of temperature and duration of wetness. Phytopathology 87:1088–1095

FAO (2014) Food Agriculture Organization of the United Nations. Faostat Database Prodstat. Available at: http://faostat.fao.org/faostat/servlet/. Accessed on 11 Dec 2014

Faoro ID, Orth AI (2010) Qualidade de frutos da pereira-japonesa colhidos em duas regiões de Santa Catarina, Brasil. Rev Bras Frutic 32:308–315

Fioravanço JC (2007) A cultura da pereira no Brasil: situação econômica e entraves para o seu crescimento. Inf Econ 37:52–60

Gonçalves MJG, Bogo A, Rufato L, Jesus WC Jr, Casa RT, Weber GC, Correa D (2014) Behavior of European pear cultivars under different quince rootstocks to 'Entomosporium' leaf spot in southern Brazil. Crop Prot 49:26–30

Holstag QA, Remphrey WR, Fernando WGD, St-Pierre RG, Panicker S (2003) Leaf-wetness duration and temperature required for infection of saskatoon leaves by *Entomosporium mespili* under controlled conditions. Can J Plant Pathol 25:168–173

Horie H, Kobayashi T (1979) Entomosporium leaf spot of Pomoideae (Rosaceae) in Japan I. Distribution of the disease; morphology and physiology of the fungus. Eur J Forest Pathol 9:366–379

IBGE (2015) Instituto Brasileiro De Geografia E Estatística - Sistema IBGE de Recuperação Automática – SIDRA. Available at: http://www.sidra.ibge.gov.br/bda/tabela/protabl.asp?c=1613&z=t&o=11&i=P. Accessed on 07 Jan 2015

IBRAF (2014) Instituto Brasileiro de Frutas. Frutas frescas - Importação. Available at: http://www.ibraf.org.br/estatisticas/Exportação/Comparativo_das_Exportações_Brasileiras_de_Frutas_Frescas_2012-2013.pdf. Accessed on 10 Dec 2014

Madden LV (2007) The study of plant disease epidemics. APS Press, St Paul

Margarey RD, Sutton TB, Thayer CL (2005) A simple generic infection model for foliar fungal plant pathogens. Phytopathology 95:92–100

Nunes CC, Alves SAM (2012) Elaboração e validação de escala diagramática para quantificação da severidade de entomosporiose em folhas de pereira. Summa Phytopathol 38:239–244

Nunes CC, Alves SAM, Silva VC, Boneti JIS, Katsurayama Y (2013) Epidemiologia da entomosporiose da pereira em pomares no Sul do Brasil.Boletim de Pesquisa e Desenvolvimento no. 18. Embrapa Uva e Vinho, Bento Gonçalves

Pascholati SF, Leite B, Stangarlin JR, Cia P (2008) Interação planta-patógeno: fisiologia, bioquímica e biologia molecular. Piracicaba, Fealq

Rosenberger DA (1981) Fabraea leaf blight of pear. In: Proceedings of apple and pear disease workers. Bad Apple 2: 81–86

Van der Zwet T (1990) Fabraea leaf spot. In: Jones AL, Aldwinckle HS (eds) Compendium of apple and pear diseases. APS Press, St. Paul, pp 23–24