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Seasonal susceptibility of citrus scions to *Phytophthora citrophthora* and *P. nicotianae* and the influence of environmental and host-linked factors on infection development

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Abstract Three citrus scions were evaluated to determine seasonal changes in susceptibility to infections by *Phytophthora citrophthora* and *Phytophthora nicotianae*. In a period of 24 months, the Clementine mandarin cv. Hernandina, the hybrid Fortune mandarin and the sweet orange cv. Lane-Late were branch-inoculated under field and laboratory conditions. Field studies showed that the cultivars inoculated with *P. citrophthora* developed the highest lesion areas during March–June (spring) and September–October (autumn) and with *P. nicotianae* from June to August (summer). However, lesion areas on detached citrus branches did not show a definite pattern of infection because lesion sizes fluctuated irregularly during the study. The lesion area caused by *P. nicotianae* in different citrus scions correlated significantly with the monthly mean maximum values of temperature, relative humidity, and the percentage of the relative water content in the 24-month period of inoculations. In contrast, there was no correlation between these variables and the extent of colonisation by *P. citrophthora*. Nevertheless, a significant relationship was observed between lesion areas caused by *P. citrophthora* from October to May of each year and the same variables that were significant in inoculations with *P. nicotianae*. Seasonal changes in the susceptibility of citrus cultivars to *P. citrophthora* and *P. nicotianae* may facilitate timing of disease control measures to coincide with periods when disease development is greatest.

Keywords Aerial infections · Seasonal variation · Branch canker · Citrus cultivars

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Introduction

Phytophthora citrophthora is the causal agent of branch canker of citrus trees, a damaging disease widely disseminated in the main citrus-producing areas in Spain (Alvarez et al. 2008). This pathogen infects the main scaffold branches of the tree inducing the formation of cankers with gum exudations, giving the branches a bleeding appearance. The expansion of the lesions upwards affects secondary branches and downwards it affects the trunk. If the canker girdles the trunk or the branches, shoots above the canker die, resulting in a dieback of infected limbs or trees. Field observations of this new syndrome showed that rootstock infections are not frequent (Alvarez et al. 2008).

In surveys conducted from 2003 to 2004, the disease was detected on several citrus cultivars;

nevertheless, Clementine mandarins and their hybrids were the main affected citrus group (Alvarez et al. 2008). In this study, in addition to its association with branch infections, *P. citrophthora* was also found causing citrus gummosis (foot rot) either as the unique species or associated with *P. nicotianae* (syn. *P. parasitica*) (Alvarez et al. 2008). Trunk cankers on the scion caused by both *Phytophthora* species had a greater incidence than root infections, showing the prevalence of above-ground infections in these citrus areas. *Phytophthora citrophthora* and *P. nicotianae* are citrus pathogens which have been known for a long time in Spain (Tuset 1977); however, until the emergence of branch cankers caused by *P. citrophthora*, *P. nicotianae* had been considered the most damaging species in the Spanish citrus growing-areas, often associated with citrus root rot (Tuset 1983a).

In citrus orchards affected by citrus diseases such as root rot caused by species of *Phytophthora*, fungicide applications are recommended in spring and autumn (Tuset 1983b; Graham and Timmer 2008) to coincide with periods of susceptible root flushes in the spring (after the spring leaf flush) and autumn (Graham and Timmer 2008). These recommendations are consistent with the periods of greater susceptibility of citrus trees to below-ground infections caused by *Phytophthora* spp., known to fluctuate seasonally (Matheron et al. 1997; Dirac et al. 2003). In citrus growing-areas with Mediterranean climates in the northern hemisphere, root infections caused by *P. citrophthora* are severe during May and June (spring) and less important from December to February (winter), while root infections caused by *P. nicotianae* are severe during summer and early autumn and less important in winter (Ippolito et al. 1992; Dirac et al. 2003).

However, little is known about the periods of greater susceptibility of citrus scions to above-ground infections caused by *Phytophthora* under field conditions. This information is essential to develop an appropriate programme for the control of aerial infections caused by *Phytophthora* spp. using fungicides. This would also allow the reduction of unnecessary chemical use and consequently would decrease the probability of resistance developing. In addition, these data could be used to determine optimum periods for evaluating genetic resistance to *Phytophthora* spp. (Jeffers and Aldwinckle 1986; Browne and Mircetich 1995).

Available data on *Phytophthora* lesion development on aerial tissues of citrus trees were obtained by Matheron and Matejka (1989), who evaluated the temporal susceptibility of detached shoots or bark strips from scions to *P. citrophthora* and *P. nicotianae*. They concluded that it was not possible to determine optimum periods for fungicide applications due to the lack of consistent peaks of infection in the assayed plant material. On the other hand, Matheron and Matejka (1993) demonstrated that the periods of maximum disease development in shoot and root tissues inoculated with *P. citrophthora* and *P. nicotianae* did not coincide, due to different seasonal changes in the susceptibility to infection.

Rootstocks are commonly categorised as tolerant or moderately resistant to *Phytophthora* infections, while all citrus scions are considered as susceptible or very susceptible (Graham and Menge 1999). The degree of susceptibility of citrus shoots and bark tissues to *P. citrophthora* and *P. nicotianae* is related to the pattern of flush or shoot growth (Matheron and Matejka 1989). However, root flushes occur after shoot growth (Bevington and Castle 1985; Lutz and Menge 1986). This temporal difference in physiological activity could explain the dissimilar duration of maximum disease development in above and below-ground infections (Matheron and Matejka 1993).

Environmental parameters such as soil temperature (Matheron and Matejka 1992; Matheron et al. 1997; Dirac et al. 2003), flooding duration and water potential in the soil (Wilcox and Mircetich 1985; Woods and Duniway 1986) play a significant role in the severity and dynamics of root infections by *Phytophthora* in below-ground parts of the tree. In contrast, little is known about the influence of weather variables on the severity of infections in above-ground parts of citrus trees. Specific knowledge about environmental and host-linked factors that have an influence on infections in citrus scions through the growing season could support predictions on the onset of infection.

The purpose of this work was: (1) to evaluate the seasonal dynamics of above-ground infections of three citrus scions to *P. citrophthora* and *P. nicotianae* over a 24-month period through artificial inoculations of citrus branches under field and laboratory conditions; and (2) to determine the influence of some environmental and host-related factors on the growth and severity of the infections.

Materials and methods

Phytophthora isolates

One isolate of *P. citrophthora* (Phy-114), originally isolated from cankered branches of lemon trees in Murcia province (south-eastern Spain), and one isolate of *P. nicotianae* (Phy-047), recovered from trunk lesions of affected trees in Valencia province (eastern Spain), were used for monthly inoculations throughout the experiments. These isolates were selected in a previous study for their aggressiveness to different citrus cultivars (data not shown). Stock cultures were maintained at 6°C in flasks of 15 ml containing sterile extract of soil. Before inoculations, the growth rates and colony morphologies of the isolates were examined. No systematic differences were found over the period of study. Inoculum for each inoculation consisted of mycelium produced on PDA medium which was transferred from the stock cultures. Plates were incubated for 5 days at 24°C before inoculation.

Citrus orchards

The experiments were carried out in Monserrat (Valencia province) in three close citrus commercial orchards of around 2 to 3 ha each. They were: (1) a 10 year-old Clementine mandarin (*Citrus clementina*) cv. Hernandina orchard; (2) a 16 year-old mandarin hybrid cv. Fortune (*C. clementina x Dancy*) orchard and; (3) a 15 year-old sweet orange (*Citrus sinensis*) cv. Lane-Late orchard. All scion varieties were grafted on Carrizo citrange rootstock. Citrus trees were cultivated (i.e., irrigated, fertilised, pruned, etc.) and maintained (i.e., weed and pest control, etc.) as in most commercial citrus orchards in Spain, except from applications of antioomycete products. Irrigation was applied via a drip system, starting when soil moisture was below the water capacity level. The exact periods were dependent of the rainfall, but usually were from 4- to 10-day intervals (depending on the evaporation rate).

Field inoculation of citrus branches

In each orchard, thirty citrus trees were arbitrarily selected and marked with coloured numbered bands. A set of ten trees randomly distributed was used for inoculations with *P. citrophthora*, another group of ten trees for inoculations with *P. nicotianae* and a third group of ten trees served as a control. For each inoculation date, ten lignified branches (one branch per tree) of each group of trees were inoculated. Each branch (~25–30 mm-diam) was randomly selected from the middle third of the canopy. From October 2004 to September 2006, all citrus scions were branch-inoculated the first week of every month.

Selected branches were surface-disinfested with 70% ethanol before inoculation. With a cork borer of 8 mm-diam, a disk from the bark was removed to expose the cambium, and for placing a PDA agar plug colonised with mycelium of the isolates side-downwards on the wound. Control branches were inoculated with a plug of sterile agar. After inoculation, the wound was covered with moist cotton wool, sealed with a strip of Parafilm® and wrapped with foil paper to prevent drying.

Previous research established an incubation period of 4 weeks after inoculation as an optimal time for the determination of symptom readability under field conditions (Alvarez et al. 2008). At the end of these 4 weeks, the surface of the branch bark was scraped in order to reveal margins of healthy and necrotic tissue above and below the original wound. Canker area was traced on a transparent plastic sheet and subsequently digitalised to be quantified by means of the software Assess (American Phytopathological Society. St. Paul MN). The size of the inoculation wound was subtracted to provide the real lesion area. To confirm that cankers resulted from infections caused by each inoculated *Phytophthora* species, both pathogens were re-isolated monthly by plating colonised bark tissue onto PARBPH (Jeffers and Martin 1986) selective medium.

Laboratory inoculation of detached citrus branches

In a parallel experiment at the time of each field branch inoculation, thirty lignified branches of around 20 cm in length and 25 mm-diam were randomly collected from non-inoculated trees in each orchard. These branches were immediately stored in plastic bags, sent to the laboratory and inoculated later in the day.

Detached branches (ten per treatment) were inoculated with a 5 mm-diam plug of each *Phytophthora* species as described previously. Controls were inoc-

ulated by placing a sterile agar disk into the wounds. In order to reduce desiccation, the freshly cut ends were covered immediately with moist cotton wool held in place with a parafilm band. The branches were subsequently placed in moist chambers and incubated at 24°C. To avoid secondary colonisation of saprophytes, a benomyl solution (10 mg l^{-1}) was sprayed into the moist chambers. The extent of colonisation was assessed 7 days after inoculation as described previously. Isolations onto PARBPH were made in order to confirm that cankers were caused by each *Phytophthora* species.

Measurement of environmental variables

Environmental variables in the orchards were monitored from 1 October 2004 to 30 September 2006 with CR10 and CR10X dataloggers (Campbell Scientific Ltd., Leicester, UK) connected to combined temperature-relative humidity (RH) (Model HMP35AC), wetness (Model 237), and rainfall (Model ARG100) sensors. Sensors to measure the duration of canopy wetness, temperature and RH were mounted 1.80 cm above ground level in the canopy to avoid direct sunlight. Data on temperature and RH were recorded hourly. Rainfall and leaf wetness data were recorded every 10 min when these events occurred. Eight weather variables were considered: maximum (RMX), mean (RMM) and minimum (RMN) percentage of air RH; maximum (TMX), mean (TMM) and minimum (TMN) air temperature in °C; rainfall amount (RAIN) and hours of canopy wetness (CWP).

Determination of bark relative water content (RWC) of citrus scions

At the time of each branch inoculation, five cortical tissue strips of 5 cm long×3 cm wide were cut from branches located in the middle third of the tree canopy (~1.60 m above the ground line) in each treatment. These samples were protected from desiccation with a plastic film and stored in an icebox until processing in the laboratory later in the day. Relative water content (RWC) of the plant material used in the inoculations was calculated according to the procedure of Robin et al. (1994). The fresh weight (FW) of the samples was measured in the laboratory. Samples were incubated overnight in water, blotted dry, weighed to determine turgid weight (TW), dried at 70°C, and reweighed to determine dry weight (DW). RWC was calculated according to the formula RWC= [(FW−DW)/(TW−DW)]×100.

Data analysis

Analyses of variance were performed using a completely randomised design for each tested citrus scion to establish differences between seasonal development of lesions and the influence of the inoculated *Phytophthora* species. Means were separated according to Fisher's least significant difference test ($P<0.05$) using SAS statistical software version 9.0 (SAS Institute, Cary, NC).

Linear and multiple regression analyses were performed to explore and measure the potential relationships between lesion sizes caused by *P. citrophthora* and *P. nicotianae* and environmental and host-related variables. Data collected during the periods from October 2004 to September 2006 were used for the regression analysis. These analyses were conducted separately for each citrus cultivar tested with the SAS statistical package and significant differences among means were determined at $P<0.05$. Collinearity diagnostics were also performed on each data set to avoid unstable estimates and high standard errors due to linear dependence between predictor variables.

Because the lesion areas and rainfall heights often had zero values and also high values, they showed a skewed distribution. Both of the variables were thus transformed with the square root (sqrt) transformation (y= sqrt [lesion area], x_2= sqrt [rainfall). For the values of the other variables, square root and logarithmic transformations of the data were also evaluated to obtain a closer approximation to normal distribution of the residuals.

Results

Field inoculation of citrus branches

Data sets of lesion development for each *Phytophthora* species and citrus scions were separated in seasonal periods to identify the greatest peaks of infection: autumn (September, October and November); winter (December, January and February); spring (March, April and May); summer (June, July and August).

Significant effects ($P<0.05$) of seasonal lesion development according to the inoculated *Phytophthora* species were detected (Table 1). No necrosis developed from control inoculations with sterile agar.

All cultivars showed similar trends in lesion development. The highest average of lesion area in citrus scions inoculated with *P. citrophthora* was obtained in spring, followed by autumn, summer and finally winter (Table 1). There were large variations in the monthly area of lesion within each year (Fig. 1), e.g., in 2005 the greatest area of lesions was in May; however, in 2006 the greatest lesions were obtained in April, with the exception of the Lane-Late cultivar. The lowest averages of lesion area for all cultivars were obtained from December to January of each year.

Citrus scions inoculated with *P. nicotianae* also showed seasonal changes in lesion development. Hernandina and Lane-Late showed the largest lesions during the summer months, followed by spring and finally autumn and winter (Table 1). In contrast, Fortune showed the largest lesion areas in spring, with no significant differences from the mean values obtained in summer. Lesion areas in autumn and winter were statistically similar. There were also large variations in the monthly areas of lesion within each year. Fortune cultivar showed the highest values of lesion area in May, while Hernandina showed these in July in both 2005 and 2006 (Fig. 1). However, Lane-Late showed the highest averages of lesion area in May 2005, while in 2006 this was obtained in July.

In general, the lesion areas were greater in 2005 than in 2006 for all citrus cultivars and *Phytophthora* species. Data analyses also revealed that infections by *P. citrophthora* were greater than those of *P. nicotianae* (Table 1).

Seasonal fluctuations in detached-branch inoculations

Over a 24-month period, detached citrus scions inoculated with both *P. citrophthora* and *P. nicotianae* showed a seasonal pattern of infections ($P<0.05$) (Table 1); however, this was irregular and dependent on the year of inoculation (Fig. 2). No necrosis developed from control inoculations with sterile agar.

The Fortune cultivar inoculated with *P. citrophthora* showed the lowest lesion areas in November–December and March–May and the highest lesion sizes in January–February and August–September of each year. In Hernandina, the lowest size of lesion development took place from March to May; however, no distinguishable seasonal pattern was observed due to the irregularity in lesion development. In Lane-Late, the lowest lesion sizes were recorded from March to May and the highest from June to October of each year (Table 1).

Inoculations with *P. nicotianae* also displayed an irregular pattern of infections. In Fortune and Hernandina, the maximum and minimum values of lesion sizes fluctuated irregularly and were dependent on the year of inoculation. In the first year of observations it

Table 1 Seasonal changes in susceptibility of three citrus cultivars inoculated with *Phytophthora* spp. under field and laboratory conditions

Seasons[a]	Area of lesion (cm^2)					
	P. citrophthora			*P. nicotianae*		
	Fortune	Hernandina	Lane-Late	Fortune	Hernandina	Lane-Late
Field assays						
Autumn	5.75[b]B[c]	5.28 B	4.89 B	2.20 A	2.86 A	1.77 A
Winter	1.67 A	2.23 A	1.68 A	0.10 A	0.36 A	0.30 A
Spring	11.40 C	10.78 C	10.03 C	8.44 B	6.14 B	5.77 B
Summer	4.78 AB	3.03 A	4.34 B	6.77 B	11.33 C	7.78 B
Average	5.90	5.33	5.24	4.38	5.17	3.91
Laboratory assays						
Autumn	0.68[b]A[c]	2.17 b	2.42 BC	0.87 AB	0.95 AB	1.36 B
Winter	1.19 AB	2.33 b	1.73 B	0.38 A	0.63 A	0.16 A
Spring	0.38 A	0.30 a	0.39 A	0.26 A	0.23 A	1.08 B
Summer	1.71 B	2.50 b	2.83 C	1.05 AB	1.72 AB	2.82 C
Average	0.99	1.83	1.84	0.64	0.88	1.36

[a] Autumn (September, October and November); Winter (December, January and February); Spring (March, April and May); Summer (June, July and August).

[b] Each value is mean of 60 replicates.

[c] Means in the same columns followed by the same letter are not significantly different according to Fishers protected LSD at $P<0.05$ level.

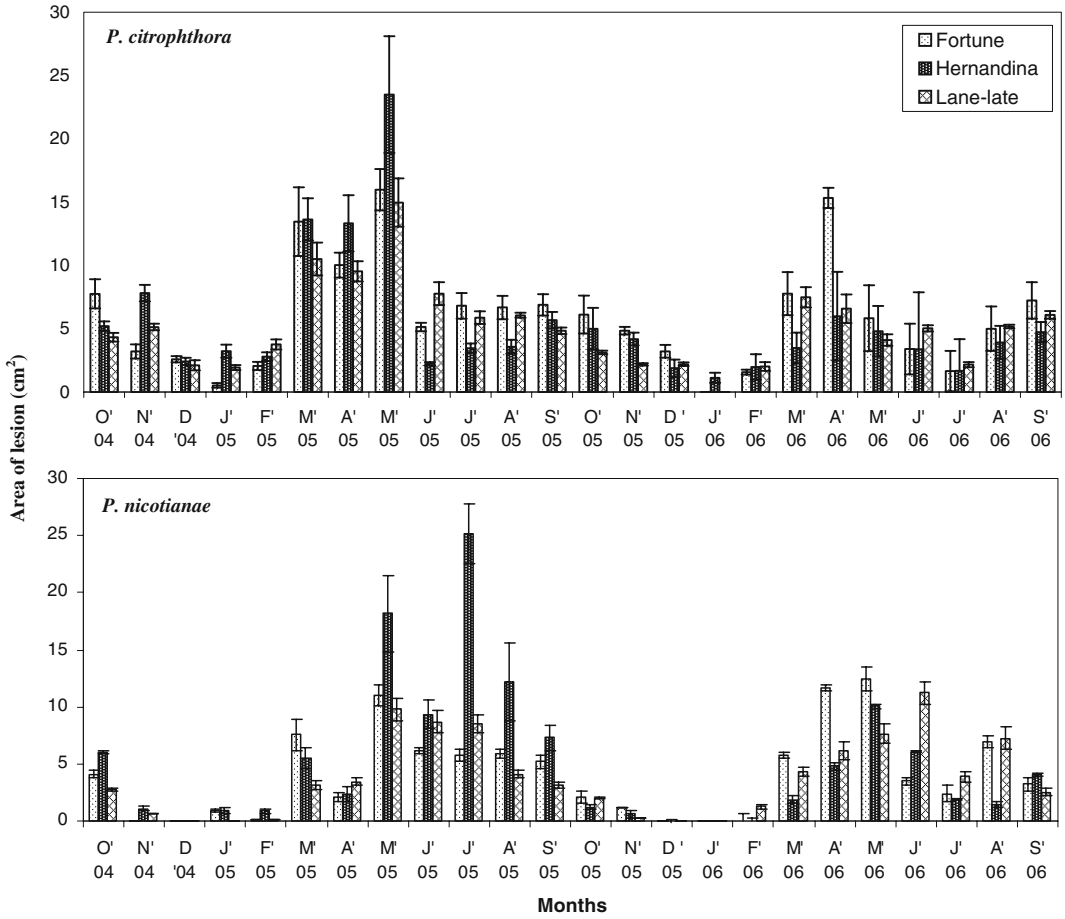

Fig. 1 Monthly changes in lesion development on citrus branches in the field on three different citrus cultivars inoculated with *P. citrophthora* or *P. nicotianae*

was not possible to establish a seasonal pattern of infections; however, in the second year, the greatest lesion areas were registered in the period from July to September in both cultivars. In Lane-Late, the lowest lesion sizes were recorded around January and the highest values in the period June–September of each year (Fig. 2).

Seasonal fluctuations in RWC

The RWC of the three citrus scions varied significantly during the study ($P<0.05$), showing a seasonal pattern; however, these results were dependent on the citrus cultivar and on the year the data were recorded (Fig. 3). In Fortune, the RWC fluctuated from 64.1% to 86.8%; the minimum percentage was observed from November to January and the maximum from March or April to June or July of each year. The RWC in Hernandina varied from 67.6% to 91.5%. In each year of study, a peak of maximum values from April to July or August, decreasing quickly to their minimum percentage between August or September to October or November was observed. The RWC in Lane-Late fluctuated from 80.1% to 91.5%, showing minimum values around December and July and maximum around March or September in each year. In general, the greatest values in RWC were shown in Lane-Late and Hernandina followed by Fortune.

Effect of environmental and RWC variables on lesion development

Multiple and simple regression analyses showed no correlation ($P>0.05$) between environmental factors, RWC and lesion size developed in the citrus scions

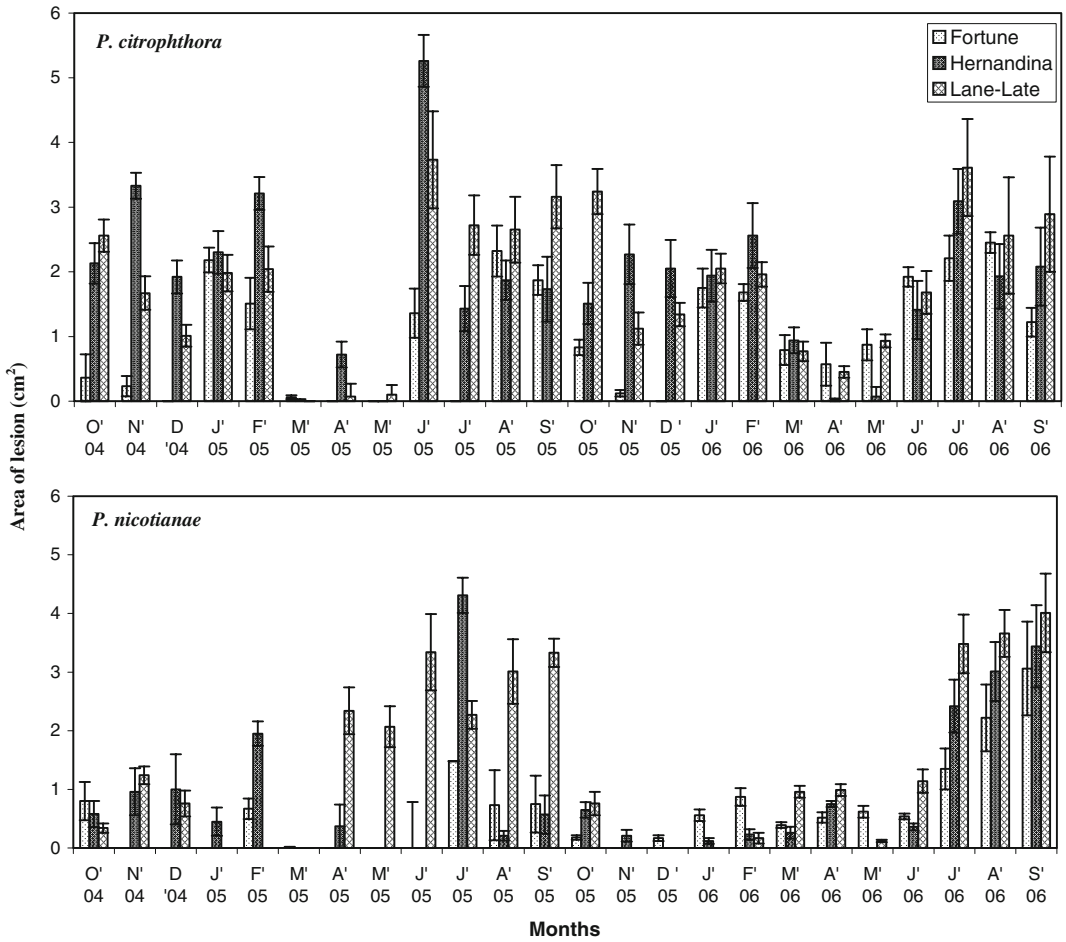

Fig. 2 Monthly changes in lesion development on excised branches in vitro on three different citrus cultivars inoculated with *P. citrophthora* or *P. nicotianae*

inoculated with *P. citrophthora* during the period of study. Several transformations of data did not greatly increase the amount of variability explained (data not shown). However, when a specific period within the annual cycle of the culture was analysed by means of a simple regression analysis, e.g., from October to May in each year, lesion areas were significantly correlated with the monthly percentage of RH, monthly air temperature and monthly percentage of RWC variables, but not with the number of hours of canopy wetness and rainfall amount (Table 2). The relationship of the variables RH and rainfall with the area of lesion was negative. The best fit variables were selected in agreement with the highest r^2 values. In the cvs Fortune and Lane-Late, the area of lesion was significantly correlated with the mean monthly maximum air temperature (TMX) and the mean monthly maximum RH (RMX) (Table 2). In Hernandina, a high correlation was observed among TMN and RMN only during the second year of study.

Examination of data plots showed that curvilinear relationships were more appropriate to explain the relationships between the studied variables. The best fit obtained to explain the relationship between lesion development and TMX in all citrus scions inoculated with *P. citrophthora* corresponded to a power function of the form $y = bx^a$, in which y is the lesion size in cm^2, x is the temperature in °C, a are units of lesion area per unit of temperature and b is a parameter that describes the steepness of the gradient (Table 3). A second grade polynomic equation ($y = ax^2 + bx + c$) was found, which closely correlated the RWC values with lesion size. The percentage of variation explained by the models of each regression equation

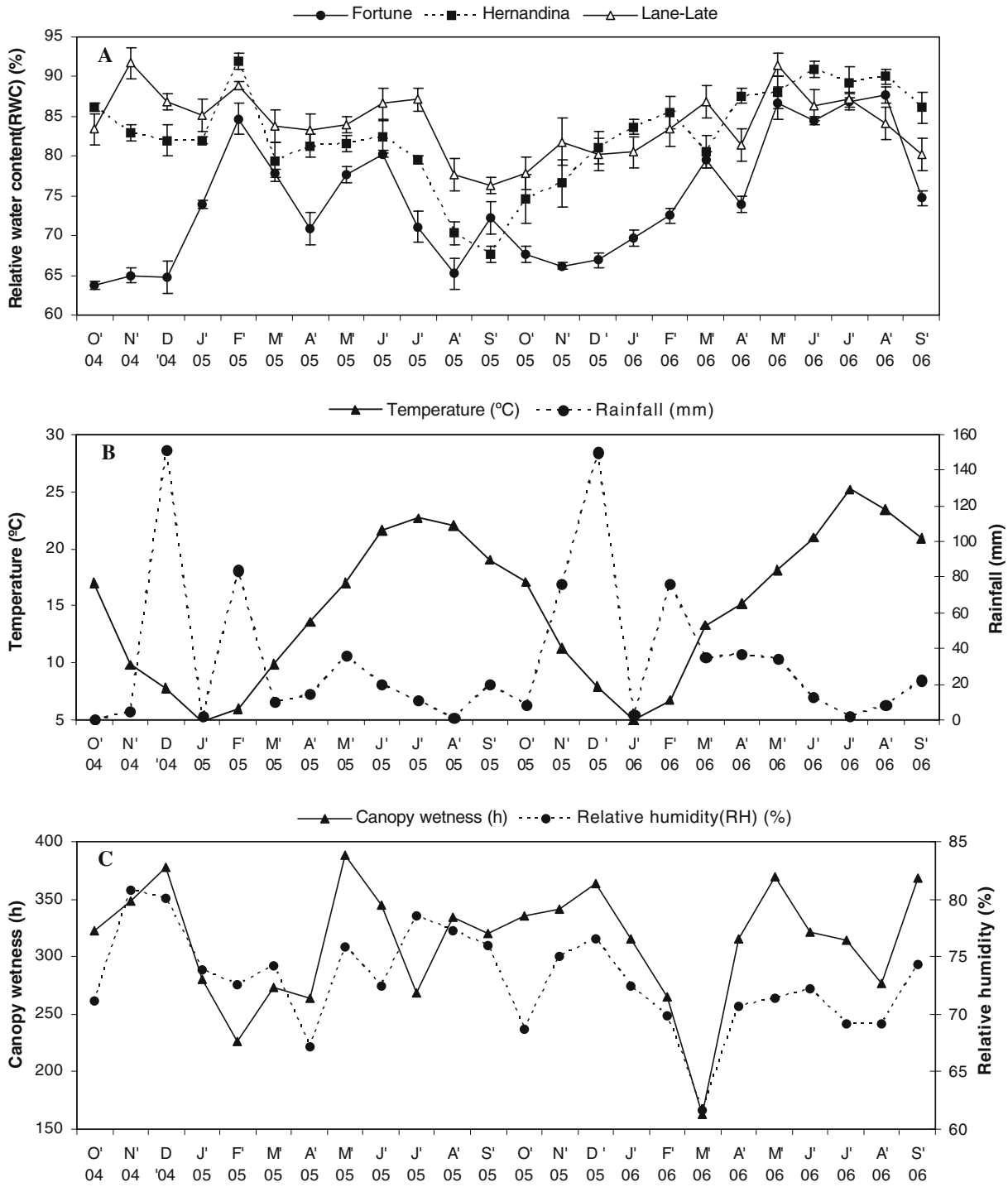

Fig. 3 **a** Relative water content (RWC) of excised bark examined monthly, **b** average monthly temperature and monthly rainfall, and **c** average monthly RH and canopy wetness recorded at the weather station nearest the field of study

Table 2 Simple regression analysis between lesion size and environmental variables on citrus branches inoculated with *P. citrophthora* during October 2004–May 2005 and October 2005–May 2006

Independent variables	Simple regression analyses[a]					
	October 2004–May 2005			October 2005–May 2006		
	Slope	r^2	*P* value	Slope	r^2	*P* value
Fortune cultivar						
CWP	0.02105	0.045	0.6119	−0.00731	0.011	0.8035
RMX	−1.32325	0.719	0.0078	−0.97689	0.413	0.0432
RMM	−0.35119	0.074	0.5120	−0.30925	0.088	0.4744
RMN	−0.52191	0.220	0.2407	−0.32220	0.252	0.2041
RAIN	−0.03312	0.095	0.4576	−0.00782	0.043	0.6214
TMX	1.39318	0.741	0.0060	0.69652	0.479	0.0478
TMM	0.92300	0.566	0.0312	0.63909	0.453	0.0572
TMN	0.70231	0.430	0.0773	0.53358	0.368	0.1104
RWC	0.54230	0.779	0.0085	0.82463	0.791	0.0027
Hernandina cultivar						
CWP	0.03881	0.094	0.4591	0.00389	0.022	0.7213
RMX	−1.33654	0.447	0.0697	−0.17793	0.294	0.1650
RMM	−0.21343	0.017	0.7592	−0.08007	0.042	0.6235
RMN	−0.63797	0.201	0.2656	−0.11026	0.862	<0.001
RAIN	−0.04477	0.105	0.4317	−0.00536	0.146	0.3499
TMX	1.49884	0.523	0.0426	0.41225	0.602	0.0235
TMM	1.03267	0.432	0.0764	0.32213	0.828	0.0017
TMN	0.83046	0.367	0.1114	0.30412	0.862	0.0009
RWC	−1.09566	0.221	0.2860	0.19720	0.519	0.0437
Lane-Late cultivar						
CWP	0.02107	0.080	0.4952	0.00700	0.010	0.8094
RMX	−0.87602	0.557	0.0332	−0.70306	0.653	0.0152
RMM	−0.25318	0.068	0.5301	−0.30507	0.088	0.4752
RMN	−0.46696	0.312	0.1501	−0.35857	0.321	0.1434
RAIN	−0.02604	0.104	0.4361	−0.01252	0.113	0.4148
TMX	1.00941	0.688	0.0108	0.85727	0.775	0.0023
TMM	0.73771	0.640	0.0171	0.81634	0.757	0.0049
TMN	0.61797	0.589	0.0260	0.62966	0.527	0.0418
RWC	0.75969	0.856	0.0028	0.82947	0.587	0.0265

RMX maximum percentage of air RH, *RMM* mean percentage of air RH, *RMN* minimum percentage of air RH; *TMX* maximum air temperature in °C, *TMM* mean air temperature in °C, *TMN* minimum air temperature in °C; *RAIN* rainfall amount, *CWP* hours of canopy wetness

[a] *P* value, ANOVA probability for the regression equation; r^2. Proportion of explained variation

ranged from 66.3% to 86.8% for TMX and from 52.4% to 89.8% for RWC (Table 3).

Multiple regression analysis showed significant correlations ($P<0.05$) between several environmental factors and the lesion size in all citrus scions inoculated with *P. nicotianae* over a 24-month period (Table 4). Temperature, RH and RWC variables were highly significant in all citrus cultivars; however, there was no significant effect of rainfall or wetness of canopy. RH and rainfall variables were again negatively related to the lesion area. Overall, the model for each citrus cultivar accounted for 76% of the variation in Fortune, 81% in Hernandina and 82% in Lane-Late (data not shown).

Using a simple regression analysis, the TMX and the RMX variables were strongly associated with the lesion size of *P. nicotianae*, showing the highest r^2 values (Table 5). Curvilinear relationships also best explained the relationship between variables. For the temperature variable, the best fit obtained to describe the infection by *P. nicotianae* corresponded to a power function ($y = bx^a$). The percentage of variation

Table 3 Regression data for the relationship between temperature, relative water content (x) and lesion size (y) on branches of three citrus scions inoculated with *P. citrophthora* during October 2004–May 2005 and October 2005–May 2006

Variables, citrus scions and regression model	Regression data[a]							
	a		*b*		*c*		r^2	
	Oct. 2004–May 2005	Oct. 2005–May 2006	Oct. 2004–May 2005	Oct. 2005–May 2006	Oct. 2004–May 2005	Oct. 2005–May 2006	Oct. 2004–May 2005	Oct. 2005–May 2006
Temperature ($y=bx^a$)								
Fortune	5.089	6.397	2^{-6}	4^{-8}				
Hernandina	3.090	2.586	0.001	0.002				
Lane-Late	3.079	7.474	0.000	2^{-9}				
Relative water content ($y=ax^2+bx+c$)								
Fortune	0.002	0.047	0.241	−5.910	−24.99	186.91	0.689	0.869
Hernandina	0.239	−0.005	−41.080	0.994	1770	−43.72	0.169	0.525
Lane-Late	0.090	0.061	−14.543	−9.780	584.6	388.60	0.898	0.608

[a] a, b, c are regression coefficients; r^2 is the proportion of explained variation.

explained by the models of each regression equation for this variable ranged from 70.9 to 73.4 (Table 6).

Despite some apparent relationship between the RWC values and monthly lesion size, when determined by regression analysis was weak and was only found in the cvs Fortune and Lane-Late (Table 4). However, simple regression analysis in annual intervals (from October to September of each year) revealed that the relationship between these variables was dependent on the year of evaluation and the citrus cultivar (Table 6). Curvilinear relationships showed that all citrus cultivars fitted a power equation model in both years of study. In the period from October 2004 to September 2005, the model explained 40.1% and 62.1% of the variation, and in the period from October 2005 to September 2006, 26.2% and 85.8% of the variation (Table 6).

Discussion

The most accurate conclusions concerning the seasonal susceptibility of the citrus hosts to infections by *P. citrophthora* and *P. nicotianae* were obtained only from field experiments. Lesions caused by both pathogens fluctuated greatly throughout the year and showed a distinctive seasonal pattern on the inoculated citrus cultivars. Seasonal changes in lesion size on detached branches were recorded, but fluctuated irregularly during the study and did not correlate with the infection pattern developed under field conditions.

In field experiments, maximum invasion of tissues by *P. citrophthora* usually occurred from March to May and from September to October and, for *P. nicotianae*, from March or April through July or August and was dependent upon the citrus cultivar and year of observation. A disease management programme that optimises or minimises the number of fungicide applications for control of aerial infections by *Phytophthora* should be scheduled in these periods. This finding is somewhat obvious because this temporal pattern appears to be similar to that observed for underground infections by *Phytophthora* (Ippolito et al. 1992; Matheron et al. 1997; Dirac et al. 2003); however, this has never been demonstrated previously in experiments under field conditions and for above-ground infections.

The onset of infections recorded under field conditions was related to shoot development and the tested *Phytophthora* species. Citrus branches became more susceptible to *Phytophthora* after growth flush, previously reported (Matheron and Matejka 1989). Weather conditions occurring before this growth stage did not influence the severity of infected branches. Shoot growth flush can be affected by climatic factors such as the temperature, the content of tree carbohydrates (Noling 2003), or the physiological process associated with an alternate bearing tendency (Sauls 2008) in the assayed citrus cultivars. Differences found between the months of greater susceptibility of the cultivars to infections by *Phytophthora* species

Table 4 Multiple regression analyses between lesion size and environmental variables on citrus branches inoculated with *P. nicotianae* during the 24-month period of study

Cultivars and independent variables	Slope	r^2	P^a
Fortune			
CWP	0.01167	0.023	0.4473
RMX	−0.29470	0.358	0.0010
RMM	−0.25329	0.075	0.1665
RMN	−0.22858	0.017	0.0307
RAIN	−0.01647	0.101	0.1049
TMX	0.40913	0.429	<0.001
TMM	0.56811	0.418	<0.001
TMN	0.33041	0.325	0.0019
RWC	0.39716	0.451	0.0001
Hernandina			
CWP	0.00139	0.001	0.9499
RMX	−0.53977	0.587	<0.001
RMM	−0.05190	0.015	0.8459
RMN	−0.29043	0.136	0.0576
RAIN	−0.02470	0.111	0.0833
TMX	0.99211	0.622	<0.001
TMM	0.66731	0.557	<0.001
TMN	0.64746	0.610	<0.001
RWC	0.11451	0.015	0.5325
Lane-Late			
CWP	0.00588	0.080	0.6555
RMX	−0.31978	0.577	<0.001
RMM	−0.20813	0.069	0.1844
RMN	−0.16568	0.124	0.0709
RAIN	−0.01500	0.115	0.0829
TMX	0.42765	0.641	<0.001
TMM	0.59877	0.635	<0.001
TMN	0.36248	0.535	<0.001
RWC	0.51836	0.324	0.0019

RMX maximum percentage of air RH, *RMM* mean percentage of air RH, *RMN* minimum percentage of air RH; *TMX* maximum air temperature in °C, *TMM* mean air temperature in °C, *TMN* minimum air temperature in °C; *RAIN* rainfall amount, *CWP* hours of canopy wetness

[a] ANOVA probability for the regression equation.

in each year of observation could be associated with some of these factors. Cultural practices such as ringing of branches, extensively used to improve fruit set in Clementine mandarins in Spain (Agustí 2000) could also have an influence in some physiological process of the tree. However, this practice was not used in the field experiments of our study.

The greatest size of lesions in the cultivars inoculated with *P. citrophthora* was observed in Fortune, followed by Hernandina, with the lowest severity in Lane-Late. However, with *P. nicotianae* the highest averages of affected tissues were obtained in Hernandina, followed by Fortune and Lane-Late. Differences in lesion severity found between cultivars are consistent with observations of natural infections of the trees, where Clementines and their hybrids are the citrus group more susceptible to infections. This result could indicate that genetic factors of the host may have influence on the severity of lesion development by *Phytophthora* species.

Results of our study also showed that *P. citrophthora* was more aggressive than *P. nicotianae* and had greater periods of activity during the year. These results are consistent with previous studies of citrus (Matheron and Matejka 1989) and with other work that corroborates the dominant status of *P. citrophthora* in Mediterranean regions (Ricci et al. 1990; Alvarez et al. 2008). Although *P. nicotianae* has not previously been associated with branch infections in citrus trees (Alvarez et al. 2008), results on the

Table 5 Simple regression analysis between several meteorological variables and lesions size on branches of citrus cultivars inoculated with *P. nicotianae*

Independent variables	Regression analysis[a]	
	P value	r^2
Fortune cultivar		
RMX	0.0010	0.358
RMN	0.0307	0.173
TMX	0.0003	0.418
TMN	0.0019	0.326
Hernandina cultivar		
RMX	<0.001	0.587
RMN	0.0496	0.136
TMX	<0.001	0.622
TMN	<0.001	0.611
Lane-Late cultivar		
RMX	<0.001	0.577
RMN	0.0309	0.124
TMX	<0.001	0.635
TMN	<0.001	0.535

RMX maximum percentage of air RH, *RMN* minimum percentage of air RH; *TMX* maximum air temperature in °C, TMN minimum air temperature in °C.

[a] *P* value, ANOVA probability for the regression equation; r^2. Proportion of explained variation

dynamics of infection would be valuable when attempting to determine economic treatment thresholds for this pathogen in infected citrus scions.

Detached branches inoculated with both *Phytophthora* species showed seasonal changes despite being maintained under standard temperature and humidity conditions in the laboratory. This result suggests that the seasonality in infections could be attributed to host physiology and other factors related to the host. Dirac et al. (2003) observed seasonal changes in infections on excised roots inoculated with *P. citrophthora* and *P. nicotianae* incubated under the same temperature in the laboratory. They argued that temperature should be eliminated as a primary cause of seasonal variation in root infections by *Phytophthora*.

Irrespective of the citrus scion and the species of *Phytophthora* inoculated, the lowest lesion areas in detached branches were found from March to May of each year. In contrast, lesion sizes in field inoculations showed the highest averages in this period. This discrepancy could be related to the constant temperatures in which the in vitro experiments were conducted, which were in contrast to the regular changes in this parameter under field conditions between night and day, especially in spring. Lesion sizes on detached branches of Lane-Late orange were greater than those of Fortune mandarin; however, field studies showed a

Table 6 Regression data for the relationship between temperature, relative water content (x) and lesion size (y) on branches of three citrus scions inoculated with *P. nicotianae*

Variables, regression model and citrus scions	Regression data[a]					
	a		*b*		r^2	
Temperature[b] ($y = bx^a$)						
Fortune	7.0208		1^{-9}		0.709	
Hernandina	6.1991		2^{-8}		0.709	
Lane-Late	6.1135		2^{-8}		0.734	
Relative water content[c] ($y = bx^a$)						
	O 2004–M 2005	O 2005–M 2006	O 2004–M 2005	O 2005–M 2006	O 2004–M 2005	O 2005–M 2006
Fortune	13.50	16.08	1^{-25}	2^{-30}	0.401	0.639
Hernandina	7.29	19.10	3^{14}	5^{-37}	0.111	0.858
Lane-Late	33.96	19.80	3^{-66}	2^{-38}	0.621	0.262

[a] *a*, *b*, regression coefficients; r^2, proportion of explained variation

[b] Regression coefficients are values corresponding to data sets of 24 months

[c] Regression coefficients are values corresponding to intervals of October–May of each year.

converse situation. Similar results were obtained from previous studies where detached plant material was used (Browne and Mircetich 1996; Matheron and Mircetich 1985; Luque et al. 2002). Resistance of excised vegetative material may be altered by the physical detachment of plant parts from the growing plant or by changes in the physiology of the excised stem pieces (Matheron and Mircetich 1985; Matheron and Matejka 1989, 1993). As a consequence, excised plant material may not show the true dynamics of lesion development compared to studies in which living plant material is used.

Results of our study also showed that the temperature and RH were weather variables positively associated with the lesion size by both *Phytophthora* species. Temperature has been previously cited as the environmental parameter of greatest influence in lesion development (Matheron and Mircetich 1985; Feld et al. 1990; Matheron and Matejka 1992; Robin et al. 1994; Luque et al. 2002). However, there are no previous reports on the influence of RH on the development of lesions. The average of the maximum values recorded for temperature and RH variables (TMX and RMX), correlated significantly with lesion growth. Luque et al. (2002) found a positive correlation between lesion growth and the mean minimum temperature, but not with the RH values in inoculations with *P. cinnamomi* in *Quercus suber*. Although results of our study suggest that temperature and RH are weather factors strongly related to aboveground infections by *P. nicotianae*, RH levels are strongly influenced by temperature and periods of rainfall and field irrigation.

The variables TMX and RMX correlated significantly with lesion development in citrus scions inoculated with *P. nicotianae* when the whole data set (24 months) was analysed. However, there showed no correlation with lesion development in inoculations with *P. citrophthora* during this period. This lack of correlation could be explained by the low averages of lesion size recorded in the summer months (June to August), and the high values of temperature and RH recorded in these months. However, lesion growth was highly correlated with these variables from October to May of each period. The lack of correlation in annual or biannual regressions suggest that other unmeasured variables could have influenced lesion development of *P. citrophthora* in the summer months.

The lack of natural infections of *P. citrophthora* in citrus in the summer months has already been cited in previous work, suggesting dormancy in propagules (Matheron et al. 1997), rhizosphere microorganisms (Dirac and Menge 2002; Dirac et al. 2003) or phytoalexin secretion (Dirac et al. 2003). In this latter case, citrus trees can secrete scoparone, a phytoalexin produced by the bark of citrus trees that may inhibit infections by *P. citrophthora* in the summer (Afek and Sztejnberg 1988, 1989; Dirac et al. 2003). Afek and Sztejnberg (1993) also studied the production of scoparone and the resistance to gummosis caused by *P. citrophthora*. They found that citrus trees produced the most scoparone at 25°C, production decreasing progressively as the temperatures declined. We consider that the lack of correlation between environmental variables and lesion development in *P. citrophthora* could be the consequence of the effect of scoparone on the severity of lesions or fluctuations in the relative content of carbohydrates in the bark. Although the activity of scoparone has been successfully tested in in vitro assays against *P. nicotianae* in citrus fruit infections (Ortuno et al. 1997), there are no previous studies on its effect on this species on the scion bark. The scoparone is produced by the bark of citrus trees and contradictions about its production (Fourie et al. 2000) or not (Dirac et al. 2003) by the roots have been cited. Results of our study would indicate that *P. nicotianae* is weakly affected by the production of this phytoalexin or its concentration, because lesion sizes did not diminish in summer months in contrast to *P. citrophthora*. However, the seasonality of the infections could also be related to the carbohydrate concentrations in the tree. Dirac et al. (2003) indicated that *P. nicotianae* infections were more abundant in summer roots when sugar levels increased, whereas *P. citrophthora* infection appears to be more abundant in winter roots when the content in starch was high and low in sugars. Studies to establish this variation in levels of the scion bark have not been conducted. Further research is needed to confirm these hypotheses.

A significant positive correlation was also found between lesion size and RWC values in *P. nicotianae*, which agree with previous studies on its influence in lesion development by *Phytophthora* species (Tippet et al. 1987; Robin et al. 1994; Okey et al. 1996). In our study, this parameter was also important in the development of infections by *P. citrophthora* in defined

periods within the cycle of culture of citrus. Okey et al. (1996) observed that cocoa clones with high moisture content were more susceptible to infections by *P. palmivora* than those with low moisture content. Orchard (1985) suggested that bark moisture content in cocoa clones may be influenced by weather conditions such as the peaks of dry and wet periods. Inconsistencies in the correlations between lesion size caused both by *P. citrophthora* and *P. nicotianae* and RWC values of the citrus cultivars could be related to rainfall episodes or drought and flood periods influencing in the values of RWC.

The results from this study suggest that the development of a forecasting system for the onset of infections by species of *Phytophthora* in the field is feasible. Relationships between temperature, RH, and RWC of the bark required for lesion development derived in this study provide a basis for prediction. However, field results showed that there are still some other outstanding effects of environmental factors, or factors associated with the host, to be understood before an accurate prediction of the onset of infection can be achieved. The extent to which environmental conditions affect lesion development by *Phytophthora* species between years highlights the importance of conducting resistance trials over several seasons, and the value of using well-defined control isolates in pathogenicity tests.

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