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FULL RESEARCH PAPER

Effect of temperature, relative humidity, leaf wetness and leaf age on *Spilocaea oleagina* conidium germination on olive leaves

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Abstract The effects of temperature, relative humidity (RH), leaf wetness and leaf age on conidium germination were investigated for *Spilocaea oleagina*, the causal organism of olive leaf spot. Detached leaves of five ages (2, 4, 6, 8 and 10 weeks after emergence), six different temperatures (5, 10, 15, 20, 25 and 30°C), eight wetness periods (0, 6, 9, 12, 18, 24, 36 and 48 h), and three RH levels (60, 80 and 100%) were tested. Results showed that percentage germination decreased linearly in proportion to leaf age ($P<0.001$), being 58% at 2 weeks and 35% at 10 weeks. A polynomial equation with linear term of leaf age was developed to describe the effect of leaf age on conidium germination. Temperature significantly ($P<0.001$) affected frequencies of conidium germination on wet leaves held at 100% RH, with the effective range being 5 to 25°C. The percent germination was 16.1, 23.9, 38.8, 47.8 and 35.5% germination at 5, 10, 15, 20 and 25°C, respectively, after 24 h. Polynomial models adequately described the frequencies of conidium germination at these conditions over the wetness periods. The rate of germ tube elongation followed a similar trend, except that the optimum was 15°C, with final mean lengths of 175, 228, 248, 215 and 135 μm at 5, 10, 15, 20 and 25°C, respectively after 168 h. Polynomial models satisfactorily described the relationships between temperature and germ tube elongation. Formation of appressoria, when found, occurred 6 h after the first signs of germination. The percentage of germlings with appressoria increased with increasing temperature to a maximum of 43% at 15°C, with no appressoria formed at 25°C after 48 h of incubation. Increasing wetness duration caused increasing numbers of conidia to germinate at all temperatures tested (5–25°C). The minimum leaf wetness periods required for germination at 5, 10, 15, 20 and 25°C were 24, 12, 9, 9 and 12 h, respectively. At 20°C, a shorter wetness period (6 h) was sufficient if germinating conidia were then placed in 100% RH, but not at 80 or 60%. However, no conidia germinated without free water even after 48 h of incubation at 20°C and 100% RH. The models developed in this study should be validated under field conditions. They could be developed into a forecasting component of an integrated system for the control of olive leaf spot.

Keywords *Cycloconium oleagina* · *Olea europaea* · Peacock spot

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Introduction

Olive leaf spot (OLS), also called peacock spot, is caused by the fungus *Spilocaea oleagina* (syn. *Cycloconium*

oleagina). The disease is widespread in all olive-growing regions of the world, and has been recognized in Mediterranean areas for over a century (Bernès 1923). In warm and dry climates the disease is not usually an important problem because it needs cool and moist weather for its development. Olive leaf spot symptoms usually occur on the upper surface of the leaves (Graniti 1993), expanding and coalescing to cover a large proportion of leaf area, often causing premature leaf-fall. Spots are usually more abundant on foliage from the lower parts of olive trees, and many twigs in these parts become completely defoliated. Over successive seasons, the disease causes poor growth and dieback of the defoliated twigs (Miller 1949; Laviola 1992; Azeri 1993; López-Doncel et al. 2000). Under very wet conditions, small sunken brown lesions may be found on the petioles, fruit peduncles and fruit (Graniti 1993), resulting in fruit drop and decreased oil yields (Verona and Gambogi 1964).

In New Zealand, about 40% of olive trees assessed in a survey (MacDonald et al. 2000) had OLS, suggesting that it may play a major role in the reported low productivity. In California, Wilson and Miller (1949) reported severe outbreaks of OLS in the period 1941 to 1949, with yield losses of up to 20% in some areas. In the Mediterranean olive-growing regions, which are characterized by long dry summers, OLS is controlled by application of copper-containing fungicides prior to winter rains (Teviotdale et al. 1989). However, timing of the fungicide applications was reported to be critical for effective control of the disease (Graniti 1993).

The influence of temperature and leaf wetness duration on conidium germination and infection has been well studied for a number of pathosystems, and such information has been used to develop weather-based systems for timing of fungicide sprays (Jones et al. 1984; Xu et al. 1995). However, despite the yield losses caused by OLS in some areas very little is known about the biology of the pathogen and some results appear to be contradictory. For example, the temperatures for conidium germination on agar were reported by Wilson and Miller (1949), Dzaganiya (1967), and Kashy et al. (1991) to be a minimum of 5°C, an optimum of 20°C, and a maximum of 30°C. Chen et al. (1981) also showed that conidium germination on agar occurred in the temperature range 6 to 28°C, with an optimum at 16 to 20°C. However, on olive leaf surfaces Saad and Masri (1978) demonstrated that germination occurred over temperatures of 8 to 24°C with an optimum of 20°C.

Environmental factors such as temperature and moisture appear to be the driving force of the infection and spread of OLS (Graniti 1993); however, most of the reported studies were based on field observations, with few experimental studies into the effects of environmental variables on *S. oleagina* conidium germination. Salerno (1966) reported that free moisture was required for the germination of conidia and that they germinated poorly, if at all, at <90% RH. In contrast, Saad and Masri (1978) found that on detached olive leaves 62% of the conidia had germinated when the leaves were incubated at 20°C under 100% RH for 48 h. The effect of leaf age on conidium germination and the impact of environmental conditions on germ tube elongation have not been previously studied for *S. oleagina*. Moreover, models incorporating various environmental variables have not been developed for *S. oleagina* conidium germination.

Considering the importance of conidium germination for infection by *S. oleagina* and subsequent OLS development, further investigation is required to elucidate the effects of temperature and moisture on conidium germination and to address the inconsistencies in the literature. Hence, this paper describes experiments to investigate the effects of leaf age, temperature, leaf wetness duration, and relative humidity (RH) on conidium germination, appressorium formation and germ tube elongation for *S. oleagina*. Regression models were developed to describe the observed effects.

Materials and methods

Plant production

The experiments were conducted at Lincoln University on detached olive leaves obtained from 1 to 2-year-old olive trees ('Barnea') grown in a greenhouse maintained at 22±5°C and 30 to 60% RH under natural daylight. The plants were grown in plastic pots (13 cm diam) containing a mixture of composted bark and pumice (4:1, *v*/*v*) with 8 to 9 months slow release fertilizer (N/P/K, 15:4:7.5). The first pair of leaves to reach the early bud stage was tagged on each plant after 2 months of growth and thereafter at 2-week

intervals for 10 weeks. The marked leaves were detached and used in the detached leaf experiments described below.

Humidity chambers

Humidity chambers were constructed which consisted of plastic containers 22×15×6 cm, each having a wire gauze placed over a plastic grate supported on four pillars about 4 cm high. The three different RH levels required were created by filling the bottom of the containers with 500 ml saturated sodium bromide (60% RH) or ammonium sulphate (80% RH) solutions or water (100% RH) and tightly sealing with a lid. After allowing the solutions to equilibrate for 14 days to ensure saturation, their RHs at two temperatures (10 and 20°C) were measured using Hobo temperature and RH sensors (Onset Computer Corp., Pocasset, MA). At both temperatures, RH was within ± 2% of the expected, published values (Winston and Bates 1960). The humidity chambers were used for incubation in all detached leaf assays.

Inoculum preparation and inoculation

Spilocaea oleagina was found to grow very slowly on artificial media, on which it produced no conidia. Consequently, when conidia were needed for inoculation they were obtained from leaves with characteristic OLS lesions picked from naturally infected olive trees ('Barnea') grown in a commercial grove in Canterbury, New Zealand. The leaves were agitated in distilled water and the conidial suspension filtered through a double layer of cheesecloth to remove leaf debris. On the day of each experiment, ten leaves with actively sporulating OLS lesions were picked and washed together to produce the inoculum suspensions. For all experiments, inoculum suspensions were adjusted to 5×10^4 conidia ml^{-1} using a haemacytometer, the conidia being identified by their characteristic morphology (Graniti 1993). Conidium viability of all conidial suspensions was determined according to the methods of Kashy et al. (1991) and found to be similar (55 to 60%).

Preliminary experiment

In a preliminary experiment, six olive leaves of the same age were detached from each of six different greenhouse plants and were each inoculated with three drops (10 μl) of a conidial suspension deposited on the upper leaf surface. After inoculation, the leaves were arranged in a randomised design on wire gauze within six replicate humidity chambers (RH 100%), which were sealed and incubated at 20°C for 24 h in the dark. After 24 h, the leaves were removed from the humidity chambers and dried gently with air from a fan set at slow speed and placed 30 cm from the leaves at room temperature (about 20°C and 50% RH).

To evaluate conidium germination and appressorium formation, the dried leaves were cut into 1×1-cm pieces such that each contained the site of the conidium droplets, and cleared in a 1:1 solution of glacial acetic acid and 95% ethanol for 24 h. They were then stained with aniline blue (Saad and Masri 1978) and examined using a light microscope at ×200 magnification. The total number of germinated and ungerminated conidia was counted in each of the 12 microscope fields observed for each droplet. A conidium was considered germinated if the length of the germ tube exceeded half the length of the conidium. The number of germinated conidia with appressoria was also recorded. Analysis of variance was conducted on percent germination data. The variation in percentage of germination between the leaves and droplets was found to be low (±1%), and when the number of leaves was reduced to three, the mean percent germination could be estimated to within ±2%. Therefore, all subsequent inoculations were performed with six or three leaves according to level of variations to be tolerated in the experiments.

Effect of leaf age on conidium germination and appressorium formation

Five leaf ages (2, 4, 6, 8 and 10 weeks) were tested. Six leaves per age group were inoculated, allocated to blocks, arranged in humidity chambers, and incubated as described for the preliminary experiment above. After 24 h, the leaves were removed from the humidity chambers, dried and examined for conidium germination and appressorium formation as described previously. The experiment was conducted twice.

Effect of temperature on conidium germination and germ tube elongation

Six temperatures (5, 10, 15, 20, 25 and 30°C) were tested. Fully expanded leaves (4 weeks old) were

excised from the olive plants grown in the greenhouse by cutting at the stem end of the petiole. The leaves were inoculated as described for the preliminary experiment. After inoculation, the leaves were arranged randomly on wire gauze and sealed in the six humidity chambers (100% RH), one for each incubation at each of the five temperatures. For each temperature, there were 36 leaves of which six leaves were removed from the humidity chamber after 48 h for assessing percentage conidium germination and appressorium formation as described for the preliminary experiment.

Three of the remaining leaves in each humidity chamber were randomly selected after incubation of 12, 18, 24, 36, 48, 72, 96, 120, 144 and 168 h for determining germ tube length. The recorded lengths were from the longest germ tube for each of 10 randomly selected conidia per droplet (total of 30 conidia per replicate leaf for each treatment). The germ tubes were measured using image analysis software (analySIS*B, Soft Imaging System GmbH, Munster, Germany). The experiment was conducted three times.

Effect of leaf wetness duration on conidium germination

Seven leaf wetness periods (6, 9, 12, 18, 24, 36 and 48 h) at six temperatures (5, 10, 15, 20, 25 and 30°C) were tested. The second pairs of fully expanded leaves (4 weeks old) per shoot were excised from olive plants grown in the greenhouse. For each temperature, there were two identical humidity chambers each containing 24 olive leaves. The leaves were inoculated, allocated to blocks, and arranged in humidity chambers (100% RH).

At the end of each leaf wetness period, three leaf samples were selected randomly from each of the replicate humidity chambers and air-dried. The designated wetness periods included the time required for leaves to dry after removal from the humidity chambers (approximately 30 min). The dry leaf pieces containing the conidium droplets were cut, cleared and assessed for conidium germination and appressorium formation. The experiment was conducted three times.

Effect of RH on conidium germination following different periods of leaf wetness

Detached olive leaves (4 weeks old) were obtained from the plants grown in the greenhouse and inoculated. After inoculation, the leaves were arranged randomly on wire gauze in three identical humidity chambers (100% RH) to ensure continuous leaf wetness and incubated at 20°C in the dark. All leaves were inoculated with the conidia within 30 min after harvesting. After each wetness period (0, 6, 9, 12, 18, 24 and 48 h), 18 randomly selected leaves were removed from the humidity chamber and air-dried. They were then allocated to the three different humidity chambers (60, 80, and 100±2% RH) (six leaves each) for a period that provided a total of 48 h incubation for all leaves (48, 42, 39, 36, 30, 24 and 0 h, respectively). At the end of the second incubation, the leaves were cleared and assessed for conidium germination. The experiment was conducted twice.

Data analysis and model development

Analysis of variance (ANOVA) and model fitting was conducted using Genstat GLM (Genstat 7.2, Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, UK) and R 2.0.1 (R Development Core Team 2004) to assess the effects of leaf age, temperature, RH and leaf wetness duration on percent conidium germination, appressorium formation and germ tube length. A preliminary *F*-test was conducted to determine whether observations of repeated experimental trials could be pooled. Pairwise comparisons were conducted using Fisher's least significant difference tests to identify the source of the heterogeneity between trials. In instances where no significant ($P>0.05$) differences were found between trials, data were combined and a single regression line fitted.

The relationship between leaf age and percentage conidium germination at 20°C after 48 h of incubation was best described by the linear model: $Y = a + bA$, where Y is the percentage conidium germination, A is leaf age, a is the intercept on the y-axis and b is the slope of the regression.

For the effect of temperature on conidium germination, appressorium formation and germ tube growth, attempts were made to fit several models, but second-order polynomial models best described the relationships. They were of the form:

$Y = c + bT + aT^2$, where Y is percentage conidium germination or appressorium formation, T is temperature and a, b, c, are parameters to be estimated. For the germ tube data, the models were fitted separately for each temperature and were

described by $Y = c + bt + at^2$, where Y is germ tube length and t is the incubation time and a, b, c, are parameters to be estimated.

For the effect of leaf wetness duration and RH on conidium germination, both polynomial function and asymptotic regression $[Y = a(1 - \exp(-bw))]$ were used to explore the data at each temperature and RH level using R version 2.0.1 (R Development Core Team 2004) with the 'nlme' statistical package (Pinheiro et al. 2004). The Akaike information criterion (AIC) was used for model selection (Sakamoto et al. 1986), which was calculated as: $\mathrm{AIC} = -2\log[L(\theta/y)] + 2K$, where $\log[L(\theta/y)]$ is the log-likelihood function at its maximum point and K is the number of estimated parameters in the model. The model with lowest AIC value was selected as the best model (Pinheiro and Bates 2000). The goodness-of-fit of the models was assessed based on size of the asymptote, the standard errors associated with the estimated parameters, P values of the estimated parameters and the analysis of residual plots. Before fitting the model for RH effect, conidium germination was expressed as the percentage of maximum germination at 48 h of continuous wetness.

For all regression analysis, a normal distribution was assumed because the AIC values and the form of residuals indicated that percent germination or percent maximum germination fitted a normal distribution better than a binomial distribution.

Results

Germination characteristics

Conidia were observed to be either one-celled or two-celled, but only the two-celled conidia germinated. Germination of a conidium began with splitting in the wall at the end(s) of the conidium followed soon after by emergence of a hyaline germ tube. The conidia germinated at both ends or on the sides, but any one conidium rarely had more than two germ tubes.

Effect of leaf age on conidium germination

The effect of leaf age on conidium germination after 48 h incubation at 20°C differed between two repeated experiments, but the trends were similar. Olive leaf age significantly ($P<0.001$) affected the germination of *S. oleagina* conidia. As the leaf became older, conidium germination decreased (Fig. 1). For Trial 1, the conidium germination was significantly ($P<0.001$) different between all leaf ages except between 6 and 8 week-old leaves. However, in Trial 2 the only leaf age comparison that was not significantly different for conidium germination was between 2 and 4 week-old leaves. Although other models were fitted for both trials, a simple linear model best described the data. The estimates of the parameters from the linear models and their standard errors are presented in Table 1.

The percentage of germinated conidia which formed appressoria within 48 h differed between trials, although leaf age had no significant ($P=0.082$) effect on appressoria formation. In Trial 1, the percentage of germinated conidia that had formed appressoria on the 2, 4, 6, 8, and 10-week-old leaves was 34.0, 25.0, 30.9, 33.5, and 23.5%, respectively. In contrast, in Trial 2 it was 49.1, 47.4, 43.8, 35.4 and 37.1%, respectively.

Effect of temperature on conidium germination, appressorium formation and germ tube length

Temperature significantly ($P<0.001$) influenced conidium germination. After 48 h of continuous wetness, conidia germinated at temperatures from 5 to 25°C, with maximum germination being observed at 20°C. The effect of temperature on conidium germination

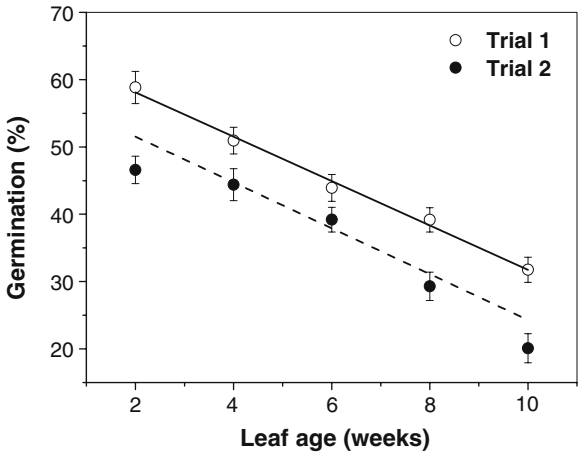

Fig. 1 Effect of leaf age on the germination of *Spilocaea oleagina* conidia inoculated onto detached olive leaves. Data presented (*symbols*) are the means of six replicate leaves with three inoculum droplets per leaf, whereas *lines* are predicted values, based on regression equations derived from point data

Table 1 Estimated parameters and associated standard errors (SE) for the polynomial models described in Figs. 1, 2, 3, 4, and 5

	Estimated parameters						R^2
	a		*b*		*c*		
	Value	SE	Value	SE	Value	SE	
Fig. 1 $Y = a + bA$							
Model							
Trial 1	58.3	2.25	−3.41	0.339	–	–	0.945
Trial 2	64.7	2.09	−3.30	0.315	–	–	0.994
Fig. 2 $Y = a + bT + cT^2$							
Germination	−11.2	2.68	8.46	0.351	−0.27	0.009	–
Appressorium	3.3	4.97	7.17	0.587	−0.29	0.019	–
Fig. 3 $Y = a + bt + ct^2$							
Temp. (°C)							
5	−9.6	4.24	1.54	0.120	−0.0005	0.00007	–
10	−7.8	1.86	1.95	0.089	−0.0053	0.00409	–
15	2.6	3.61	1.79	0.078	−0.0018	0.00059	–
20	29.3	4.96	1.48	0.112	−0.0025	0.00052	–
25	20.3	1.93	0.87	0.059	−0.0014	0.00033	–
Fig. 4 $Y = a + bw + cw^2$							
Temp. (°C)							
5	−10.7	1.28	1.12	0.122	−0.002	0.0022	–
10	−12.8	1.31	2.17	0.125	−0.022	0.0020	–
15	−9.1	1.63	2.67	0.155	−0.027	0.0020	–
20	−18.7	1.48	4.13	0.141	−0.051	0.0030	–
25	−17.9	1.65	2.96	0.157	−0.033	0.0030	–
Fig. 5 $Y = a + bw + cw^2$							
RH (%)							
60	−66.6	7.30	14.25	1.119	−0.370	0.0365	–
80	−69.4	7.27	15.11	1.114	−0.394	0.0363	–
100	0.9	6.17	6.98	0.946	−0.146	0.0309	–

Fig. 1: Effect of leaf age (*A*) on *S. oleagina* conidium germination; Fig. 2: Effect of temperature (*T*) on *S. oleagina* conidium germination and appressorium formation; Fig. 3: *S. oleagina* germ tube elongation over time (*t*) at different temperatures; Fig. 4: Effect of leaf wetness duration (*w*) on *S. oleagina* conidium germination at different temperatures; Fig. 5: *S. oleagina* conidium germination at different RHs after initial leaf wetness periods (*w*).

followed a similar trend in all repeated trials. There was no significant (P=0.123) difference between the trials, except at 15°C in Trial 3 in which germination was significantly (P<0.05) higher than in the other two trials. The highest percentage of germination was observed at 20°C and the estimated optimum temperature from the quadratic response function was 15.9°C (Fig. 2a). The estimates of the parameters from the quadratic function and their standard errors are presented in Table 1.

Many germ tubes of *S. oleagina* formed amorphous appressoria that were visible as swellings at the tip of the germ tubes. This occurred within 6 h of germ tube emergence irrespective of the incubation temperature. Appressorium formation appeared to be favoured by cooler temperatures than conidium germination. The formation of appressoria increased gradually from 33% at 5°C, to a maximum of 43% at 15°C after 48 h of continuous wetness, and then declined with further increase in temperature (Fig. 2b).

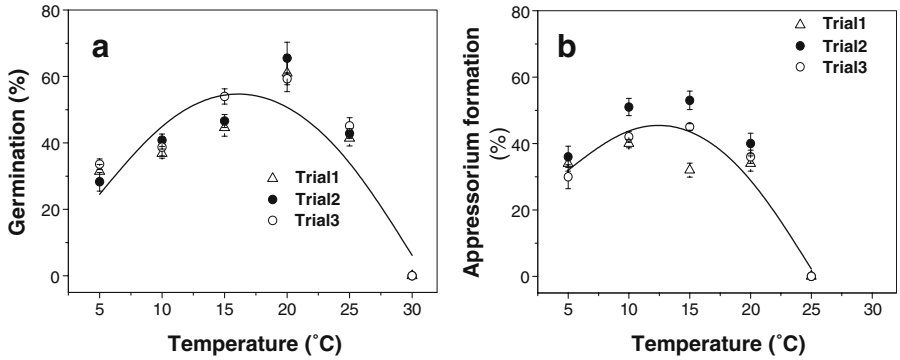

Fig. 2 Effect of temperature on germination (**a**) and appressorium formation (**b**) 48 h after *Spilocaea oleagina* conidia were inoculated onto detached olive leaves and kept wet continuously. *Symbols* represent the means of 18 observations for each data point and the *lines* represent the predicted values

Although approximately 40% of the conidia had germinated at 25°C after 48 h of incubation, none produced appressoria. Appressorium formation at 10 and 20°C was significantly ($P<0.05$) lower in Trial 1 than in Trial 3 but similar at all other temperatures. The relationship between temperature and appressorium formation followed a similar trend as conidium germination except that the highest percentage of

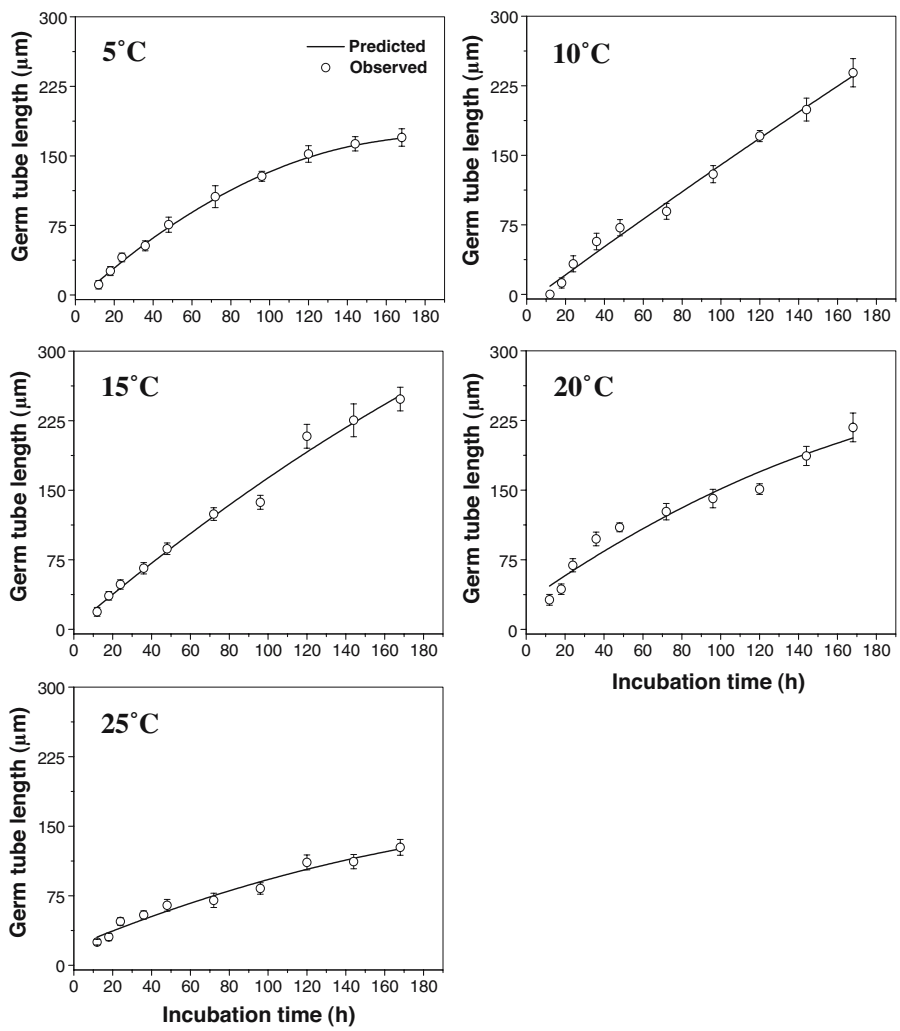

Fig. 3 Influence of temperature on germ tube elongation of *Spilocaea oleagina* on detached olive leaves at various incubation periods. *Symbols* represent the means of three trials with 30 observations for each incubation time. The *lines* represent the predicted values

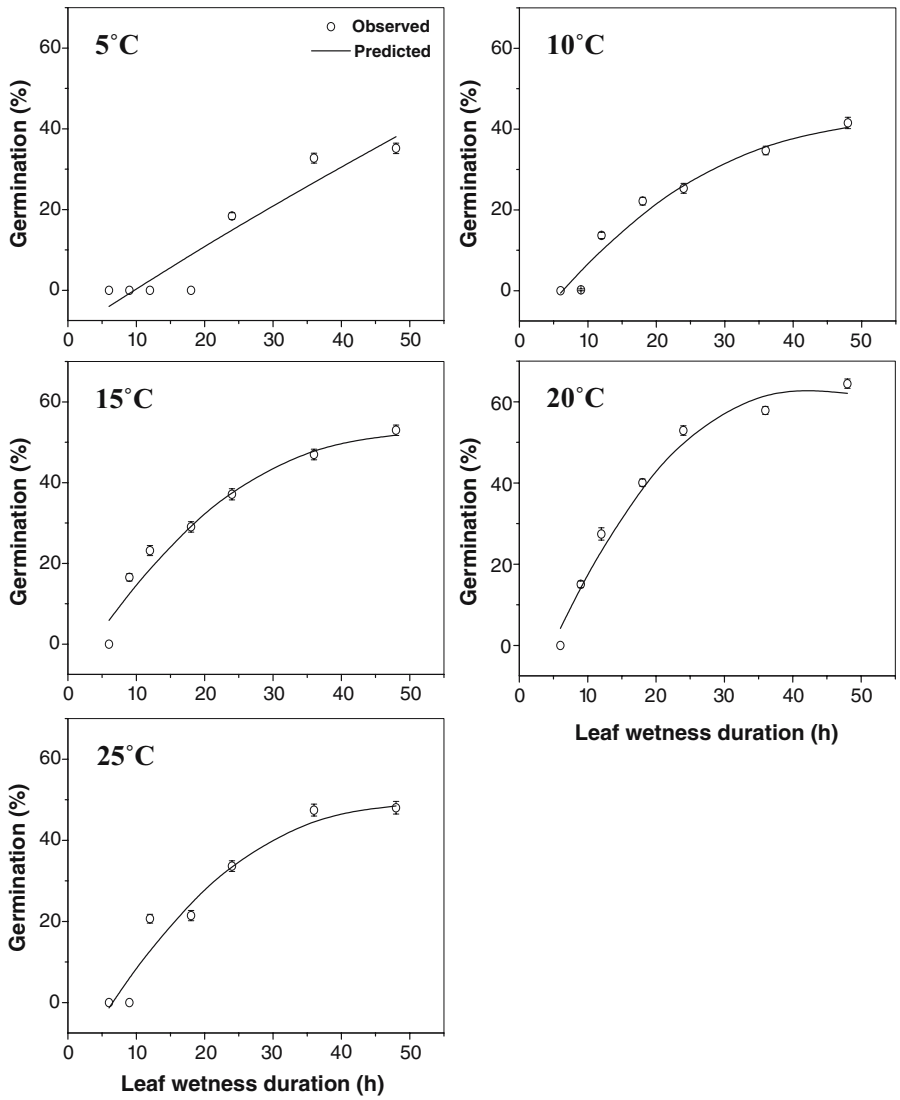

Fig. 4 Effect of leaf wetness duration on *Spilocaea oleagina* conidium germination on detached olive leaves at various temperatures. *Symbols* represent the means of three trials with 54 observations for each combination of temperature and incubation time and the *lines* represent the predicted values

germinated conidia with appressoria was observed at 15°C and the estimated optimum temperature from the quadratic response function was 12.5°C (Fig. 2b). The estimates of the parameters from the regression models and their standard errors are presented in Table 1.

Germ tube length increased with increasing incubation period. Temperature made little difference to the mean rate of germ tube elongation except that after longer incubation periods it was less at the higher temperatures (20 and 25°C) than at the other temperatures tested (Fig. 3). For example, although the mean germ tube lengths at 15 and 20°C were similar (110 μm) after 48 h of incubation, after 168 h the mean length was significantly ($P<0.001$) greater at 15°C than at 20°C. The mean length of germ tubes after 168 h of incubation was greatest at 15°C (245 to 260 μm) followed by 10°C (220 to 245 μm) and at 20°C it ranged from 150 to 228 μm. The length of the germ tubes was consistently less at 25°C compared with the other temperatures tested, and at 25°C they were observed to be narrower than at the other temperatures. However, the mean number of germ tubes per conidium was higher (1.6) at 25°C compared with the other temperatures tested (1.1 to 1.3). No germ tube branching was observed after 168 h of incubation at all temperatures.

Although logistic and polynomial regressions were explored to determine whether a single or series of lines would adequately describe the effect of temperature

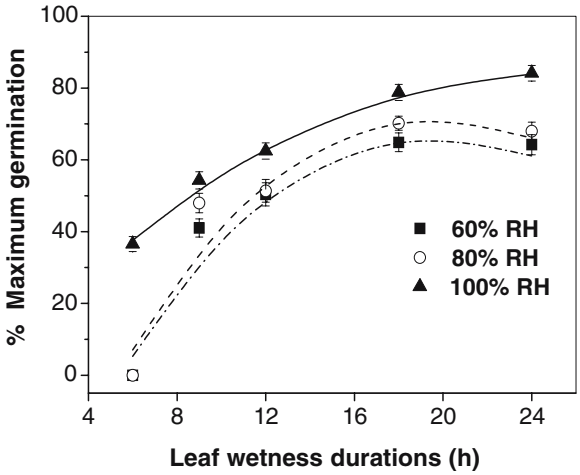

Fig. 5 *Spilocaea oleagina* conidium germination at different RHs following different initial leaf wetness periods. *Symbols* represent the means of observations for Trials 1 and 2 expressed as the percentage of maximum germination (60 and 57.9%, respectively) at 48 h continuous wetness and 20°C. *Values* are means of two trials with 18 observations each and the *lines* represent the predicted values

on germ tube elongation at various incubation times, germ tube growth at each temperature was best described by a second-order polynomial regression (Fig. 3). The logistic model consistently underestimated the germ tube length at 15 and 25°C, whereas at 10 and 5°C it overestimated the germ tube length.

Effect of leaf wetness duration on conidium germination

Generally, percent germination increased with increasing wetness duration at all temperatures tested. Conidium germination was first observed 24, 12, 9, 9, and 12 h after inoculating the leaves at 5, 10, 15, 20, and 25°C, respectively (Fig. 4). The mean percentage of germination after 24 h was 16.1, 23.9, 38.8, 47.8, and 35.5% at 5, 10, 15, 20, and 25°C, respectively. At all temperatures, no conidium germinated without free moisture. The polynomial models at each temperature consistently had lower AIC values (2,585–2,777) compared to the asymptotic models (2,777–2,865), and at 5 and 10°C the asymptotic models failed to converge. The relationship between temperature and leaf wetness duration in relation to conidium germination at each temperature was best described by: $Y = a + bw + cw^2$, where Y is the predicted percentage germination and w is leaf wetness duration (Fig. 4). The estimates of the parameters and their standard errors are presented in Table 1.

Effect of initial leaf wetness duration and RH on conidium germination

At 20°C, percent germination increased with increasing initial wetness duration and appeared to reach an asymptote in the range of 30 to 50% germination. Conidium germination was significantly ($P<0.001$) affected by the duration of initial leaf wetness and the RH. The RH treatments after initial wetness showed that without free moisture (0 h), no conidia germinated after 48 h of incubation, even at 100% RH. However, after 6 h initial wetness, 20% of conidia germinated at 100% RH but no germination occurred when the subsequent incubation was at 60 or 80% RH. Higher RH (100%) following the end of the wetness periods increased ($P<0.05$) conidium germination compared with 80% RH. However, there was no significant ($P=0.364$) difference between levels of conidium germination at 60 and 80% RH, irrespective of the initial leaf wetness duration (Fig. 5). Visual observation of the inoculated leaves showed no water condensation on the leaves at the end of the second period of incubation.

Although both polynomial and asymptotic regressions were explored to evaluate the effect of RH on *S. oleagina* conidium germination on detached olive leaf following initial wetness duration at each RH level, the polynomial regression functions were considered to be the best because they had lower AIC values (1,450–1,513), higher P values of estimated parameters and satisfactory residual plots. Consequently, only the results obtained for this model are presented here. The predicted germination was given by: $Y = a + bw + cw^2$, in which Y is the percentage of maximum germination, and w is the initial wetness duration (Fig. 5). The estimates of the parameters and their standard errors are presented in Table 1.

Discussion

This study was the first comprehensive investigation of the combined effects of temperature, leaf wetness, and leaf age on germination, germ tube, and appressorium development of *S. oleagina* conidia on detached olive leaves. Percent germination was found

to decrease with increasing leaf age, which had not been reported previously for *S. oleagina*. However, similar results have been reported for other pathogenic fungi (Bentes and Matsuoka 2002). For example, a higher percentage germination of *Colletotrichum guaranicola* conidia was reported on young leaves (94.7%) than old leaves (38%) of susceptible clones of *Paullinia cupana* var. *sorbilis* 24 h after inoculation. In a similar study, Viljanen-Rollinson et al. (1998) showed that more *Erysiphe pisi* conidia germinated on 5-day-old leaflets (79.8%) than on 15-day-old leaflets (69.7%) of pea plants. It was also demonstrated that on apple leaves, the germination and infection by *Venturia inaequalis* conidia (*Spilocaea pomi*) was higher on younger leaves than older ones (Schwabe 1979; MacHardy 1996). The causes of the differences in conidium germination at the various leaf ages are probably associated with the availability of nutrients on the surfaces of the leaves, although other mechanisms of spore activation may be involved. Young olive leaves have thinner cuticles compared with older ones and, as a result, are more likely to leak nutrients to the surfaces of the leaves. *Spilocaea oleagina* conidia germinated more slowly on water agar (25%) compared with nutrient-containing media (60%) at 48 h (F. O. Obanor, unpublished), suggesting that nutrient availability is important in their activation and subsequent germination.

Several substances can regulate spore germination, including endogenous self-inhibitory chemicals and components of plant epicuticular wax that stimulate germination (Podila et al. 1993; Kolattukudy et al. 1995). The surface wax encountered by spores that land on plants may also contain chemical components that act as signals for the beginning of a plant-fungus interaction and the ratio of these components changes with leaf age (Jetter and Schäffer 2001). Podila et al. (1993) showed that the epicuticular wax component, fatty alcohol, from avocado fruit stimulated germination of *Colletotrichum gloeosporioides* conidia and appressorium formation in various conditions. In addition, Prusky et al. (1991) reported an involvement of a chemical signal from the epicuticular wax during the interaction of avocado and *C. gloeosporioides*, the cause of avocado anthracnose. Therefore, differences in levels of epicuticular wax chemicals at various olive leaf ages could have contributed to the higher germinability of *S. oleagina* conidia on young leaves compared with older ones. The higher levels of conidium germination on young olive leaves could explain, in part, the higher levels of OLS infection on young leaves under field conditions.

The temperature range of 5 to 25°C observed for conidium germination on detached olive leaves is similar to that reported previously for *S. oleagina* conidium germination by Saad and Masri (1978). However, other researchers have reported conidium germination on agar to occur at a minimum of 5°C, with an optimum of 20°C, and a maximum of 30°C (Wilson and Miller 1949; Dzaganiya 1967; Kashy et al. 1991), although Mijuskovic (1969) reported germination occurring on agar at 7 to 28°C with an optimum of 14 to 19°C. The wide range of temperatures at which *S. oleagina* conidia germinate suggests that infection may occur throughout the year in olive-growing regions with mild temperatures leading to high OLS disease levels since young susceptible olive leaves are always available in groves.

The germ tube length increased with increasing leaf wetness duration, although temperature had no significant effect on germ tube development except with prolonged incubation. Interestingly, however, more germ tubes per conidium were observed at 25°C and they were narrower than at temperatures <25°C. The apparent deformity in the germ tube structure could be associated with its inability to penetrate and infect the host tissues. This may explain the lack of new infection and slow lesion development on olive leaves, which was observed when the temperature was >25°C in the field (Laviola and Scarito 1993; Guechi and Girre 1994).

In this study, *S. oleagina* formed appressoria over a wide range of temperatures although formation was favoured by cool temperature (<20°C) and severely reduced at 25°C. In addition, the time taken for appressoria to first appear was not affected by temperature. In contrast, Saad and Masri (1978) observed no appressoria at temperatures <16°C, and at higher temperatures appressorium formation time was temperature-dependent. For example, they observed appressorium formation 8 and 16 h after conidia had germinated at 20 and 24°C, respectively. This differed from other pathogens in which the optimum temperature for appressorium formation coincides with that for spore germination. For example, Miehle and Lukezic (1972) showed that the optimum temperature for *Colletotrichum trifolii* conidium germination and appressorium formation were similar (24°C) after 24 h

of incubation. However, our study demonstrated that the optimum temperature for appressorium formation was different from that of conidium germination.

The relationship between conidium germination and leaf wetness duration was demonstrated by Saad and Masri (1978) who reported that on detached olive leaves, a minimum of 42 h leaf wetness was required for *S. oleagina* conidia to germinate at 12°C, while at 20°C, 18 h was required. In the present study, percent conidium germination increased with increasing wetness duration at all temperatures tested. For the first signs of conidium germination, different periods of leaf wetness were required at different temperatures, such that at 5°C germination of conidia required 24 h of leaf wetness, whereas at 20°C, 9 h were required. The differences in that report and the results of the present study could be attributed to various intrinsic and extrinsic factors. For example, they could be associated with differences in isolates used in the two reports as well as the capacity of the conidia to absorb water from the surrounding environment. The report of Saad and Masri (1978) provided no information on time of year conidia were collected or on storage conditions. Our previous report showed that the viability of *S. oleagina* conidia was significantly reduced during the summer months in New Zealand olive groves (Obanor et al. 2005). The shorter minimum leaf wetness at all temperatures required for conidia to germinate suggests that several generations of infection may occur in olive-growing regions with cool, moist conditions. This is consistent with the observed high OLS disease levels in New Zealand olive groves.

In this study, high RH alone was not sufficient to induce conidium germination on leaves incubated at 20°C for 48 h, indicating that free moisture is essential. Approximately 6 h of initial leaf wetness followed by 100% RH was required for conidium germination. This result is in agreement with Salerno (1966) who reported that free moisture was required for the germination of conidia and that they germinated poorly, if at all, at <90% RH. However, Saad and Masri (1978) found that on detached olive leaves 62% of the conidia had germinated when the leaves were incubated at 20°C under 100% RH for 48 h. In that study, no information was given on either spore imbibing time or on potential for water condensation on the leaf surface. In the experiments reported here, water condensation on leaves was not observed, thus demonstrating that conidia of *S. oleagina* cannot germinate and infect at high RH at 20°C in the absence of free moisture. Our results also indicated that high RH periods could be important after rainfall events of short duration.

The results of this study have the potential to be used in development of an OLS prediction model, but would need to be validated in the field before being incorporated into disease management strategies. This may improve the timing of fungicide spray applications to better target high risk periods and may allow a reduction in the number of sprays whilst maintaining protection of susceptible host tissues. However, before a prediction model can be developed, further research should be carried out to investigate the effects of environmental factors on OLS infection and disease severity under controlled conditions.

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