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OLIVE LEAF SPOT: EPIDEMIOLOGY AND CONTROL
OLIVE LEAF SPOT: EPIDEMIOLOGY AND CONTROL

A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy at
Lincoln University,
Canterbury, New Zealand

FRIDAY OSAS OBANOR

2006
DEDICATION

This thesis is dedicated to the underprivileged children
around the world.
Olive fruits of the cultivar ‘Barnea’
The overall goal of this research was to improve the understanding of the environmental factors affecting development of olive leaf spot (OLS) in New Zealand olive groves and to develop strategies for the disease management.

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 leaf 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 ($P<0.001$). Temperature significantly ($P<0.001$) affected conidium germination, with the effective range being 5 to 25°C and the optimum being 20°C. The rate of germ tube elongation followed a similar trend, with the optimum being 15°C. Formation of appressoria, when found, occurred 6 h after the first signs of germination and their frequency followed the same temperature trends with a maximum of 43% at 15°C and none formed at 25°C. The minimum leaf wetness periods for germination at 5, 10, 15, 20, and 25°C were 24, 12, 9, 9, and 12 h, respectively. Studies on the pre-penetration and infection processes on olive leaves by *S. oleagina*, using scanning electron microscopy, showed that the penetration pegs allowed direct penetration of the cuticle, but could not penetrate the host through the trichomes.

A study of the effects of temperature, leaf age, conidial concentration, continuous and interrupted leaf wetness periods and relative humidity (RH) during the dry periods on whole plants showed that OLS severity decreased ($P<0.001$) with increasing leaf age at the time of inoculation. The severity of OLS also increased as inoculum concentration increased from
1.0 × 10² to 2.5 × 10⁵ conidia per mL at all five temperatures (5-25°C), with most lesions formed at 15°C and least at 25°C. The minimum leaf wetness periods for infection at 5, 10, 15, 20, and 25°C were 18, 12, 12, 12, and 24 h, respectively. Infection frequencies during wet periods interrupted with dry periods of 70 and 100% RH depended on the RH and length of the dry period and the length of the initial wet period, with the lowest OLS severity after 12 h wetness and 12 h at 70% RH.

Sporulation on olive leaf spot lesions followed similar trends over the same temperatures and moisture conditions, with maximum sporulation at 15°C and 100% RH but none at 25°C and 70% RH. Lesion expansion was affected (P<0.001) by the same conditions, with 15°C and continuous wetness being optimum and very little expansion occurring at 25°C.

Sequence analysis of rDNA (ITS1-5.8S-ITS2), restriction fragment length polymorphism (RFLP), and universally primed-polymerase chain reaction (UP-PCR) techniques were used to study the genetic structure of S. oleagina populations using 98 isolates from New Zealand olive groves along with one isolate each from Australia and Italy. Alignment of the cloned sequences of four New Zealand isolates with the isolates from Australia and Italy showed great similarity with similarity indices for ITS1, ITS2 and 5.8S coding regions of the isolates being 96-100, 99.8-100 and 99-100%, respectively. RFLP analysis revealed no differences between the New Zealand isolates but UP-PCR found low levels of gene and genotypic diversity in all populations. Analysis of molecular variance (AMOVA) showed small but significant (P = 0.001) variations among regions, and most of the molecular variability (87%) was found within populations.

Greenhouse studies of the fungicides, boscalid, captan, carbendazim, copper hydroxide, copper sulphate, difenoconazole, dodine, kresoxim-methyl, and a kresoxim-methyl/copper hydroxide mixture, showed that all significantly (P<0.001) reduced OLS severity, with highest efficacy when applied up to 3 days pre- and 3 days post-inoculation. In field trials, most of the fungicides reduced disease incidence when applied twice in spring and autumn. Of the fungicides tested, copper sulphate and a mixture of kresoxim-methyl/copper hydroxide were the most effective, reducing disease severity by 85 to 96% and 63 to 93%, respectively.
This research has provided three models relating climatic parameters to OLS development that focus on conidium germination, infection and sporulation models which should be field-tested with the more promising fungicides. They could then be incorporated into a disease forecasting system, which may be used in conjunction with a spray schedule that could improve disease control for New Zealand growers by optimising the number and timing of fungicide applications.

Keywords: Peacock spot, Cycloconium oleagina, Fusicladium oleagina, Olea europaea, olive leaf spot, germination, infection, sporulation, fungicides, and population diversity.
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"If we are only for ourselves, what are our lives worth?"

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CHAPTER 1

Introduction

1.1 OLIVE PRODUCTION

The cultivated olive, *Olea europaea* L., is among the oldest known cultivated tree species in the world (Figure 1). In the past several hundred years the olive has been spread from the Mediterranean region to North and South America, Japan, New Zealand and Australia (Hartmann et al., 1980). Although olives have been grown in New Zealand for over a century, there has been resurgence in interest since the mid to late 1980’s which can be attributed to a greater awareness of the health and culinary benefits of olive oil (Richard, 2000).

![Figure 1](image)

*Figure 1*: An olive grove in Blenheim, New Zealand, planted with the cv. ‘Barnea’.

The productivity of olive trees is influenced by several factors, including environmental conditions and potential damage by pests and diseases. Climate is the most important environmental variable affecting the production of olives since it determines when or if fruit
will ripen, the degree to which pests and diseases may affect crops and the amount of irrigation that will be required. Olive production thrives in climates with warm and dry summers, but very high summer temperatures adversely affect yield, as does high rainfall (Grigg, 2001). The trees can tolerate some frost, but not sustained low temperatures. The highest olive productivity occurs in areas that have mild winters and long, warm, dry summers, such as in the Mediterranean countries (Spain, Italy and Greece). These climatic requirements for olive cultivation contrast sharply with some other olive producing countries such as New Zealand, Chile, and Argentina where the temperate climates are dominated by cool, wet springs and summers, and frosty winters. Olive productivity in the New Zealand climate is at best ‘marginal’ but olive oil produced in New Zealand is of very high quality, with most oil meeting the “extra virgin” criteria.

In traditional cultivation systems, the productivity of olive trees is relatively low. It often involves mixed cropping systems, in which long term average yields of 2.5 tonnes per hectare are considered high. However, the production potential of many olive cultivars is 4-5 times higher when grown under intensive, irrigated, modern growing conditions (Hartmann, 1983; Crescimanno, 1965).

Pests and diseases can reduce the potential productivity of olives. Several diseases, such as olive leaf spot (OLS), and olive knot, have been recorded in most olive growing regions worldwide. OLS is one of the most serious diseases in all olive-growing regions in the world. However, few severe outbreaks of the disease have been reported in the major olive producing countries, such as Spain and Italy (Tosi and Zazzerini, 2000). In California, for example, little damage was attributed to olive leaf spot until the period 1941 through 1949 when severe outbreaks resulted in yield losses estimated to be as high as 20% in some areas (Wilson and Ogawa, 1979). However, there have been no reports with specific data on the relationship between OLS severity, incidence and olive yield losses, information which is vital in establishing the economic impact of OLS disease on olive production, and the need to develop sound control strategies that can maximise growers’ profits.
1.2 SYMPTOMS OF OLIVE LEAF SPOT

Olive leaf spot usually occurs on the upper surface of the leaf (Figure 2A). Spots on the lower leaf surface are rare, and when they occur, spots are masked by the thick layer of peltate hairs (Graniti, 1993). Leaf lesions are initially inconspicuous sooty blotches but later develop into muddy green to almost black circular spots 2 to 10 mm in diameter. Old spots may show necrotic, olivaceous-green centres with one or a few brownish concentric rings (Chen et al., 1981), sometimes with a faint peripheral, yellowish, violet or pale-brown halo. The dark green to black spots on the yellow background of the leaf blade are thought by some researchers to resemble the spots on a peacock’s tail, hence the alternative names ‘peacock’s eye’ or ‘peacock spot’.

As the spots expand and coalesce to cover a large proportion of the leaf area, leaves often senesce turning yellow and being shed from the tree prematurely. Recurrent infections cause poor growth and dieback of the defoliated twigs (Miller, 1949; Laviola, 1992; Azeri, 1993; López Doncel et al., 2000). Since flushes of new leaves are greatest in spring and these new leaves are highly susceptible to the disease, disease outbreaks at this time can cause severe defoliation and so limit photosynthesis during the remainder of the year (Graniti, 1993). However, the direct causes of defoliation, whether due to petiole infections, threshold sizes of lesions or a combination of stress factors, have not been reported.

Occasionally under very wet conditions, the pathogen may penetrate young petioles, peduncles and fruit, causing small, sunken brown lesions (Figure 2B). However, these are uncommon except on susceptible cultivars such as ‘Barnea’, in which fruit peduncles are frequently infected, sometimes causing fruit drop (Prota, 1995).

Figure 2: Olive leaf spot symptoms on olive leaves (A) and fruits (B), cv. Barnea.
Olive leaf spot can cause reduced growth and yield in olive trees, as well as poorer olive oil quality (Verona and Gambogi, 1964). Apart from the dieback of twigs and branches defoliated by recurrent leaf spot infection, losses arise mostly from the reduction in leaf area (Wilson and Miller, 1949). In addition, the shedding of auxiliary leaf buds in severely infected trees means that fewer can be converted to flowering shoots, probably causing still lower yield (Castellani, 1952). Infection of fruit can also cause unacceptable blemishes on table olives, and when it occurs on oil-producing cultivars, infection may cause a delay in ripening and decreases in oil yield (Verona and Gambogi, 1964).

No estimates of losses due to OLS effects have been reported in most olive-growing regions worldwide where serious outbreaks are usually only sporadic, but the damage done is considered significant enough to warrant control measures (Wilson and Miller, 1949; Tosi and Zazzerini, 2000).

1.3 CAUSAL ORGANISM

1.3.1 Name and description of the pathogen
The causal organism of OLS was first described in southern France by Castagne (1845) as Cycloconium olaeaginum, but Boyer (1891) introduced the Latinized spelling of the species name, oleaginum. However, Hughes (1953) placed the fungus in the genus Spilocaea, hence the current name Spilocaea oleagina. Although no sexual stage has been found, recent phylogenetic analysis has shown that S. oleagina belongs to the Dothideomycetes class of the Ascomycotina, and is an anamorphic phase of a yet unidentified Venturia species (González-Lamothe et al., 2002). However, very recently the three anamorph genera of Venturia (Fusicladium Pollaccia and Spilocaea) were merged into the well-known and more conserved genera, Fusicladium (Schubert et al., 2003). Hence, the new name for Spilocaea oleagina is Fusicladium oleagina. However, the old name, Spilocaea oleagina, is the one used in all of the literature cited and so will be used throughout this thesis.

The fungus lives parasitically on olive leaves forming subcuticular, flat, radiating colonies. Reproduction occurs when numerous hyphal branches arise from this subcuticular mycelium, pierce the thick cuticle and enlarge above the leaf surface to produce short ampulliform, olivaceous-brown, monoblastic conidiophores. The bulbous, one-celled conidiophores are
10-30 µm in length by 8-15 µm in width. The tip of each short globose conidiophore is surmounted by an apical scar through which conidia are pushed. The conidia are greenish-yellow to yellowish-brown, elongate-ovoid to pyriform with a truncate base and may be one-celled or two-celled. Conidia are 14-27 µm in length and 9-15 µm thick at their broadest part. They may be straight or with the terminal cell somewhat curved (Wilson and Miller, 1949; Graniti, 1993).

1.3.2 Growth characteristics of the fungus on artificial media

The growth characteristics of *S. oleagina* are very similar to those of *Venturia inaequalis* (Cooke) G. Wint., the causal organism of apple black spot (also known as apple scab), on both host tissue and in culture. Both fungi are difficult to culture on artificial media and have relatively slow growth rates. For *S. oleagina*, the most favourable solid medium for growth so far discovered is olive leaf extract (OLE) agar (Appendix 1.1) (Wilson and Miller, 1949). On OLE agar, early growth is in the form of olive brown submerged hyphae, which produce numerous intercalary, sometimes terminal, chlamydospores. The chlamydospores are spherical to oval in shape, dark brown, thick-walled and filled with refractive granules of fatty origin. The pathogen does not sporulate *in vitro*. However, in very few cases it has been observed that the end of a hyphal branch may form a conidium which usually germinates *in situ* giving rise to a continuation of the hyphal strand (Wilson and Miller, 1949).

![Figure 3: Spilocaea oleagina colonies growing on olive leaf extract agar after 6 months of incubation at 20°C.](image)

The fungal colonies are visible to the naked eye as dark, olive-brown spots after 2 to 3 weeks of growth at 20°C. After two months of growth, the aerial mycelium of the colony appears as a hemispherical greyish green, felt-like stromatic body of about 1.5 cm in diameter. These older colonies are differentiated into three layers: a basal submerged layer of loosely interwoven hyphae rich in chlamydospores, a medial layer above the surface of the agar formed of dark-brown closely interwoven hyphae, and an upper aerial layer of light brown felt-like mycelium (Figure 3). It has also been observed that some submerged hyphae may develop minute black sclerotia which are composed of a hard outer rind surrounding a small hyaline softer pith (Miller, 1949).

1.4 LIFE CYCLE OF SPILOCAEA OLEAGINA IN RELATION TO DISEASE DEVELOPMENT

1.4.1 Olive growth stage in relation to OLS disease
The olive is an evergreen perennial crop and the leaves generally live for 2-3 years, dropping when the tree is putting on new growth, or when they are shaded. The annual growth cycle of olives depends on the climatic conditions. In cooler climates, such as occurs in New Zealand, olive trees show rapid growth in spring, with a summer peak in activity, followed by a decline in development of new leaves in autumn and no new leaves appearing in winter (Renowden, 1999). However, in regions with hotter climates the trees produce few or no new leaves in mid-summer, and resume growth again in the autumn, giving two peaks of leaf production (Martin, 1994). It is these new leaves that are most susceptible to OLS disease (Miller, 1949).

The susceptibility of an olive tree to *S. oleagina* appears to be affected by its nutritional status. Soil deficient in calcium was reported to increase the susceptibility of the tree to OLS (Petri, 1913, cited in Miller, 1949). The disease was also reported to be most prevalent on heavy soils where nitrogenous fertilizers were used (Foëx, 1924). In addition, olive groves interplanted with cereals or fodder crops, in which the soil moisture and organic matter were depleted, were reported to suffer increased damage by *S. oleagina* in comparison with trees surrounded by bare soil (Anagnostopoulos, 1938).
OLS infection is most frequently found on leaves in the lower canopy of olive trees and many twigs in these parts become completely defoliated. After rain, these leaves remain wet for longer than those in the upper parts where air circulates more freely. Since rain also washes conidia downward, the leaves in the lowermost branches are exposed to more inoculum than those on branches higher in the tree (Graniti, 1993; Guechi and Girre, 1994). In California, Wilson and Miller (1949) reported that location of the trees with respect to exposure to sunlight and air movement influenced disease severity, with trees along the east or south borders of olive grove being more exposed and so, less diseased than those inside the grove. Guechi and Girre (1994) also reported that in Algeria the damage caused by *S. oleaginosa* on leaves facing north was much greater than on those facing south.

Figure 4: The proposed life cycle of *S. oleaginosa*.

1.4.2 Source of conidia for new infections

Although infection by the pathogen causes leaves to drop prematurely, some infected leaves remain on the tree providing a source of inoculum for new infections in spring (Wilson and Miller, 1949) (Figure 4). Unlike *V. inaequalis*, the main source of *S. oleaginosa* inoculum for
primary infection is the sporulating lesions on leaves that have oversummered on the trees. A new crop of conidia can readily be produced on the same leaf spots after rain or a period of high humidity (Prota, 1995). When most leaves with visible lesions have dropped, for example in hot, dry summers, new inoculum may arise either from leaves with latent infections that resume growth and become manifest, or from minute spots bearing a number of actively sporulating conidiophores (Viruega et al., 1999).

Conidium production by lesions on leaves which remain on the tree, have been reported to vary with the season of the year. In Mediterranean olive-growing regions such as southern Italy and Lebanon, the production of conidia is at a maximum during October and November (autumn and early winter), and in March and April (spring), whereas the inoculum density in summer is low. However, sporulation was reported to be dependent on climatic conditions, especially rain events, humidity, and temperature, which were similar to the optimum conditions for the growth of the fungus during autumn and spring (Saad and Masri, 1978; Viruega et al., 1999; Tosi and Zazzerini, 2000). Under the relatively cool, moist New Zealand field conditions, the seasonal trend of conidium production by \textit{S. oleagina}, conidium viability and development of infections needs to be investigated.

Environmental conditions may influence \textit{S. oleagina} conidium viability. The conidia formed on OLS lesions may be viable for several months, however, once detached from the conidiophores, conidia lose their viability in less than a week (Laviola, 1966; Tosi and Zazzerini, 2000). In central California, it was found that the viability of conidia produced by \textit{S. oleagina} in summer was low (Miller, 1949). In contrast, Saad and Masri (1978), found no significant difference in the viability of conidia produced by \textit{S. oleagina} in a Lebanon olive grove throughout the season.

1.4.3 Dissemination of conidia
Many studies have shown that \textit{S. oleagina} conidia from lesions in the upper part of the trees are usually carried downward by rain water and this downward dispersal accounts, at least in part, for the greater severity of the disease in the lower parts of the tree (Wilson and Miller, 1949; Tenerini, 1964; Laviola, 1968). Although it is generally assumed that conidia are dispersed by rain splash, there is little data available on the distance from its source which a conidium travels during a rain event. However, some recent research has also shown that
wind dissemination of conidia can occur up to 5 m from source without a rain event if relative humidity is high (>90%) (Lops et al., 1993; Frisullo et al., 1994). Furthermore, De Marzo et al. (1993) reported that in olive groves in southern Italy, S. oleagina conidia could be disseminated by insect vectors such as Ectopsocus briggsi Mac Lachlan (Psococottero).

1.4.4 Conidium germination

When a conidium germinates a hyaline to light brown germ tube emerges through a split in the conidium wall at either end after 36-48 hours under favourable conditions (Wilson and Miller, 1949). Research trials on the effect of the various environmental conditions on conidium germination have been poorly reported and results appear to be contradictory. 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, optimum of 20°C, and maximum of 30°C. However, Saad and Masri (1978) demonstrated that on olive leaf surfaces, germination occurred at temperatures ranging from 8 to 24°C with an optimum of 20°C. These authors did not include a minimum temperature lower than 8°C in the range they tested, but did demonstrate a lower maximum range to that reported for conidium germination on agar.

Relatively few field studies have reported an association between temperature, leaf wetness and OLS disease development. Salerno (1966) reported that moisture is required for the germination of conidia and that they germinated poorly, if at all, at low relative humidity (<90%). Although it has been recognised that moisture is essential for conidium germination (Graniti, 1993), to date only one report has been found which demonstrated a relationship between conidium germination and leaf wetness duration under controlled conditions. Saad and Masri (1978) showed that different periods of leaf wetness were required at different temperatures, such that at 12°C germination of conidia required 42 hours of leaf wetness, whereas at 20°C, 18 hours was required (Table 1). However, the study did not include duration of leaf wetness at lower (<12°C) and higher (>24°C) temperatures. Considering the importance of conidium germination to OLS development, further investigation is required to address the inconsistencies in the reported results of the effect of temperature and moisture on conidium germination, on agar and on leaf surfaces, both in laboratory and field conditions. Such information is essential for identifying possible infection periods especially in cooler olive growing regions such as New Zealand. This could improve the timing of
fungicides spray applications to better target high risk periods, potentially allowing a reduction in the number of spray applications, compared to the number used at present, while still maintaining protection against the disease.

**Table 1**: Leaf wetness durations (in hours) required for conidium germination and appressorium formation at different temperatures (Saad and Masri, 1978)

<table>
<thead>
<tr>
<th>Incubation Temperature (°C)</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Process</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conidia germination</td>
<td>42</td>
<td>36</td>
<td>18</td>
<td>24</td>
</tr>
<tr>
<td>Appressoria formation</td>
<td>*</td>
<td>48</td>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td>*Not determined</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.4.5 Infection and lesion development

The mode of infection of *S. oleagina* is similar to that of *V. inaequalis*. During the early stages of infection, the germ tubes of the germinating conidia of both pathogens develop appressoria, which attach the pathogens to the leaf surface. Leaf infection is through the cuticle, which is pierced and enzymatically degraded by the infection hyphae. Further growth of the pathogen is by the radiating mycelia, composed of branched hyaline, septate hyphae, that expand to form round, flat submerged colonies between the outermost portions of the epidermal cell wall and the cuticular layer (Graniti, 1962; Graniti, 1993; MacHardy, 1996).

The moisture periods needed for conidium germination limits the periods of disease development. In central California, where mild rainy winters and long, hot, dry summer occur, OLS is prevalent only during the winter months (Miller, 1949; Teviotdale and Sibbett, 1995). In Mediterranean areas, such as Italy, OLS infection occurs mostly in autumn through winter, or in spring, with no infection occurring in the hot, dry summers (Tosi and Zezzerini, 2000). However, in regions where rain is frequent even during the mild summers, such as in New Zealand, infection may occur throughout the year.
The calculation of infection periods in olive groves is often difficult because periods of interrupted wetness are common during spring and early summer, and conidia on leaf surfaces are often subjected to alternating wet and dry periods before infection occurs (Prota, 1958). The duration of a dry period, interrupting a continuous wet period, has been reported to reduce disease severity and conidium survival of *V. inaequalis* (Becker and Burr, 1994; Ayor and Sanogo, 1997). However, there have been no investigations reported on the effect of interrupted wet periods on conidium germination and infection for *S. oleagina*.

The duration of the incubation period needed before expression of OLS symptoms depends on the olive cultivar, environmental conditions, leaf age and the seasonal growth of the trees. In Italy, the incubation period is about 15 days under favourable temperature (<20°C) and
moisture conditions (Salerno, 1966), but if infection is followed by a hot, dry period, it may take several weeks or even months for lesions to become evident (Andreucci and Bonifacio, 1962; Graniti, 1993). For example, the lesions that appear in autumn may be due to spring or summer infections, which became latent and, so were not visible at that time (Figure 5).

In California, it was observed that lesions, which appeared in winter and early spring, continued to enlarge and develop through spring until early summer (Miller, 1949). These lesions, which initially appeared as well-defined, small, dark spots in winter and spring, continued to produce abundant conidia as they expanded. The lesions often coalesced to cover large areas of the leaf surface (Figure 6A), from which abundant conidia were produced. In early summer, the same lesions developed faint haloes around the spots (Figure 6B), but few conidia were produced from them. During, hot, dry summers the lesions stopped expanding and became dry, hardened, cracked or blistered. Miller (1949) reported that in some lesions, the cuticle broke away from the epidermal cells and in the centres of the lesions the outer epidermal cell walls sloughed off.

Figure 6: Olive leaves with the different stages of OLS development at different periods of the year in New Zealand. Early stage of lesion development, winter or spring (A) and old lesions (arrows) that oversummered on the trees (B).

These dried and heat damaged lesions did not reactivate in autumn. It is unclear whether the behaviour of OLS lesions during summer months is a result of the high temperature or low humidity, or a synergistic effect of both factors. Severely infected leaves fell during late spring and early summer, but those with few lesions remained on the trees (Miller, 1949).
Although it has been recognised that lesion development is associated with climatic conditions, no study has demonstrated the precise effect of temperature and moisture on the various stages of lesion development under controlled and field conditions. Improved understanding of the disease cycle can provide opportunities for the development of effective control strategies.

1.4.6 Survival of the pathogen

The activity of the pathogen is high throughout the year except in hot, dry summer months during which it survives as mycelium in diseased leaves in the tree canopy (Viruega et al., 1999). Azeri (1993) observed that the fungus did not die when the leaves fell, but remained alive for some time growing as a saprophyte although the author failed to provide information on the duration of the period. Miller (1949) reported that on fallen diseased leaves, the dark mycelium of the fungus, which usually occupies the subcuticular tissue of the upper epidermis, ramified throughout the leaf tissue even to the lower epidermis, but appeared to produce few conidia. With time, the fallen diseased leaves were found to be invaded by other saprophytic fungi which gradually replaced the pathogen (Miller, 1949). In France, Bernès (1923) concluded that fallen diseased leaves might contribute significantly to new infections of OLS, hence the recommendations of collecting and burning of fallen leaves for control of leaf spot. However, there was no substantial evidence to support this practice. More recent studies have demonstrated that *S. oleagina* may remain viable in fallen leaves for some time and produce abundant conidia, but the conidia produced by lesions on fallen diseased leaves do not constitute an important source of inoculum for new infection (Prota, 1958; Assawah, 1967; Guechi and Girre, 1994).

1.5 GENETIC DIVERSITY OF *SPILOCAEA OLEAGINA*

Pathogen populations must constantly adapt to changes in their environment to be successful. In agricultural ecosystems, the crop management practices that limit the success of pathogens impose strong selection pressures that may enhance the evolution of pathogen populations. For example, repeated use of fungicides to control *V. inaequalis*, has resulted in the emergence of new genotypes that are fungicide-resistant (MacHardy, 1996).
A number of factors may contribute to genetic change within populations including mutation, mating systems, gene flow or migration, as well as population size and selection pressures (McDonald, 1997). Although *S. oleagina* was first described by Boyer in 1891 (Miller, 1949), very little is known about the pathogen itself, despite the loss of crop yield caused. Recently, however, the DNA sequences of the 18S and 28S ribosomal RNA genes (rDNA), and the internal transcribed spacers (ITS) and 5.8S rDNA region of the fungus have been determined (González-Lamothe et al., 2002). Since the non-coding ITS sequences evolve faster than the coding sequences, and may vary among species and populations, variations in ITS sequence have been employed to study the genetic diversity and virulence variations of many plant pathogens such as *V. inaequalis* (Sierotzki et al., 1994; Tenzer and Gessler, 1997). Genetic studies of this nature have not been reported for *S. oleagina*.

### 1.6 OLIVE LEAF SPOT MANAGEMENT

#### 1.6.1 Resistance varieties

Olive cultivars and clones show differing susceptibilities to OLS, and local selections are known to have some resistance to OLS, at least under certain environmental conditions (Graniti, 1993). Although little has been done to improve the resistance of valuable but susceptible cultivars through selection or breeding programmes, a recent identification of genetic markers in olives, which are linked to OLS resistance and susceptibility (Mekuria et al., 2001) should prove to be a powerful screening tool in breeding programmes.

#### 1.6.2 Cultural practices

Olive trees need to be pruned to maintain good health and productivity. Over-pruning lowers crop yields while no pruning, or only light pruning, disrupts the leaf-wood ratio, makes trees more likely to revert to biennial bearing and makes them less productive in the long term (Bernardino, 2002). Pruning methods, aimed at reducing the humidity and shading that favours the growth of *S. oleagina*, are recommended for olive trees in groves prone to recurrent infection of OLS disease. Pruning should produce open, airy trees that allow penetration of light and heat, enabling leaf surfaces to dry out rapidly and, as a consequence, reducing disease development. MacDonald et al. (2000) reported that olive tree age influenced the incidence of OLS since older trees, with more dense canopies had greater disease incidence than younger trees, whose canopies were less dense. In addition, open trees
allow better penetration and coverage by fungicides, thereby improving control (Holb et al., 2001). The direct effect of pruning on the severity and incidence of apple diseases, such as black spot, sooty blotch and flyspecks have been demonstrated (Ocamb-Basu et al., 1988; Holb et al., 2001).

1.6.3 Chemical control

Chemical fungicides are the principal method of control for olive leaf spot throughout olive-growing regions of the world (Lopez-Villata et al., 1989). Fungicide application schedules include use of preventive treatments just before the main infection seasons, which often coincide with the main shoot-growth seasons (spring and/or autumn). In California and Mediterranean regions, Bordeaux mixture, a product resulting from the reaction of copper sulphate, calcium hydroxide, and lime, has been the most commonly used fungicide to control OLS. Teviotdale and Sibbett (1995) reported that in California, Bordeaux mixture was usually applied at 6.9-17.5 kg/ha in autumn before winter rains, and in spring to coincide with new shoot, although the efficacy of the second application was considered questionable. Several other copper-containing fungicides, such as tribasic copper sulphate, copper oxychlorides, copper hydroxide and copper oxide, have also been used to control OLS (Wilson and Miller, 1949; Teviotdale et al., 1989; Bourbos and Skoudridakis, 1993; Domingo-Garcia, 1996).

Systemic fungicides such as benomyl, fenarimol, difenoconazole and thiophanate-methyl have also been used to control OLS during the incubation period or when the infection is quiescent. Systemic fungicides are usually more effective at lower doses than the protectant fungicides (Siegel, 1981) and since plants can absorb them, total spray coverage of foliage is not required for optimum activity (Jones, 1981; Shabi et al., 1994). As infection of leaves occurs during favourable periods of wet weather, the successful prevention of infection by protective fungicides largely depends upon timely applications of the spray. Wilson and Miller (1949) observed that in Californian olive groves, unsatisfactory control of OLS resulted with a single application of Bordeaux mixture if it was not applied prior to protracted rains in autumn or early winter. Thus, the number and timing of the fungicide applications may vary considerably according to local seasonal conditions. Application frequency depends on the persistence of the fungicide used and the length of the favourable weather conditions. For example, in regions with a dry Mediterranean climate
such as Cordoba, in Spain, three spray applications (at winter end, summer end and late autumn) are recommended (Graniti, 1993; Tosi and Zazzerini, 2000). However, there have been no reports on the control of OLS using copper-containing fungicides under high disease pressure as may occur in wet or humid climates.

Disease and treatment history may have cumulative effects on OLS severity in any given season. In California, some researchers reported that OLS severity gradually decreased in trees treated annually. In one experiment, trees treated in the first year had less OLS than non-treated trees, even in the second year when all the trees were left untreated (Wilson and Miller, 1949; Teviotdale and Sibbett, 1995).

Table 2: Levels of copper residues on olive leaves treated with copper-containing fungicides once annually in autumn in Tulare County, California (Teviotdale et al., 1989)

<table>
<thead>
<tr>
<th>Material, pounds copper per acre</th>
<th>1984-85 Days after treatment</th>
<th>1985-86 Days after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35</td>
<td>115</td>
</tr>
<tr>
<td>Check</td>
<td>0.7</td>
<td>1.9</td>
</tr>
<tr>
<td>NordoX, 7.5</td>
<td>18.9</td>
<td>13.7</td>
</tr>
<tr>
<td>Bordeaux, 12.5</td>
<td>17.0</td>
<td>11.1</td>
</tr>
<tr>
<td>COCS, 12.5</td>
<td>9.3</td>
<td>6.9</td>
</tr>
<tr>
<td>Kocide, 13.2</td>
<td>7.3</td>
<td>6.0</td>
</tr>
<tr>
<td>Microcop, 13.2</td>
<td>5.7</td>
<td>5.2</td>
</tr>
<tr>
<td>Copper Count N, 3.9</td>
<td>5.0</td>
<td>4.7</td>
</tr>
<tr>
<td>Copper Count N, 2.5</td>
<td>5.0</td>
<td>4.7</td>
</tr>
<tr>
<td>P=0.05, LSD =</td>
<td>2.4</td>
<td>4.9</td>
</tr>
</tbody>
</table>

*Materials applied Nov. 2, 1984, and Nov. 7, 1985 (day 0) by handgun sprayer to runoff, approximately 500 gal water/acre.

50 Fifty leaves per tree analysed for amount of copper on leaf surfaces, five replications of single-tree plots.

Teviotdale et al. (1989) studied the persistence of some copper-containing fungicides on olive leaves in California. They were able to detect copper residues on olive leaves treated once annually (autumn) even after 360 days (Table 2) although the concentrations remaining were likely to be ineffective. Autumn or early-winter applications, which give satisfactory control of OLS, may create a residue problem on harvested fruit especially when two or more sprays are used as these fruits are not picked until late winter. Thus, if Bordeaux or any other residue-depositing fungicide is to be used against the disease before harvest, this problem must be solved by either removing the residue from the fruit or by delaying application of the spray until the fruit is picked.
With New Zealand environmental conditions favouring OLS development, copper-containing fungicides may neither be sufficiently effective nor sustainable for complete dependence on them alone because of the ecological impact of copper deposits in the soil. Although several copper-containing fungicides are used in New Zealand, only Cuprofix is registered for use on olives, and its efficacy in controlling OLS has not been fully established. There is a need, therefore, to investigate the rate, application number and timing, as well as efficacy of copper-containing and other fungicides in controlling OLS under New Zealand field conditions.

One of the main implications arising from the use of any fungicides is the behaviour of their residues, which give rise to important health considerations, especially in products destined for human consumption. The metabolic pathways of these compounds in plant tissues often differ according to the species of plant or the organ of concern (Siegel, 1981). Thus, there is a need for careful investigation into the toxicological aspects and residue degradation of the potentially useful systemic fungicides before they can be recommended for registration in olives.

1.7 THE ROLE OF OLIVE LEAF SPOT IN THE NEW ZEALAND OLIVE INDUSTRY

The New Zealand olive industry is a new, fast growing industry with potential for high returns from premium oil. In the last 10 years, it was estimated that over 2,000 hectares of land from Northland through to central Otago have been planted with olives (Statistics New Zealand, 2005). OLS is one of the major problems threatening the olive industry in New Zealand because the cool, moist weather is ideal for its development. The disease can be devastating in some groves, with almost all the leaves on olive trees dropping prematurely, particularly during the winter months (Figure 7).
A preliminary survey on the prevalence of OLS during the summer of 1999/2000 revealed that OLS is widespread throughout New Zealand with all regions and cultivars affected (MacDonald et al., 2000) (Figure 8). About 40% of all olive trees assessed were found to be infected with OLS, which indicates that the disease is widespread in New Zealand olive groves and may play a major role in the low productivity of olives. Thus, research into the factors which affect the development of OLS in New Zealand olive groves and the development of effective control strategies, is important in the growth of this industry.
Olive leaf spot has not been well studied overseas probably because it does not pose a serious problem to their olive industry, and in New Zealand, there have been no investigative reports on OLS. Of the overseas reports found, many were in languages other than English, including Spanish, Italian and Arabic. In addition, most of the work on *S. oleagina* has been reported in growers’ manuals, with little or no scientific data to support conclusions drawn, and in some cases the research trials were superficial in nature. These factors have highlighted the need for quality research into the epidemiology and control of this damaging disease.

Although it has been recognised that OLS development is associated with climatic conditions, no study has demonstrated the precise effect of temperature and moisture on the various stages of lesion development under controlled and field conditions. There have also been no studies on the control of OLS under conditions of high disease pressure. Unlike the Californian olive-growing regions, which have long, dry summers, OLS in New Zealand cannot be controlled by a single application of copper-containing fungicides prior to winter rains. Investigations need to be conducted to determine the most effective type and application timing of fungicides under New Zealand conditions so that OLS may be economically managed.

1.8 AIMS AND OBJECTIVES

The overall goal of this PhD research was to improve the understanding of the factors affecting development of OLS in New Zealand olive groves and to develop strategies for disease management. A section on OLS control and management will then be developed for a growers’ manual whose purpose is to provide comprehensive sound guidelines for olive growers to ensure the availability of high quality New Zealand olive products in the international and local marketplaces of the future.

Specific objectives were:

1. To investigate the effect of different environmental factors on:
   - *S. oleagina* conidium germination and infection processes,
   - OLS lesion development and sporulation under controlled conditions.

2. To investigate the effects of biological factors, including leaf age, season and conidium concentration on conidium germination and disease development.
3. To develop predictive models that may be used in development of a disease forecasting system.
4. To examine infection and sporulation processes by scanning electron microscopy.
5. To examine the DNA profiles of *S. oleagina* isolates to determine genetic variability of *S. oleagina* populations from New Zealand and if possible overseas.
6. To test the efficacy of different fungicides on *S. oleagina* conidium germination and OLS development in greenhouse and field trials.

1.9 THESIS OUTLINE

Each of the research chapters (Chapters 2-9) is written in the format suitable for scientific peer-reviewed publications. Thus, each chapter is a stand-alone piece of research, with its own Abstract, Introduction, Materials and methods, Results, Discussion, Acknowledgement and Reference sections. A Concluding Discussion (Chapter 10) is presented to discuss the significance of the overall research outcomes. All references cited are also listed in the Bibliography. This format has, invariably, resulted in some repetition of content; however, where possible this has been kept to a minimum.

1.10 REFERENCES


CHAPTER 2

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

2.1 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 leaf 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). Temperature significantly (*P*<0.001) affected conidium germination, with the effective range being 5 to 25°C and the optimum being 20°C. The rate of germ tube elongation followed a similar trend, with the optimum being 15°C. 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 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 in 80% or 60% RH. However, no conidia germinated without free water even after 48 hours of incubation at 20°C and 100% RH. These results may be useful in screening olive cultivars for resistance.

2.2 INTRODUCTION

Olive leaf spot (OLS), also called peacock spot, is caused by the fungus *Spilocaea oleagina* Castagne (Hughes) (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, dry climates the disease is not usually an important
problem because it needs cool, moist weather for its development. Olive leaf spot symptoms usually occur on the upper surface of the olive leaf. Spots on the lower leaf surface are rare, but when they occur, they are masked by the thick layer of peltate hairs (Graniti, 1993). As the spots expand and coalesce to cover a large proportion of the leaf area, leaves often turn yellow and are shed from the tree prematurely. Spots usually are more abundant on foliage from the lower parts of olive trees, and many such twigs 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). Occasionally under very wet conditions, small, sunken brown lesions can be found on the petioles, fruit peduncles, and fruit (Graniti, 1993), usually on susceptible cultivars such as ‘Barnea’, in which fruit peduncles are frequently infected. Infection of fruit peduncles can result in fruit drop because the peduncles may break or wither (Prota, 1995). Infection of fruit can cause unacceptable blemishes on table olives, and when it occurs on oil-producing cultivars, infection may cause a delay in ripening and a decrease in oil yields (Verona and Gambogi, 1964).

During the summer of 1999/2000, a New Zealand survey to determine prevalence of OLS revealed that the disease was widespread, with all regions and cultivars being affected (MacDonald et al., 2000). About 40% of all olive trees assessed had symptoms of OLS, and in some cases complete defoliation of the trees were observed, suggesting that it is a serious disease in New Zealand olive groves and may play a major role in the low productivity of olives. In California, Wilson and Miller (1949) reported severe outbreaks of OLS in the period 1941 through 1949, which resulted in yield losses estimated to be as high as 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 fungicide sprays (Hindorf et al., 2000). However, despite the loss of crop yield caused by OLS, very little is known about the biology of the pathogen, *S. oleagina*, and some results appeared 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. However, Saad and Masri (1978) demonstrated that on olive leaf surfaces, germination occurred at temperatures ranging from 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, and information on the precise effects of environmental variables on *S. oleagina* conidium germination and infection have not been fully investigated. Although moisture is known to be essential for conidium germination, to-date only Saad and Masri (1978) have reported trials that demonstrated a relationship between conidium germination and leaf wetness duration in controlled conditions. Considering the importance of conidium germination to 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 chapter describes experiments to investigate the effects of temperature, leaf wetness duration, and relative humidity on conidium germination, appressorium formation and germ tube elongation for *S. oleagina*.

2.3 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 (cv. ‘Barnea’) grown in a greenhouse maintained at 22 ± 5°C and 30 to 60% relative humidity (RH) under natural daylight. The plants were grown in plastic pots (13 cm in diameter) containing a mixture of composted bark and pumice (4:1, v/v) with 8 to 9 months dose of slow release fertilizer (N:P:K = 15:4:7.5). The first pair of leaves to reach the early bud stage, principal growth stage 07 (Sanz-Cortés et al., 2002), was tagged on each plant after 2 months of growth and thereafter at 2-week intervals for 10 weeks. The marked leaves were then detached and used in the detached leaf experiments described below.
Humidity chambers
Humidity chambers were constructed from plastic containers 22 x 15 x 6 cm. In the bottom of each, a wire gauze was 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 sulfate (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 relative humidities at two temperatures (10 and 20°C) were measured using Hobo temperature and relative humidity 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
*S. oleagina* was found to grow very slowly on artificial media, on which it produced no conidia. Consequently, OLS lesions from naturally infected olive leaves ('Barnea') grown in a commercial grove in Canterbury, New Zealand, were picked from the trees when needed to provide conidia for inoculation. Conidial suspensions were obtained from the leaves by agitating them in distilled water and filtering the suspension 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 $5 \times 10^4$ conidia/mL using a haemacytometer. Conidium viability of all conidial suspensions was determined by placing 100 μL of the suspension onto three replicate OLE agar (Appendix 1.1) plates and incubating them at 20°C for 48 h in the dark. The plates were then examined using a light microscope at ×200 magnification. The percent germinated conidia were calculated from 500 conidia and the viability found to be similar in all samples (55 to 60%).

2.3.1 Preliminary experiment
In a preliminary experiment, six olive leaves of the same age were detached from 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 block 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. Under these conditions, the
droplets did not dry out. 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, immediately after leaves had dried, they were cut into 1 x 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 hours. 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. The variation in percent 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 levels of variation to be tolerated in the experiments.

2.3.2 Effect of leaf age on conidium germination and appressorium formation

Five different 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 48 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.

2.3.3 Effect of temperature on conidium germination, appressorium development and germ tube elongation

Six different 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 previously for the preliminary experiment. After inoculation with the conidial suspension of S. oleagina, the leaves were arranged randomly on wire gauze and sealed in the six humidity chambers.
(100% RH), one for each temperature tested. For each chamber, there were 36 leaves of which six sets of leaves were removed from the humidity chamber after 48 h to assess percentage conidium germination and appressorium formation as described for the preliminary experiment. The remaining 30 leaves were used for determining germ tube length as described below.

Three of the leaves in each humidity chamber were randomly selected after incubation times 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.

2.3.4 Effect of temperature and 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) as described in previous experiments.

At the end of each leaf wetness period, three leaf samples were selected randomly from each of the replicate humidity chambers and air-dried as in the preliminary experiment. 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 as described previously. The experiment was conducted three times. The incubators were reallocated to different temperatures at each experiment repeat.
2.3.5 Effect of relative humidity 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 as in the preliminary experiment. 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 as described above. 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 hours, respectively). At the end of the secondary incubation, the leaves were cleared and assessed for conidium germination as previously described. The experiment was conducted twice.

2.3.6 Data analysis and model development

Multivariate analysis of variance (MANOVA) 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 experiment trials could be pooled. 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, appressorium formation or germ tube length, \( T \) is temperature, and \( a, b, c \), are parameters to be estimated. For the germ tube data, the models were fitted separately for each temperature.

For the effect of leaf wetness duration on conidium germination, the proportion of germinated conidia \( (p) \) was arcsine-transformed \((\text{arcsin}\sqrt{p})\) or logit-transformed \((\log[p/(1-p)])\) to stabilize the variance. Because there were no significant differences between experiments, the data were pooled before model fitting. The model tested was of the form: \( Y = f(T, W) \), where \( Y \) is the logit or arcsine-transformed proportion of conidium germination and \( f(T, W) \) is a linear function of temperature \( (T) \) and leaf wetness duration \( (W) \). The terms \( T, T^2, T^3, W, W^2, TW, TW^2, T^2W \) were tested in all possible combinations. The model selection was based on the significance of the \( F \) value for each model, the significance of estimated parameters, randomness and normality of residuals, as well as the coefficient of determination \( (R^2 \text{ and } R^2 \text{ adjusted for degree of freedom}) \).

A nonlinear mixed model was developed to explain the influence of RH (transformed as relative dryness) on percent conidium germination at 48 h after the initial wetness duration using R 2.0.1 and assessed based on size of the asymptote, the standard errors associated with the estimated parameters, and the analysis of residual plots. Because the initial scatter plots indicated similar variation, both experimental repeats were analyzed together, with experiment as a random effect. Asymptotic lines were fitted by non-linear mixed effect models (Pinheiro and Bates, 2000). The general form of the asymptotic line used was:

\[ f(x) = a + (b - a) \{\exp \{-\exp \gamma \} \cdot x\} \], where \( x \) is the wetness duration, \( a \) is the asymptote, \( b \) is the intercept on the vertical axis, and \( \gamma \) is the logarithm of the rate constant. Before model fitting, conidium germination was expressed as the percentage of maximum germination at 48 h of continuous wetness, and the variable relative dryness \((101 - \text{RH})\) was logarithmically transformed \([\log(101 - \text{RH})]\).

2.4 RESULTS

2.4.1 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 from either end or on the sides, but any one conidium rarely had more than two germ tubes.

2.4.2 Effect of leaf age on conidium germination

The effect of leaf age on conidium germination after 48 h incubation at 20°C differed among repeated experiments, but the trends for the experiments were similar. Olive leaf age significantly \((P<0.001)\) affected the germination of \(S.\) \(oleagina\) conidia. It was observed that as leaves became older, percent conidium germination decreased (Figure 9). 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.

![Figure 9: Germination of Spilocaea oleagina conidia inoculated onto detached olive leaves aged 2-10 weeks. The regression equations for Trials 1 and 2 are \(Y = 58.35 - 3.405A (R^2 = 0.9449)\) and \(Y = 64.63 - 3.285A (R^2 = 0.9939)\), respectively, where \(Y\) is percent conidium germination and \(A\) is leaf age. 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. Bars are the standard errors of the means.](image)

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 appressorium formation in all trials. In Trial 1, the percentage of germinated conidia that 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.

2.4.3 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 followed a similar trend in all the experimental repeats. For each temperature, 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 germinated conidia was observed at 20°C but the estimated optimum temperature from the quadratic response function was 15.9°C (Figure 10A).

![Figure 10](image)

**Figure 10:** Germination (A) and appressorium formation (B) 48 h after *Spilocaea oleagina* conidia were inoculated onto detached olive leaves and kept wet continuously at 5-30°C. Data presented (symbols) are the means of six replicate leaves with three inoculum droplets per leaf. The line in A represents germination calculated with the equation \( Y = -11.2 + 8.46T - 0.263T^2 \) \( (R^2 = 0.8289) \), and the line in B represents appressorium formation calculated with the equation, \( Y = 3.33 + 7.17T - 0.287T^2 \) \( (R^2 = 0.8914) \), where \( T \) is the temperature and \( R^2 \) is the coefficient of determination from the combined data of the three trials. Bars are the standard errors of the means.
Many germ tubes of *S. oleagina* formed amorphous appressoria (Appendix 2.1) that were visible as swellings at the tip of the germ tubes. This was observed 6 h after the first sign of conidium germination irrespective of the incubation temperature. Appressorium formation was affected by temperature (*P*<0.01) being 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 increases in temperature (Figure 10B). Although approximately 40% of the conidia had germinated at 25°C after 48 h of incubation, none produced appressoria. The relationship between temperature and appressorium formation followed a similar trend as conidium germination except that the highest percentage of germinated conidia with appressoria was observed at 15°C and the estimated optimum temperature from the quadratic response function was 12.5°C (Figure 10B).

Germ tube length increased with increasing incubation period. Temperature made little difference to the mean rate of germ tube elongation in the first 48 h of incubation, whereas after longer incubation periods it was less at the higher temperatures (20 and 25°C) than at the lower temperatures tested (Figure 11). For example, although the mean germ tube lengths at 15°C and 20°C were similar (110 µm) after 48 h of incubation, by 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 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 even after 168 h of incubation at all temperatures.

Although both logistic and polynomial regressions were explored to determine whether a single or series of lines would adequately describe the effect of temperature on germ tube elongation at various incubation times, germ tube growth at each temperature was best described by a second-order polynomial regression equation (Figure 11). 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.
Figure 11: Germ tube elongation of *Spilocaea oleagina* on detached olive leaves at various temperatures and incubation periods. Symbols represent the means of three trials with 3 leaves and 30 conidia per leaf. The lines represent the predicted values calculated from combined data of the three trials with the following equations, 5°C: $Y = -9.64 + 1.54t - 0.00049t^2$ ($R^2 = 0.9910$), 10°C: $Y = -7.82 + 1.95t - 0.0053t^2$ ($R^2 = 0.9980$), 15°C: $Y = 2.59 + 1.79t - 0.00182t^2$ ($R^2 = 0.9840$), 20°C: $Y = 29.3 + 1.48t - 0.0025t^2$ ($R^2 = 0.9390$), 25°C: $Y = 20.31 + 0.865t - 0.001415t^2$ ($R^2 = 0.9640$), where $Y$ is the mean of the longest germ tube for each of 10 randomly selected conidia and $t$ is the incubation time. Bars are the standard errors of the means.
2.4.4 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 (Figure 12). 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.

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Figure 12: *Spilocaea oleagina* conidium germination on detached olive leaves at various temperatures and leaf wetness durations. Symbols represent the means of six replicate leaves.
with three inoculum droplets per leaf for each combination of temperature and incubation
time, and the lines represent the predicted values calculated with \( \text{Logit}(Y) = b_0 + b_1T^2 + b_2T^3 
+ b_3TW^2 + b_4TW^3 + b_5T^2W^2 + b_6W^2 + b_7W^3 + b_8WT^2 \), where \( Y \) is the predicted percentage
germination, \( T \) is temperature, \( W \) is leaf wetness duration, and \( b_0 \ldots b_8 \) are the parameters to be
determined (Table 3). Bars are the standard errors of the means.

The relationship between temperature and leaf wetness duration in relation to conidium germination was best described by:
\[
\text{Logit}(Y) = b_0 + b_1T^2 + b_2T^3 + b_3TW^2 + b_4TW^3 + b_5T^2W^2 + b_6W^2 + b_7W^3 + b_8WT^2,
\]
where \( Y \) is the predicted percentage germination, \( T \) is temperature, and \( W \) is leaf wetness duration.
The significant parameter estimates for the equation for the pooled data are listed in Table 3.

Table 3: Estimated parameters (\( P<0.001 \)) and associated standard errors (SE) for a logistic model to describe the effect of temperature (\( T \)) and leaf wetness duration (\( W \)) on the germination of \( \text{Spilacaea oleagina} \) conidia on detached olive leaves based on pooled data from three experiments

<table>
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<th>Value</th>
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</tr>
<tr>
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<td>5.61 \times 10^{-5}</td>
</tr>
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<tr>
<td>( R^2_a )</td>
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</tbody>
</table>

*Response variable = logit \( (Y) \), where \( Y \) = percent germinated conidia, \( R^2 \) = coefficient of determination and \( R^2_a \) = coefficient of determination adjusted for degrees of freedom
2.4.5 Effect of initial leaf wetness duration and relative humidity 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 hours 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 (Figure 13). Higher RH (100%) following the end of the wetness periods increased \( (P=0.05) \) conidium germination compared with 60 and 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 (Figure 13). Visual observation of the inoculated leaves showed no water condensation on the leaves at the end of the secondary incubation.

![Figure 13: Effect on Spilocaea oleagina conidium germination at different relative humidities (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. Values are means of two trials, each with six replicate leaves and three inoculum droplets per leaf. Bars are the standard errors of the means.](image-url)
A model was developed to evaluate the influence of RH on S. oleagina conidium germination on detached olive leaf tissue following initial wetness duration. The predicted germination was given by: 

\[ Y = \left( \alpha + d \cdot \alpha_d \right) + \left( \beta + d \cdot \beta_d \right) - \left( \alpha + d \cdot \alpha_d \right) e^{\left( -d \cdot \gamma \cdot w \right)} \]

in which \( Y \) is the percentage of maximum germination, \( w \) is the initial wetness duration, \( d \) is the logarithm of relative dryness \( \log(101 - RH) \), and the estimated parameters are \( \alpha \) (asymptote), \( \alpha_d \) (log dryness on asymptote), \( \beta \) (intercept), \( \beta_d \) (log dryness on intercept), \( \gamma \) (log rate constant), and \( \gamma_d \) (log dryness on rate). The parameter estimates and the standard errors are given in Table 4, and Figure 13 demonstrates the fit of the model to the data.

**Table 4:** Estimated parameters and associated standard errors for a non-linear mixed effects model \( Y = \left( \alpha + d \cdot \alpha_d \right) + \left( \beta + d \cdot \beta_d \right) - \left( \alpha + d \cdot \alpha_d \right) e^{\left( -d \cdot \gamma \cdot w \right)} \) to describe the effect of RH following initial leaf wetness on the germination of *Spilocaea oleagina* conidia

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )</td>
<td>84.3</td>
<td>4.99</td>
</tr>
<tr>
<td>( \alpha_d )</td>
<td>-5.12</td>
<td>0.524</td>
</tr>
<tr>
<td>( \beta )</td>
<td>-57.2</td>
<td>13.72</td>
</tr>
<tr>
<td>( \beta_d )</td>
<td>-75.7</td>
<td>9.19</td>
</tr>
<tr>
<td>( \gamma )</td>
<td>-1.75</td>
<td>0.158</td>
</tr>
<tr>
<td>( \gamma_d )</td>
<td>0.154</td>
<td>0.0420</td>
</tr>
</tbody>
</table>

\( \alpha = \) asymptote, \( \alpha_d = \) log dryness on asymptote, \( \beta = \) intercept, \( \beta_d = \) log dryness on intercept, \( \gamma = \) log rate constant, and \( \gamma_d = \) log dryness on rate.

**2.5 DISCUSSION**

This study showed that temperature, leaf wetness, and leaf age affect germination as well as 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. 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 (Bentes and Matsuoka, 2002). 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. 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 conidium 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. A lower proportion of Spilocaea oleagina conidia germinated on water agar (20%) compared with nutrient-containing media, potato dextrose agar (60%), at 20°C after 48 h (Appendix 2.2), suggesting that nutrient availability is important in their activation and subsequent germination.

Several substances can regulate conidium 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. The major components of leaf epicuticular wax of most plants include hydrocarbons, fatty alcohols, alkyl acetates, fatty acids, aldehydes and wax esters. The ratio of these compounds changes with leaf age. Jetter and Schäffer (2001) reported major changes in the distribution of the main components of Prunus laurocerasus waxes during leaf development, such that within 10 days of leaf development the wax fractions were dominated by alkyl acetate, and then after 18 days, alcohols dominated these fractions. However, after 60 days the alkanes accumulated steadily in the epicuticular wax film, accompanied by small amounts of fatty acids, aldehydes, and alkyl esters. Podila et al. (1993) showed that fatty alcohol, the epicuticular wax component from avocado fruit, stimulated germination of conidia and appressorium formation of Colletotrichum gloeosporioides, the cause of avocado anthracnose, under various conditions. In addition, Prusky et al. (1991) reported an involvement of a chemical signal from the epicuticular wax during the interaction between avocado and C. gloeosporioides. 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 *S. oleagina* 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 levels since young susceptible olive leaves are almost always available in those groves.

Germ tube lengths increased with increasing leaf wetness duration, although temperature had no significant effect on germ tube development except with prolonged incubation. However, at 25°C more germ tubes per conidium were observed 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).

Appressoria are integral parts of the infection process of several pathogenic fungi. In some fungi, appressorium formation may be essential for infection and survival, whereas in others it may be unnecessary. Appressorium formation by fungi is influenced by several factors including temperature, host surface characteristics, nutrient stress, and light intensity (Emmett and Parbery, 1975). The results reported here showed that *S. oleagina* formed appressoria over a wide range of temperatures although formation was favored 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 they reported that 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. In some pathogenic fungi the optimum temperature for appressorium formation coincides with that for spore germination. 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, this study demonstrated that the optimum temperature for appressorium formation was different from that of conidium germination.

This study demonstrated a relationship between conidium germination and leaf wetness duration in controlled conditions, such that 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. In contrast, Saad and Masri (1978) who researched this aspect in Lebanon 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 were required. The differences between that report and the present study could be attributed to various intrinsic and extrinsic factors. For example, they could be associated with differences in local conditions, such as leaf and conidium adaptations, which may have affected the capacity of the conidia to absorb water from the surrounding environment or the leaf surface characteristics. The report of Saad and Masri (1978) provided no information on the time of year conidia were collected or on storage conditions. In another experiment (Chapter 7), the viability of *S. oleagina* conidium was found to be significantly reduced during the summer months in New Zealand olive groves, indicating heat damage, which may also have affected germination processes.

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 the conidium imbibing time or on potential for water condensation on the leaf surface. Experiments to determine the effect of high RH in the absence of wetness must be conducted with precise control of temperature and RH, since any slight decrease in temperature may cool air below the dew point, which will result in condensation of water on the leaf surface. Given that *S. oleagina* conidia are mainly
dispersed by rain splash (Wilson and Miller, 1949; Tenerini, 1964), it is likely that under field conditions free water remains around them long enough for germination at least to be initiated. The results of this study also indicated that high RH periods could be important after rainfall events of short duration. The conditions found to be required for germination and infection suggests that several infection cycles may occur annually in a cool, moist climate, explaining the higher levels of OLS in New Zealand groves than in other olive-growing areas, such as the Mediterranean regions and California.

This study showed that a logistic model was adequate for describing percent conidium germination at different combinations of wetness duration and temperature from 5 to 25°C. The coefficient of determination ($R^2$) for the pooled data (0.8887) indicates that a moderately high proportion of the variability of logit of percent germination was accounted for by temperature, wetness durations and their interactions. The coefficient of determination adjusted for degrees of freedom ($R^2_a$) was close to the $R^2$ value (0.8877), indicating that the estimated parameters were significant. The results of this study have the potential to be used to develop an OLS prediction model, which may be incorporated into disease management strategies. This may improve the timing of fungicides spray applications to better target high risk periods and may allow a reduction in the number of commonly used sprays whilst maintaining protection of susceptible host tissues. However, before a prediction model can be developed, further research should be carried out under controlled conditions to investigate the effects of environmental factors on OLS infection and disease severity on whole plants.

2.6 ACKNOWLEDGEMENTS

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2.7 REFERENCES


CHAPTER 3

Ultrastructural studies on the penetration and infection process of *Spilocaea oleagina* on olive leaves

3.1 ABSTRACT

The pre-penetration and infection processes on olive leaves by the fungus, *Spilocaea oleagina*, the cause of olive leaf spot was studied by scanning electron microscopy. Conidia began germinating within 24 h post inoculation, forming appressoria under which penetration pegs were produced to penetrate the cuticle directly. At the penetration site, breakdown and deformity of the cuticle could be seen, which indicated that the fungus penetrated the cuticle mechanically and enzymatically. On the surface of trichomes, the same effect was also observed, but to a lesser extent, and the fungus did not penetrate the host tissue beneath the trichomes. After penetrating the cuticle, the fungus developed an entirely subcuticular growth during early stages of parasitism.

3.2 INTRODUCTION

Olive leaf spot (OLS), caused by the fungus *Spilocaea oleagina* (syn. *Cycloconium oleagina*), is a serious disease in all olive growing regions of the world, especially in areas with moist and humid weather conditions. The fungus infects fruits, leaves and shoots. Fruit infection can cause unacceptable blemishes on table olives, and when it occurs on oil-producing cultivars, may cause a delay in ripening and a decrease in oil yields (Verona and Gambogi, 1964). OLS commonly occurs on the upper surfaces of leaves but not on the lower surfaces except along the midrib area. Infected leaves often turn yellow and are shed from the tree prematurely and shoots may be stunted or die back. In addition to reducing the potential crop during the current season, reduction in leaf area and premature defoliation also reduce bloom and fruit set the following year (Miller, 1949; Laviola, 1992).

The fungus lives parasitically on olive leaves and produces abundant conidia under favourable conditions for new infection. *S. oleagina* conidia germinate over a wide range of
temperatures, 5 to 25°C, with the optimum at 20°C needing at least 9 h continuous leaf wetness or 6 h of continuous leaf wetness followed by 100% relative humidity, depending on the temperature (Chapter 2). Appressorial formation and penetration may occur 6 h after the first signs of conidium germination. On olive leaves, S. oleagina conidium germination was shown to be higher on young leaves compared to older ones (Chapter 2), possibly due to the differences in leaf surface components.

Leaf surface structures and chemical components, including stimulatory and inhibitory compounds on the phylloplane, can affect the infection processes of fungal pathogens (Inyang et al., 1998). Several modes of penetration are possible: through natural openings (e.g. stomata), through wounds and by direct penetration of the cuticle through mechanical or enzymatic processes. Of these, direct cuticle penetration is the most common means of tissue penetration (Bailey et al., 1992). The cuticle of higher plants is an extracellular membrane consisting of a polymeric cutin matrix, interspersed with soluble cuticular waxes (Jetter and Schäffer, 2001), and covered with a thin surface film of epicuticular waxes (Baker and Hunt, 1981). The cuticle characteristics of several plant species were identified as being important features in their disease resistance (Roberts and Martin, 1963). For example, Conn and Tewari (1989) showed that removing epicuticular wax from canola leaves increased the rate of Alternaria brassicae conidium germination. The cuticle provides a chemical and/or mechanical barrier to pathogen penetration and reduces surface wettability. The reduction in wettability and permeability of the cuticle caused by the epicuticular wax reduces the amount of leaching from a plant (Blakeman and Sztejnberg, 1973). This may affect germination indirectly by reducing the diffusion of leaf exudates into droplets of water containing the conidia.

Dense masses of trichomes on the leaf surface have been reported to reduce leaf wettability, but to increase humidity at the leaf surface, thereby affecting conidial penetration (Campbell et al., 1980; Wetzstein and Sparks, 1983; Grauke et al., 1988). The pathogen itself may also respond to the plant surface, at various developmental stages, before penetration, including germination, germ tube growth and formation of infection structures (Wynn, 1981). On olive leaf surfaces, trichomes (peltate hairs) have been reported (Graniti 1993) but their involvement in the penetration and infection of S. oleagina has not been studied. The objective of the present study was to describe these pre-penetration and infection processes.
and to examine the possible role of the trichomes in inhibiting the penetration of the pathogen.

3.3 MATERIALS AND METHODS

Plant material and inoculation
Two-year old plants of the susceptible olive cultivar, ‘Barnea’, grown in a greenhouse (Chapter 2) were inoculated with a *S. oleagina* conidium suspension (5 × 10⁴ conidia/mL), obtained by washing symptomatic olive leaves in distilled water as previously described in Chapter 2. The youngest fully expanded (2 weeks old) and old leaves (12 weeks old) were inoculated by application of three droplets (10 μL) of the conidium suspension on the abaxial or adaxial surfaces of leaves. A total of six plants and four leaves per plant were inoculated. Two plants inoculated with water were used as the control. After inoculation, the plants were immediately placed in a humid chamber in the dark, set to provide 100% relative humidity at 20°C, and incubated for 48 h to allow infection to occur. Subsequently, the plants were transferred to the shadehouse for disease development.

Scanning electron and light microscopy
Scanning electron microscopy (SEM) was used to study the processes of germination, penetration and infection by *S. oleagina* conidia on olive leaves. The inoculated leaves taken from plants 24 and 48 h after inoculation, and after the appearance of early symptoms on the leaves, were cut into 1 × 1-cm pieces, each of which contained the site of a conidium droplet. The leaf pieces, with or without symptoms, were mounted onto copper stubs with double-sided sticky tape, with the adaxial or abaxial surfaces facing upward. To investigate the effects of penetration events on leaf surfaces, germlings and waxes were removed from some leaf segments, by lightly pressing one-sided sticky tape onto the surface. Mounted specimens were coated with gold in a sputter coater (Polaron 5000), and examined with a LEICA S440 scanning electron microscope operating at 5.0 kV. At least five observations were made on each leaf segment and the one-sided sticky tape.

For the light microscopy, both healthy and infected leaf samples were obtained from the plants 12 weeks after inoculation when OLS symptoms were visible. Leaf segments were mounted with a 30% aqueous solution of gum arabic and cut into 0.5 to 1 μm sections using a
freezing microtome. They were stained with aniline blue (Saad and Masri, 1978) for at least 24 h and examined using a light microscope at ×200 magnification.

3.4 RESULTS

3.4.1 Leaf surface structure
Trichomes, which were peltate with broadly expanded heads and attached to the leaf surface by a short stalk, were apparent on the adaxial (Figure 14) and abaxial (Figure 15) leaf surfaces. The trichomes were sparsely distributed on the adaxial leaf surface; however, the abaxial surface was covered with overlapping layers of trichomes except for the area around the midrib where they were sparser.

Figure 14 and 15: General olive leaf anatomy 14. Adaxial surface showing sparsely distributed glandular peltate trichomes. 15. Abaxial surface with overlapping layers of glandular peltate trichomes. TR = trichomes, MR = midrib. Scale bars on Figures 14 and 15 are 20 and 30 μm, respectively.

3.4.2 Germination and penetration
Within 24 h post inoculation on young leaves conidia germinated to produce short germ tubes with appressoria, which were characterized by swellings at the tips of the germ tubes (Figure 16). Germ tubes emerged from the tips of the conidia, usually extending the length of the conidium, but occasionally growing perpendicular to the conidium. Figure 17 shows both configurations from a conidium, a situation observed sometimes, but then only one germ tube
produced an appressorium for penetration (Figure 17). In some cases, conidia produced long germ tubes that failed to produce appressoria 48 h after inoculation (Figure 18) an event that was quite common on old leaves, but uncommon on young leaves. Around the penetration site of young leaves, there was a ring of what appeared to be degraded cuticle indicating production of degrading enzymes to assist penetration of the pathogen (Figure 19).

**Figure 16-19:** Germination of conidia and penetration of *Spilocaea oleagina* on an olive leaf surface post inoculation. 16. Germinated conidium with one germ tube on the upper leaf surface, 24 h post inoculation. 17. Germinated conidium with two germ tubes on the upper leaf surface, 24 h post inoculation. 18. Conidium with long germ tube that failed to produce an appressorium by 48 h post inoculation. 19. Cuticle degradation around a penetration site, 48 h after inoculation. CO = conidium, GT = germ tube, AP = appressorium, CD = cuticle degradation. Scale bars on Figures 16, 17, 18 and 19 are 10, 20, 20 and 2 μm, respectively.
Figure 20-23: Scanning electron micrographs of *S. oleagina* conidia and appressoria detached from the adaxial leaf surfaces show the germling structures 24 h after inoculation and a penetration hole into an olive leaf. **20.** Penetration hole (PH) on the cuticle. **21-23.** Early to late stages of penetration peg development. PP = penetration pore, CO = conidium, PS = penetrating structure developing, PG = penetration peg, AD = adhesive layer. Scale bars on Figures 20, 21, 22 and 23 are 1, 2, 1 and 3 μm, respectively.

Wherever an appressorium and the germ tube had been lifted off the leaf surface with sticky tape, a hole made by the penetration peg in the cuticle was visible (Figure 20), demonstrating that penetration had taken place. The penetration holes were usually spherical to ellipsoid, and their diameters were 0.9-1.3 μm. On some old leaves an area of slight degradation of the cuticle could also be seen around the penetration hole with deformity of the leaf surface, which seemed to indicate that physical force played an important role. On the sticky tape, the appressoria and germ tubes could be seen (Figures 21, 22, and 23). The adhesive layer of
mucilaginous substance under each appressorium and the germ tube clearly demonstrated the different stages of development after conidium germination, namely mucilage production, appressorium formation and direct penetration through the cuticle (Figure 21).

Figure 24-26: Germination of *Spilocaea oleagina* conidia on the olive leaf trichomes 48 h after inoculation. 24. Germinating conidium with a germ tube 25. Adaxial leaf surface showing the area where the germlings and the trichomes were removed with a sticky tape. 26. Germinating conidium with germ tube apparently selecting a penetration site between the trichomes. TR = trichomes, TS = trichomes stalk, AT = area where trichome was removed with no penetration hole under it, GT = germ tube, CO = conidium. Scale bars on Figures 24, 25, and 26 are 2, 20 and 10 μm, respectively.

To investigate the role of trichomes in fungus penetration, the surfaces of the trichomes under germinating conidia were also examined (Figure 24).
Occasionally, penetration holes appeared on the surface of the trichomes, but when these were stripped off, holes were never observed on the cuticle under the trichomes (Figure 25), suggesting that the pathogen could produce penetration pegs but that they were unable to penetrate successfully through the trichomes. In some cases, the growing germ tube appeared to avoid the trichomes, penetrating the cuticle in a gap between them (Figure 26). On the abaxial surface of leaves where there were overlapping layers of trichomes, no penetration was detected even at 48 h after inoculation.

**Figure 27-30:** Light and scanning electron micrographs of a cross-section of olive leaves. 27 and 28: Light micrographs of a cross-section of olive leaves. 27. Healthy leaf. 28. Infected leaf with sporulating lesion showing mycelium restricted to the space between the cuticle and the periclinal wall of the epidermal cells. 29 and 30: Scanning electron micrographs of a cross-section of olive leaves. 29. Uninfected leaf. 30. Necrotic leaf lesion showing the cavity possibly caused by the invading mycelium between the cuticle and the walls of the upper epidermal layer as shown in Figure 28. C = cuticle, EW = epidermal cell wall, UE = upper epidermal cells, MY = mycelium, IT = intact junction of the cuticle and epidermis, CD = conidiophore, CO = detached conidium, CV = cavities caused by the invading mycelium. Scale bars on Figures 29 and 30 are 10 and 2 μm, respectively.
Light microscopy was used to study cross-sections of both uninfected (Figure 27) and infected (Figure 28) olive leaves when OLS symptoms were visible. The results showed that after penetrating the cuticle, the fungal growth was entirely subcuticular (Figure 28) and that the hyphae never penetrated into the upper epidermal cells. Scanning electron microscope examination of cross-sections of healthy (Figure 29) and infected symptomatic (Figure 30) leaves revealed some cavities between the cuticle and the upper epidermis of the infected leaves, perhaps caused by the invading hyphae. No evidence was found of hyphae below the epidermal cells.

3.5 DISCUSSION

The ultrastructural study of the infection by *S. oleagina* conidia of olive leaves showed the occurrence of cell surface attachment, germination, mucilage production, appressorium formation and direct penetration through the cuticle. The present study also reports the presence of subcuticular growth of hyphae in leaves infected with *S. oleagina* conidia.

Fungal attachment to the host surface is an essential prepenetration process that determines the success of infection and disease development (Isaac, 1992). It seems that the germ tubes of the germinating conidia of *S. oleagina* adhered tightly to the leaf surface by adhesive substances but the constituents of these substances are unknown. Some fungal species produce mucilage (polysaccharides, glycoproteins and hexosamine) for adhesion to the host (Laborda and Maxwell, 1976; Isaac, 1992). Gold and Mendgen (1984) suggested that the role of fungal exudates could be multifunctional. They may attach the germling to the plant surface, seal up penetration sites, protect appressoria against drying and act to store enzymes required for penetration. Exudates of some fungal species, such as *Ascochyta rabiei* (Höhl et al., 1990), *Phomopsis phaseoli* (Kulik, 1988), and *Phyllosticta amplicida* (Kerchung and Hoch, 1995), have been reported to contain polysaccharides. But for others, such as *Fusarium oxysporum* f. sp. *lycopersici*, *Verticillium albo-atrum* and *F. oxysporum* f. sp. *pisi*, the mucilaginous substances were also thought to contain cell wall-degrading enzymes (Bishop and Cooper, 1983). It is also possible that besides providing adhesion, the mucilage may contain substances used for recognition of the host surface (Roustae et al., 2000).
In this study, it seems that the observed cuticle degradation around a penetration site may have been due to the production of hydrolytic enzymes, such as cutinase. However, some deformity observed on the cuticle at the penetration sites and the formation of penetration pegs suggests that penetration also occurred through mechanical pressure. These results support the observation of Graniti (1962) on naturally infected olive leaves which showed that leaf infection by *S. oleagina* conidia was through enzymatic degradation and mechanical piercing of the cuticle by the infection hyphae. Direct cuticle penetration has also been reported for other fungi including *V. inaequalis* (Köller et al., 1991), *Fusarium solani* f. sp. *pisi* (Woloshuck and Kolattukudy, 1984), *Phoma macdonaldii* (Roustaee et al., 2000) and *Rhizoctonia solani* (Kenning and Hanchey, 1980). For both *V. inaequalis* and *F. solani* f. sp. *pisi*, cutinase was reported to be involved in cuticle degradation during penetration whereas with *P. macdonaldii* and *R. solani* direct penetration was achieved when the infection peg broke the cuticle by mechanical pressure.

The unsuccessful penetration by *S. oleagina* of abaxial leaf surfaces and through the trichomes suggests that they are an important defence feature against this pathogen. Diehl and Graves (1994) reported that in mockernut hickory, the number of glandular trichomes present on the leaf surface was related to its resistance to scab caused by *Cladosporium caryigenum* infection, with the most resistant cultivar having the greatest number of vesicular, discoid, and fasciculate trichomes. The failure of *S. oleagina* to penetrate these surfaces also suggests that wax-degrading enzymes were not produced in large quantities. Alternatively, it may be that the trichomes produced some chemicals which inhibited the penetration activities of the pathogen. This hypothesis is supported by a previous report on scab resistance in varieties of pecan in which the collapsed trichomes were shown to release phenols that inhibited *C. caryigenum* spore germination and hyphal growth (Wetzstein and Sparks, 1983). These authors showed a significant correlation between resistance of pecan leaves to *C. caryigenum* infection and a greater frequency of collapsed trichomes. Thus, structural and chemical evaluations of the trichomes of olive are needed to ascertain if they truly influence the OLS infection process.

Subcuticular growth of germ tubes occurs in a range of plant pathogens including *V. inaequalis* (Preece, 1962), *P. macdonaldii* (Roustaee et al., 2000), and several species of *Ascochyta* (Pandey et al., 1987; Clulow et al., 1991; Roundhill et al., 1995). In the present study, the light microscopy observations revealed that after penetration of the cuticle, growth
of the pathogen was entirely subcuticular during the early stages of parasitism. This result is consistent with the study of Graniti (1962) on naturally infected olive leaves, which showed that the invading hyphae grew in a tunnel bored by dissolution of the mostly cutinised, hydrophobic portion of the epidermal cell wall. This behaviour of S. oleagina can be seen as providing both nutritional and environmental benefits. The fungus is able to degrade and utilize as a source of food the main components of the cutinised cell wall, namely cutin, wax, cellulose and pectin (Graniti, 1965) and it can absorb water and nutrients from the apoplast through the permeable cell wall structures of the upper epidermis. Moreover, the thick overlying cuticle protects the mycelium from desiccation and some UV radiation.

It is concluded that penetration and infection of olive leaves by S. oleagina most likely involves both enzymatic and mechanical processes, and that trichomes on olive leaves have a general function of inhibiting penetration. These results have some implications in the control of olive leaf spot. The density of trichomes on leaf surfaces of susceptible and resistance olive cultivars in relationship to disease resistance should be investigated. The subcuticular mycelium growth of the pathogen may indicate that systemic fungicides, such as kresoxim-methyl and difenoconazole, could be used to control the disease during the incubation or latent periods. Future research should focus on the interaction between the fungus and different resistance cultivars to investigate the morphology and physiology of the interactions on disease development, possibly by using cytochemical-labelling techniques (Guérin et al., 1998).

3.6 ACKNOWLEDGEMENTS

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3.7 REFERENCES


CHAPTER 4

Effects of temperature, inoculum concentrations, continuous and wetness periods on infection of olive plants by conidia of *Spilocaea oleagina*

4.1 ABSTRACT

Experiments were conducted on olive plants in controlled environments to determine the effect of temperature, leaf age, conidial concentration, continuous and interrupted leaf wetness periods and relative humidity during dry interrupted wet periods on olive leaf spot severity. The results showed that for the three leaf age groups tested (young, intermediate and old), severity decreased significantly \(P<0.001\) with increasing leaf age at the time of inoculation. As inoculum concentration increased from \(1.0 \times 10^2\) to \(2.5 \times 10^5\) conidia per mL, the severity of olive leaf spot increased at all five temperatures (5, 10, 15, 20, and 25°C). Temperature also affected \(P<0.001\) the numbers of lesions, with the lesion numbers increasing gradually from 5°C to a maximum at 15°C, and then declining to a minimum at 25°C. When nine leaf wetness periods (0, 6, 12, 18, 24, 36, 48, 72 and 96 h) were tested at the same temperatures, olive leaf spot severity increased with increasing leaf wetness period at all temperatures tested. The minimum leaf wetness periods for infection at 5, 10, 15, 20, and 25°C were 18, 12, 12, 12, and 24 h, respectively. When early infection periods were composed of wet periods interrupted with dry periods (0, 3, 6, 12, 18, and 24 h) at two levels of relative humidity (70 and 100%), the length of the dry period had a significant \(P<0.001\) effect on disease severity. The effect depended on the relative humidity during the interruption, with high relative humidity (100%) resulting in higher disease severity in interrupted periods than low relative humidity (70%). A polynomial equation with linear and quadratic terms of temperature, wetness and leaf age was developed to describe the effects of temperature, wetness, and leaf age on olive leaf spot infection, which could be incorporated as a forecasting component of an integrated system for the control of olive leaf spot.
4.2 INTRODUCTION

The principal method used to control OLS throughout olive-growing regions of the world is by applying chemical fungicides (Teviotdale et al., 1989; Graniti 1993), but their efficacy is dependent on correct timing of the applications (Graniti, 1993). Because disease severity is known to increase after wet periods, accurate forecasting of OLS risk periods is desirable to improve disease control, and/or reduce fungicide use, and to allay concerns about their environmental and human health effects.

Forecasting systems have not been developed for OLS, but they have been for apple scab caused by *Venturia inaequalis* (Cooke) G. Wint., a pathogen closely related to *S. oleagina*, which has a similar mode of infection (Graniti, 1993). Most apple scab-forecasting systems are based essentially on the relationships between disease levels and the temperature, amount and duration of rain or leaf wetness. They commonly allow for the reduction of fungicide input while maintaining effective control of apple scab (MacHardy, 1996; Hindorf et al., 2000; Berrie and Xu, 2003). For olive leaf spot, field observations have indicated that environmental factors, such as temperature and moisture, are the driving forces of the infection and spread of OLS (Graniti, 1993), but precise information on the effects of environmental variables on OLS infection and development has not been reported.

OLS infection can occur at any time of the year from late autumn through to early spring, if environmental conditions are conducive (Graniti, 1993; Guechi and Girre, 1994). Results in Chapter 2 showed that conidia can germinate on detached olive leaves only if supplied with free moisture, and at temperatures ranging from 5 to 25°C with an optimum of 20°C. Models describing the relationship between leaf wetness duration, conidium germination and germ tube growth at different temperatures were developed from laboratory studies using detached leaves. However, these simple models may only have limited application in the development of disease forecasting systems because the effect of dry periods interrupting germination and infection processes at different temperatures is unclear. In olive groves, interrupted wet periods are common during spring and early summer, and during the dry periods the relative humidity (RH) may vary considerably. Consequently, the calculation of infection periods is often difficult. For *V. inaequalis* duration of a dry period interrupting a continuous wet period has been reported to reduce spore survival and the development of apple scab (Becker and
The relative humidity and light intensity during the dry period has also been reported to affect the survival of *V. inaequalis* spores (Aylor and Sanogo, 1997). However, for OLS infection and disease development the effect of intermittent wetness periods and levels of RH during wetness interruptions is unknown.

In spring, the main source of *S. oleagina* inoculum for primary infection is the sporulating, overwintering lesions on the leaves that remain on the trees (Guechi and Girre, 1994). Conidia formed on these lesions may be viable for several months, but once detached from the conidiophores, they lose their viability within a week (Laviola, 1966; Tosi and Zazzerini, 2000). The variation in numbers and viability of *S. oleagina* conidia in olive groves has been demonstrated (Guechi and Girre, 1994; Chapter 7), with higher levels of conidia being produced in spring compared with summer months when there were fewer rain events (Chapter 7). A relationship between inoculum concentration and disease severity has been reported for other related pathogenic fungi, such as *V. inaequalis* (MacHardy, 1996) and *V. pirina* (Villalta et al., 2000). According to Palm (1987) (cited in MacHardy, 1996), inoculum dose in an orchard was shown to be vital in predicting levels of apple scab infection. Palm (1987), who used inoculum concentrations ranging from $5.0 \times 10^2$ to $1.0 \times 10^5$ conidia/mL, found that the resulting disease levels increased with inoculum dose up to about $5.0 \times 10^4$ conidia/mL and thereafter did not increase further. For *S. oleagina*, the relationship between inoculum concentration, temperature and OLS severity has not been reported.

This chapter describes investigations into the effects of temperature, inoculum concentration, leaf wetness periods, interrupted leaf wetness periods, RH, and leaf age on OLS incidence and disease severity for olive plants in controlled conditions. The data generated were used to develop a polynomial equation that may be useful in developing a predictive model.

### 4.3 MATERIALS AND METHODS

**Experimental plants**

One- to two-year-old 'Barnea' olive plants were used in all experiments. Plants were produced from cuttings and grown in a greenhouse ($22 \pm 5^\circ$C) as described previously (Chapter 2). The youngest pair of fully expanded leaves were marked on each plant after two months of growth, and thereafter, at fortnightly intervals for ten weeks.
Humidity tents
Two humidity tents were constructed inside a growth cabinet (Conviron PGV36; Controlled Environments Limited, Winnipeg, Manitoba, Canada). The tents (145 x 95 x 145 cm) consisted of polyvinyl chloride (PVC) pipes, which were covered with polyethylene plastic sheet. To maintain continuous leaf wetness, one of the tents was equipped with a misting unit that produced a layer of fine droplets for 30 s every 4 h. The bottom of the other tent was completely covered with moistened absorbent paper whose ends were dipped into two containers filled with water to maintain high relative humidity during interrupted wetness periods. Three 2-3 cm diameter openings in each side of the tents allowed air exchange and some escape of water vapour.

Temperature and RH inside the chambers were monitored with a Hobo temperature and RH sensors (Onset Computer Corp., Pocasset, MA) with an accuracy of ±0.2°C and 2%, respectively. Prior to inoculation, plants were placed for 24 h inside the growth room at the respective temperature to ensure that plant tissues were brought to the designated temperature.

Inoculum preparation
For each experiment, inoculum was freshly prepared as described in Chapter 2 from naturally infected ‘Barnea’ olive leaves grown in a nearby commercial grove. For the inoculum dose experiments, the stock suspension was adjusted to 2.5 x 10^6 conidia/mL using a haemocytometer. Serial dilutions of the stock suspension were then made to obtain a range of inoculum concentrations for application on the plants. For all other experiments, the inoculum suspension was adjusted to 5.0 x 10^4 conidia/mL. Conidium viability of all conidial suspensions was determined according to the methods of Saad and Masri (1978), and found to range from 50 to 60%.

4.3.1 Effect of conidial concentrations on OLS severity
Experiments investigating the effect of different conidial concentrations on disease development at various temperatures (5, 10, 15, 20, and 25°C) were conducted consecutively in the same growth chamber, with the temperature sequence selected at random. For any given temperature, 42 plants were inoculated (six plants with each of the seven different conidial concentrations) and the experiment was conducted twice. Plants were inoculated
using an atomizer to spray them with $0, 1.0 \times 10^2, 2.5 \times 10^3, 1 \times 10^4, 5.0 \times 10^4, 2.5 \times 10^5,$ or $2.5 \times 10^6$ conidia/mL, until all the leaves were completely covered but not to run-off. After inoculation, the plants were arranged in a complete randomized design inside the growth cabinet, set at 98-100% RH to maintain leaf wetness, and incubated at the designated temperature for 48 h. Subsequently, the plants were transferred to a shadehouse, fitted with an automatic overhead sprinkler system that turned on for approximately 10 min per day, to allow disease development. The mean daily temperature in the shadehouse ranged from 5 to 15°C. Plants were monitored for lesion development weekly from 4 weeks until 12 weeks, when no new lesions were observed to be developing. Because of the slow development of OLS, disease assessment was done on the plants after 12 weeks of incubation. The disease severity was assessed on the top six fully expanded leaves at the time of inoculation by recording the number of leaf lesions per plant. Since earlier studies by MacDonald et al. (2000) and in Chapter 7 found high correlations ($P<0.001$) between numbers of OLS-infected leaves per tree, numbers of leaf lesions per plant and diseased leaf areas, and OLS lesions are known to expand very slowly, assessment of disease severity as the number of leaf lesions per plant was considered to be valid for this study.

4.3.2 Effect of wetness periods, temperature, and leaf age on OLS severity

In this experiment, eight leaf wetness periods (6, 12, 18, 24, 36, 48, 72, and 96 h) were tested at five temperatures (5, 10, 15, 20, and 25°C). Each test of temperature-leaf wetness period combination was conducted three times. Plants were spray-inoculated with the conidial suspension ($5.0 \times 10^4$ conidia/mL) of *S. oleagina* described previously. Non-inoculated plants sprayed with sterilized water served as the control. Continuous wetness in the growth chamber was provided by the previously described misting system, which maintained RH at 98-100%, and the plants continuously wet.

Four replicate plants, selected at random, were removed from the chambers after wetness periods of 6, 12, 18, 24, 36, 48, 72, and 96 h incubation. Wetness period measurements included the time required (30 to 40 min) for leaves to dry after removal from the growth chamber, which was done by placing the inoculated plants 90 cm from a fan set at slow speed at room temperature (RH about 50%). After the leaves were dry, the plants were transferred to the shadehouse for disease development. After 12 weeks of incubation, disease severity was assessed as before but on the top 12 fully expanded leaves at the time of inoculation.
leaf age effect was determined by categorizing the leaves as young (4 weeks old), intermediate (8 weeks old), and old (12 weeks old) at the time of inoculation.

### 4.3.3 Effect of dry period and RH after an initial wet period on OLS severity

The effects of different dry or interrupted wet periods on OLS development was investigated on inoculated olive plants in a growth chamber maintained at 10 or 20°C. After spray-inoculation, as described previously, the plants were exposed to either continuous wetness or interrupted periods of wetness. These consisted of an initial 12 h wet period followed by a dry period (0, 3, 6, 12, 18, or 24 h) of either low (70%) or high RH (100%) and a final wet period of 24 h. The periods of continuous wetness lasted 36, 39, 42, 48, 54, or 60 h. Thus, for each interrupted wet period treatment, there was a corresponding continuous wet period treatment of the same total duration.

The effects of different periods of initial leaf wetness on conidium infection were also investigated at both 10 and 20°C. After spray-inoculation, the plants were exposed to an initial wet period of 0, 3, 6, 12, 18, or 24 h, followed by a fixed dry period of 12 h and a final wet period whose length made the total length of the cycle up to 48 h. The dry period consisted of either low RH (70%) or high (100%) RH. For both experiments, there were eight plants for each wetness period and four plants for a single dry period at high or low RH, and each experiment was conducted twice.

For the wetness treatments, the plants were placed in a humidity tent equipped with a misting unit and within a growth chamber maintained at 70% RH. Dry periods were initiated by removing eight randomly selected plants from the misting unit after the designated times and drying their leaves with a fan as described earlier. When foliage was dry (30-40 min), four plants were then placed in the growth chamber (70% RH), and four plants in the humidity tent (100% RH) constructed inside the same growth chamber. Inoculated plants were incubated in darkness during wetness periods and dry periods. At the end of the second wet period, all plants were transferred to a shadehouse to allow development of OLS symptoms. Disease severity on the plants was assessed after 12 weeks as described previously.
4.3.4 Data analysis

All experiments were arranged in completely randomized designs and the data collected was analysed using analysis of variance (ANOVA) (Genstat 7.2, Lawes Agricultural Trust, Rothamsted Experimental Station). For the inoculum concentration experiment, the concentration was log transformed and regression analysis was then used to establish a relationship between inoculum concentration and OLS severity at any given temperature.

For the effect of leaf wetness duration, leaf age and temperature on infection and disease development, the number of leaf lesions per plant ($L$) were square root transformed ($\sqrt{L}$) to stabilize the variance. Because there were no significant differences between experiments, the data were pooled before model fitting. Several models were fitted to the data before and after transformation, but the selected model was of the form:

$$Y = f(T, W, A)$$

where $Y$ is $\sqrt{L}$ and $f(T, W, A)$ is a function of temperature ($T$), leaf wetness duration ($W$), and leaf age ($A$). Linear and quadratic terms of $T$, $W$, and $A$ and their interactions were tested. The model selection was based on the significance of $F$ value for each model, the significance of estimated parameters, randomness and normality of residuals, coefficient of determination ($R^2$) and $R^2$ adjusted ($R^2_a$) for the degree of freedom as well as the Mallows $C_p$ values.

For the effect of interrupted wetness periods on disease severity, the mean number of leaf lesions per plant for each treatment was expressed as a percentage of the maximum number of lesions per plant (PML) observed on plants from the corresponding continuous wetness treatment. Regression analysis was then used to establish a relationship between PML and length of the dry period or initial wetness duration at the two relative humidity regimes and temperatures.
4.4 RESULTS

4.4.1 Effect of conidial concentrations of *Spilocaea oleagina* on disease severity

The numbers of OLS lesions on leaves of olive plants inoculated with different inoculum concentrations at different temperatures were significantly (P=0.040) lower in the second experiment (repeat) than in the first experiment. However, the trends were similar for both experiments and there was no statistically significant interaction of experiment with other factors, therefore the data were pooled. Numbers of OLS lesions per plant (disease severity) increased with increasing inoculum concentration up to about $2.5 \times 10^5$ conidia/mL (Figure 31). The disease severity at each inoculum concentration was consistently lower at 25°C than the severity at the other temperatures tested. The influence of temperature and inoculum concentrations on leaf lesions per plant was best described by the equation

$$Y = -38 + 4.8T + 7.9I - 0.17T^2,$$

where $Y$ is the number of leaf lesions/plant, $T$ is the temperature during the time of infection, $I$ is the logarithm (base 10) of the number of conidia/mL applied to the plants. The model accounted for 89.6% of the variance, and the estimated regression coefficients were each significant at $P \leq 0.001$.

![Figure 31: Olive leaf spot severity (number of OLS lesions/plant) caused by different conidial concentration and infection temperatures at 48 h of leaf wetness. Each point is the mean number of lesions/plant determined on 12 plants (two experimental replications with six replicate plants per treatment) and six leaves per plant. Bars are standard errors of the means.](image-url)
OLS lesions were observed on leaf petioles and along the midribs on the lower sides of leaves from some plants inoculated with the two highest concentrations used, at all temperatures except at 25°C. Lesions were first observed 8 weeks after inoculation at the higher concentrations (≥5.0 × 10⁴ conidia/mL) compared with 12 weeks for the lower concentrations (≤1.0 × 10⁴ conidia/mL).

### 4.4.2 Effect of wetness periods, temperature, and leaf age on OLS severity

Olive leaf age, temperature and leaf wetness duration significantly \((P<0.001)\) affected the mean number of leaf lesions per plant. Among repeated experiments, the effect of leaf age on lesions per plant was not significantly different \((P=0.788)\) except that in the second experiment, the number of leaf lesions per plant was more variable at 10°C than in the other two experiments. The young leaves developed significantly \((P<0.001)\) more lesions than either the intermediate or old leaves (Figure 32).

For temperature and leaf wetness period, there was no difference between experimental replications on the number of lesions per plant, although at 10°C the mean number of lesions on the leaves was lower (35 lesions per plant) in Experiment 2 compared with Experiment 1 (47 lesions per plant) and 3 (50 lesions per plant) after 96 h of wetness. Mean numbers of lesions increased gradually from about 20 lesions per plant at 5°C reaching a maximum of 57 lesions per plant at 15°C, and then declined to 13 lesions per plant at 25°C. Disease severity (lesions per plant) followed a quadratic response function for temperature, which estimated the optimum as 14.5°C.

Generally, olive leaf spot infection and development on the leaves of inoculated plants increased with increasing leaf wetness period at all temperatures tested. Olive leaf spot developed at all leaf ages, temperatures and leaf wetness periods tested, except that no lesion developed for the 0 h and 6 h wetness periods after inoculation, at all temperatures. At 25°C, lesions were only observed when the leaf wetness period exceeded 24 h. The minimum leaf wetness periods for lesion development at 5, 10, 15, 20, and 25°C were 18, 12, 12, 12, and 24 h, respectively (Figure 32).
Figure 32: Olive leaf spot severity (number of lesions/leaf) with different leaf wetness durations at various ages and temperatures. Symbols represent the means of observations for each combination of temperature and wetness duration, and for the three replicate experiments (six plants per treatment with six leaves per plant). The lines represent the predicted values calculated with the equation: \( Y = \beta_0 + \beta_1 A + \beta_2 T + \beta_3 W + \beta_4 (A \times W) + \beta_5 T^2 + \beta_6 W^2 \), where \( Y \) is the predicted square root of number of lesions/plant, \( A \) is leaf age (weeks), \( T \) is temperature (°C), \( W \) is wetness duration (h), and \( \beta_0 \ldots \beta_6 \) are the determined parameters. Error bars represent the standard errors of the means.
At higher leaf wetness durations (>48 h) and at 5 to 15°C, lesions were also observed on leaf petioles, and midribs on the undersides of some of the inoculated leaves, but no lesions were observed on the lower leaf blade surfaces. The time at which the first lesion was observed on each leaf vary according to temperature and leaf wetness conditions during the infection process. For instance, under optimum conditions of 15°C and 96 h of wetness, it took 8 weeks for the first lesions to appear in all experimental repeats. However, at the other conditions lesions were first observed on the leaves about 12 weeks post-inoculation.

4.4.3 Prediction model

$T$, $W$, $A$, and the interaction of $W$ and $A$ all had significant ($P<0.001$) effects on the square root of lesion number per plant. However, there was no significant interaction between $T$ and $A$ ($T\times A$) ($P=0.568$) or $T$, $W$, and $A$ ($T\times W \times A$) ($P=0.816$). Consequently, both these interactions were excluded from the model. The model that best fitted the pooled data from the three experimental repeats was:

$$Y = \beta_0 + \beta_1 A + \beta_2 T + \beta_3 W + \beta_4 (A\times W) + \beta_5 T^2 + \beta_6 W^2,$$

(2)

where $Y$ is \sqrt{number of lesions/plant}, $A$ is leaf age (weeks), $T$ is temperature (°C), $W$ is wetness period (h), and $\beta_0, \ldots, \beta_6$ are the determined parameters. Parameter estimates and their standard errors are given in Table 5. All the estimated parameters in the model were significant at $P<0.001$. The response surface of the model for the effects of temperature, wetness duration and leaf age on OLS severity is presented in Figure 32. The model accounted for 89.5% of the variance.

4.4.4 Effect of dry period and RH after an initial wet period on OLS severity

In the first experimental series, with fixed initial wetness periods of 12 h, there was a significant ($P<0.001$) difference in the numbers of leaf lesions on the leaves between the two experiment repetitions conducted at 10°C, with the mean number of lesions being consistently higher in the first experiment than in the second. For example, the numbers of lesions produced under 60 h of continuous wetness were 78 and 64 for the first and second experiments, respectively. However, at 20°C there was no significant ($P=0.420$) difference between the experiment repeats, therefore the results were pooled.
Table 5: Estimated parameters and associated standard errors for the prediction model \( Y = \beta_0 + \beta_1 A + \beta_2 T + \beta_3 W + \beta_4 (A \times W) + \beta_5 T^2 + \beta_6 W^2 \) to describe the relationship between temperature (°C; \( T \)), leaf wetness (h; \( W \)), leaf age (weeks; \( A \)) and square root of olive leaf spot lesion numbers (\( Y \)). The coefficient of determination adjusted for degrees of freedom (\( R^2_a \)) for the pooled data was 89.5%.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \beta_0 )</td>
<td>-4.42</td>
<td>0.273</td>
</tr>
<tr>
<td>( \beta_1 )</td>
<td>-0.243</td>
<td>0.0274</td>
</tr>
<tr>
<td>( \beta_2 )</td>
<td>0.943</td>
<td>0.0255</td>
</tr>
<tr>
<td>( \beta_3 )</td>
<td>0.1557</td>
<td>0.00592</td>
</tr>
<tr>
<td>( \beta_4 )</td>
<td>-0.00124</td>
<td>0.000393</td>
</tr>
<tr>
<td>( \beta_5 )</td>
<td>-0.03292</td>
<td>0.000855</td>
</tr>
<tr>
<td>( \beta_6 )</td>
<td>-0.000817</td>
<td>0.0000458</td>
</tr>
</tbody>
</table>

\( ^a \) Estimated regression parameters for the fitted terms
\( ^b \) All parameters were significant at \( P < 0.001 \)

The length of the dry period that followed the initial 12 h wet period and RH during the dry period significantly (\( P < 0.001 \)) affected the number of lesions produced on olive leaves inoculated with \( S. \) *oleagina* conidia at 10 and 20°C (Table 6). At 10°C and 20°C, plants exposed to high humidity (100% RH) during the dry periods, had higher numbers of leaf lesions with increasing dry periods, whereas at low relative humidity (70% RH) the numbers of leaf lesions decreased with increasing dry periods.

For the continuous wetness treatments at 10 and 20°C, numbers of leaf lesions increased with increasing continuous wetness period, except for the similar numbers of lesions with 36 and 39 h wetness at 20°C. Overall the rate of increase over time was much greater under wet incubation conditions than with the corresponding interrupted incubation periods that incorporated 70 or 100% RH (Table 6). Regression analysis was used to establish a relationship between PML and length of the dry period at the two relative humidity regimes and temperatures (Figure 33). The relationship was best described by:

\[ Y = c + bD + aD^2, \]

where \( Y \) is PML, \( D \) is the dry period (h), and \( a, b, \) and \( c \) are estimated parameters (Table 7).
Table 6: Effects of continuous wet periods (CWP) or interrupted wet periods (IWP) with 70 or 100% RH during the dry period, on mean numbers of olive leaf spot lesions following inoculation of olive plants with conidia of *Spilocaea oleagina*

<table>
<thead>
<tr>
<th>Treatments (h)</th>
<th>Lesions per plant c</th>
<th>IWP a</th>
<th>CWP b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp. (°C)</td>
<td>Initial</td>
<td>Dry</td>
<td>Final</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>3</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>18</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>20</td>
<td>12</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>20</td>
<td>12</td>
<td>3</td>
<td>24</td>
</tr>
<tr>
<td>20</td>
<td>12</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
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<td>12</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>20</td>
<td>12</td>
<td>18</td>
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</tr>
<tr>
<td>20</td>
<td>12</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

a Interrupted wet duration (IWP) consisted of initial and final wet durations of 12 and 24 h, respectively, separated by a dry period of different lengths and different relative humidities, 70 and 100% RH.

b Continuous wet duration (CWP) consisted of an uninterrupted leaf wetness duration equal to the total duration of the corresponding IWP treatment.

c Mean numbers of lesions of two experiment repeats with 4 plants per treatment, 10 leaves per plant for the IWP, and 6 plants per treatment, 10 leaves per plant for CWP.

d Values in parentheses are the standard errors of the means.

For the second experimental series, with varying initial wetness periods from 0-24 h, there were significant ($P<0.001$ and $P=0.004$, respectively) differences in lesion numbers between experiment replications conducted at 10 and 20°C. At both temperatures, the numbers of lesions were consistently lower in the first experiment than in the second experiment. However, the differences did not affect the consistency of the overall trends.
Figure 33: Effect of dry periods and relative humidity (RH) on olive leaf spot severity (percentage of the maximum number of lesions per plant, PML) during the interruption of wetness periods at 10°C (A) and 20°C (B). Symbols represent the means of two replicate experiments (4 plants per experiment and 10 leaves per plant), bars are the standard errors and the lines are the predicted values. The regression equations are A: \( Y = 109 - 8.75D + 0.193D^2 \) \((R^2=0.989)\), \( Y = 92.9 - 1.83D + 0.033D^2 \) \((R^2=0.797)\) for 70 and 100% RH, respectively; B: \( Y = 109 - 6.32D + 0.079D^2 \) \((R^2=0.987)\), \( Y = 101 - 3.09D + 0.059D^2 \) \((R^2=0.972)\) for 70 and 100% RH, respectively. For the equations of each RH and temperature, where \( Y \) is the percentage of the maximum number of lesions (obtained under continuous wetness) and \( D \) is the dry period between the 12 h initial and 24 h final wet periods.
Table 7: Estimated parameters and associated $R^2$ values for the regression model, $Y = c + bD + aD^2$, which describes the relationship between percent of maximum lesions ($Y$) of olive leaf spot per plant and the length of dry period ($D$) at two different temperatures and relative humidity treatments.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>RH (%)</th>
<th>Parameters</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>10</td>
<td>70</td>
<td>0.193</td>
<td>-8.75</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.033</td>
<td>-1.83</td>
</tr>
<tr>
<td>20</td>
<td>70</td>
<td>0.079</td>
<td>-6.32</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.059</td>
<td>-3.09</td>
</tr>
</tbody>
</table>

$^1$The estimated regression parameters

Initial wetness of various durations, followed by a fixed dry period of 12 h had a significant ($P<0.001$) effect on the number of lesions that developed on the inoculated olive plants. Plants subjected to a 12 h initial wetness period followed by a 12-h dry period prior to a final wet period had the significantly ($P<0.05$) lowest numbers of lesions at both temperatures and RH levels (Table 8). At both temperatures, the effects of the two RH levels (70 and 100%) during the 12-h dry periods followed the same trends observed in the previous experiment, with fewer lesions recorded for plants under low RH (70%) than for those under high RH (100%) during the dry period (Table 8).

Numbers of lesions per plant differed significantly between the continuous wetness control and treatments at low and high humidity ($P=0.0008$, 0.002 and $P=0.006$, 0.0011, respectively) for experiments conducted at 10°C and 20°C, respectively. For both humidity treatments and temperatures, the percentage of maximum lesions (PML) values decreased with increasing duration of initial wetness up to 12 h and then increased with increasing wetness period (Figures 34A and B). No disease developed on plants that were dried immediately after inoculation (0 h).

The relationship between initial wetness duration and percentage of maximum lesion (PML) was best described by: $Y = c + bW + aW^2$, where $Y$ is PML, $W$ is the initial wetness period (h), and a, b, and c are estimated parameters (Table 9).
Table 8: Effects of different wetness periods before and after a fixed 12 h dry period of 70 or 100% RH, and a 48 h continuous wet period, on numbers of olive leaf spot lesions following inoculation of olive plants with conidia of *Spilocaea oleagina*

<table>
<thead>
<tr>
<th>Treatment periods (h)</th>
<th>Lesions per plant c</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IWD a</td>
<td>CWD b</td>
</tr>
<tr>
<td>Temp. (°C)</td>
<td>Wet (initial)</td>
<td>Dry (final)</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>12</td>
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<td>10</td>
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<td>12</td>
</tr>
<tr>
<td>10</td>
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<td>12</td>
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<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
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</tr>
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<td>6</td>
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</tr>
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<td>20</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>20</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>20</td>
<td>24</td>
<td>12</td>
</tr>
</tbody>
</table>

a Interrupted wet duration (IWD) consisted of various initial wet periods followed by a 12 h dry period (70 or 100%) and a final wet period, so that the total length of the cycle was 48 h.

b Continuous wet duration (CWD) consisted of 48 h uninterrupted leaf wetness duration.

c Means of two experiment repeats with 4 plants per treatment, 10 leaves per plant for the IWD, and 6 plants per treatment, 10 leaves per plant for CWD.

d Values in parentheses are standard errors of means.
Figure 34: Effect of initial wetness period and relative humidity (RH) on olive leaf spot severity (percentage of the maximum number of lesions per plant, PML) during the interruption of wetness periods at 10°C (A) and 20°C (B). Symbols represent the means of two replicate experiments (four plants per experiment and ten leaves per plant), bars are the standard errors and the lines are the predicted values. The regression equations are A: $Y = 93.6 - 4.31W + 0.170W^2$ ($R^2 = 0.723$), $Y = 90.6 - 2.10W + 0.082W^2$ ($R^2 = 0.806$) for 70 and 100% RH, respectively; B: $Y = 94.3 - 5.13W + 0.202W^2$ ($R^2 = 0.821$), $Y = 101 - 4.05W + 0.156W^2$ ($R^2 = 0.781$) for 70 and 100% RH, respectively. For the equations of each RH and temperature, $Y$ is the percentage of the maximum number of lesions (obtained under 48 h continuous wetness) and $W$ is the initial wetness period.
Table 9: Estimated parameters and associated $R^2$ values for the regression model, $Y = c + bW + aW^2$, which describes the relationship between percent of maximum lesions ($Y$) of olive leaf spot per plant and the length of initial wetness period ($W$) at two different temperatures and relative humidity treatments

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>RH (%)</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>70</td>
<td>0.170</td>
<td>-4.31</td>
<td>93.6</td>
<td>0.723</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.082</td>
<td>-2.10</td>
<td>90.6</td>
<td>0.806</td>
</tr>
<tr>
<td>20</td>
<td>70</td>
<td>0.202</td>
<td>-5.13</td>
<td>94.3</td>
<td>0.821</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.156</td>
<td>-4.05</td>
<td>101</td>
<td>0.781</td>
</tr>
</tbody>
</table>

The estimated regression parameters

4.5 DISCUSSION

In this study, inoculum concentration played a significant role in determining disease severity (OLS lesions/leaf), which increased with increasing inoculum concentration up to $2.5 \times 10^5$ conidia/mL, at all temperatures (5-25°C) after 48 h of incubation. With the three highest inoculum concentrations ($5.0 \times 10^4$, $2.5 \times 10^5$ and $2.5 \times 10^6$ conidia/mL) initial lesions appeared 8 weeks after inoculation compared with 12 weeks for lower inoculum level, indicating the importance of high inoculum for rapid development of OLS in the field. However, in preliminary experiment (Appendix 3.1) inoculum concentrations ($>1 \times 10^6$ conidia/mL) reduced the frequency of conidium germination, suggesting that there might be some self-inhibition of $S. oleagina$ conidium germination if concentrations were very high. However, the preliminary experiment was conducted on OLE agar not olive leaves which may have influenced the outcome. A relationship between inoculum concentration and symptom development has also been reported for other closely related fungi, such as $Venturia inaequalis$ (Hartman et al., 1999). Hartman et al. (1999) demonstrated that inoculum dose of $V. inaequalis$ and leaf wetness determined the incidence of scab lesions on apple seedlings in controlled experiments. At four temperatures and using inoculum doses ranging from $1.5 \times 10^3$ to $2.5 \times 10^5$ conidia/mL, apple scab incidence increased with increasing inoculum doses up to $8.12 \times 10^4$, after which disease incidence did not increase (Hartman et al., 1999). Similarly, Palm (1987), using inoculum concentrations ranging from $5.0 \times 10^2$ to
1.0 \times 10^5 \text{ conidia/mL}, \text{ found that the resulting apple scab levels increased up to a dose of about } 5.0 \times 10^4 \text{ conidia/mL.}

Plant tissue susceptibility to a particular pathogen can vary with the age of the tissue. In the present study, it was found that on average the youngest leaves developed more olive leaf spot lesions per plant than older, more physiologically matured leaves. The difference was particularly evident when the temperature and leaf wetness durations were optimal. Typically, disease severity on the youngest leaves was about three to five times higher than on the oldest leaves. For example, at the optimum conditions (15°C and 96 h of wetness), mean severity was 75 lesions per plant for the youngest leaves and only 15 lesions per plant for the oldest leaves. In addition, there was a more rapid increase in the severity between 6 and 24 h of wetness for the youngest leaves than for the other two leaf ages. These results are consistent with those of López-Doncel and Trapero (1999) who found that younger leaves or less cutinized leaves were more susceptible to olive leaf spot than older leaves. This phenomenon has also been recognized in other plant-pathogen systems (Trapero-Casas and Kaiser, 1992; Pedersen and Morrall, 1994; Hong and Fitt, 1995).

Field reports on the effect of olive leaf age on their susceptibility to \textit{S. oleagina} are contradictory. Guechi and Girre (1994) demonstrated that in France, olive leaf spot lesions appeared on olive leaves in December and were most abundant on the first three pairs of leaves of young shoots, which had developed in the spring of the same year. However, in California Wilson and Miller (1949) found that olive leaf spot lesions were more abundant on older leaves, from which they concluded that the older leaves were more susceptible to the disease. However, the time taken for lesions to appear may explain the confusion. The olive cultivar (‘Barnea’) used in the present study is known to be highly susceptible to the disease (MacDonald et al., 2000), yet in this study olive leaf spot symptom expression was found to be slow and lesions were usually not visible before 8 weeks after inoculations. Therefore, disease symptoms are found mostly on older leaves in the field, even though infections had probably occurred on the foliage when it was young.

The development of olive leaf spot lesions on olive plants inoculated with \textit{S. oleagina} conidia was affected by temperature and duration of wetness during the germination and infection period. Lesions were observed on the plants at all temperatures tested in this study (5-25 °C).
with an optimum at 15°C and minimum at 25°C. Longer periods were required for significant infection at suboptimal temperatures (<10 and >20°C). Even in the presence of inoculum and optimal temperatures the 0 and 6 h wetness periods resulted in no lesions, indicating that there was a minimum duration of wetness required for infection. These results are consistent with a previous study, which demonstrated that on detached olive leaves the minimum wetness periods required for S. oleagina conidium germination were 24, 12, 9, 9, and 12 h at 5, 10, 15, 20, and 25°C, respectively (Chapter 2). Free surface moisture has been reported to be a requirement for many other fungal diseases including apple scab (MacHardy, 1996), spinach white rust (Sullivan et al., 2002), and grey leaf spot on perennial ryegrass (Uddin et al., 2003). The results presented here also showed that at the sub-optimal temperatures, longer wetness durations were needed for infection to occur indicating that leaf wetness is probably the most important environmental variable for infection, though temperature regulates the rapidity and level of disease development.

This study proposed a polynomial model (Equation 2) for predicting olive leaf spot severity based on leaf age, temperature and leaf wetness duration. The model gave a good fit to the observed data since 89.5% variability in mean severity was accounted for by the components of the polynomial model. Conversely, predicted disease severity after 96 h of leaf wetness duration was much lower than the observed disease severity on leaves of the intermediate age at 5 and 20°C. However, the inclusion of a leaf age variable in the model developed in this study is justifiable because olive may produce new growth at different times of the year in different climates. A similar effect was factored into a prediction model developed for Phomopsis leaf blight of strawberry, caused by Phomopsis obscurans (Nita et al., 2003). The interaction between duration of leaf wetness and leaf age in Equation 2 will have a profound influence on development of a disease forcaster that incorporates the age profile of the olive canopy. For example, when the duration of leaf wetness is short, the difference between leaves of different age in the severity will be minimal. However, as wetness periods become prolonged, the difference between younger leaves and older leaves becomes greater, with younger leaves developing significantly more lesions than older leaves. Consequently, the risk of infection in the field will need to be weighted depending on the time course of the spring or autumn flush of growth in relation to a prolonged wetness period.

The results of the present study showed that S. oleagina infection and olive leaf spot development was affected by interrupted leaf wetness at 10 and 20°C, but it depended on the
relative humidity during the dry period, with decreased disease severity in low relative humidity (70%). This has not been reported previously for olive leaf spot, but similar results have been reported for other plant pathogens. Eisensmith et al. (1982) found that interrupted wetness periods led to reduced infection by cherry leaf spot (Coccomyces hiemalis) compared with continuous wetness periods, although relative humidities (40-90%) were uncontrolled in their study. Arauz and Sutton (1990) showed that Botryosphaeria obtusa infection of apple leaves was interrupted irreversibly following dry periods as short as 1 h after an initial wetness period of 12 h. More recently, Villalta et al. (2000) demonstrated that during interrupted wet periods at high relative humidity (>90%) and 20°C, the number of Venturia piriniae lesions per cm² decreased from 2.7 to 0.15 as the dry period increased from 1 to 90 h. In the same experiment, however, no lesions developed if leaves were dry for more than 12 h at low relative humidity (<70%). In the apple scab pathosystem (Venturia inaequalis), Schwabe (1980) found that for ascospores and conidia dry intervals of less than 16 and 32 h, respectively, did not significantly interrupt the wet periods required for infection. However, Moore (1964) showed that a dry period of 48 h significantly reduced infection levels by V. inaequalis.

In the first interrupted wetness experiment, high RH (100%) during the dry periods caused disease severity to increase with increases in length of the dry interrupted period, probably because the overall study periods were also increased, but severity was still less than at the equivalent times under continuous wetness. This result agrees with previous findings that conidia of S. oleagina were able to germinate at 20°C when high relative humidity followed an initial wetness period of 6 h (Chapter 2). Similar results were also found when investigating the influence of interrupted wetness periods on infection of wheat by Mycosphaerella graminicola (Shaw, 1991), and the infection of soybean by Cercospora kikuchii (Schuh, 1992). On wheat, when periods of 100% relative humidity during infection of M. graminicola were interrupted by dry periods with 75% relative humidity, infection was not significantly affected, but relative humidities of 50% during dry periods resulted in significant reduction in infection.

Successful infection by fungal pathogens after interrupted wetness periods could indicate that the spores had the capacity for both rapid germination and penetration, or the capacity to survive intermittent drying. The results of this study suggest that S. oleagina conidia may be able to withstand some measure of drying and to exploit successive periods of leaf wetness.
cumulatively to infect olive leaves. In the second experiment and at both temperatures tested (10 and 20°C), *S. oleagina* conidium infection was lowest when a 12 h dry (70% RH) period occurred after an initial 12 h wet period. This may be because 12 h wetness is enough to initiate germination and drying damages the germinating conidia, whereas the shorter wet periods were insufficient for initiating germination. This hypothesis is supported by the results of the first interrupted wetness experiment, where all the treatments had a 12 h initial wet period, but only at 70% relative humidity did the increasing dry periods cause reducing disease severity. It is also possible that the germlings are most vulnerable to desiccation at this stage of development. A similar result was reported by Vloutoglou et al. (1999) who found that at 15°C, *Alternaria linicola* conidia applied to linseed plants were susceptible to drying after 1 to 12 h of initial wetness, particularly when the dry period was 12 h long. Although no detailed investigation was performed on penetration and survival of germlings, the results of a previous study on conidium germination (Chapter 2) and those reported here suggest that it is probably the ability of the *S. oleagina* conidia to survive drying rather than rapid germination and penetration that is responsible for successful infection under interrupted wet periods.

Under field conditions, desiccation of spores exposed to sunlight, fluctuating temperatures and relative humidity during the interrupted wet period could be much higher than with a controlled environment. Aylor and Sanogo (1997) reported that in both laboratory and field studies the detached conidia (ungerminated) of *V. inaequalis* could survive radiation doses equivalent to 12 h sunlight. However, with *Botryosphaeria obtusa* on apple leaves, Arauz and Sutton (1990) showed that in no instance did germ tubes resume growth after being rewetted following dryness periods of any duration, and that the relative humidity during the dry period did not have any effect on the ability of the germ tubes to continue their elongation upon rewetting. Further research is required to investigate the effect of a dry period during the *S. oleagina* conidia infection process at various temperatures and relative humidity, and the influence of direct sunlight on conidium survival.

In conclusion, the data presented here have demonstrated that young olive leaves are more susceptible to olive leaf spot than older leaves, but the overall effect is moderated by the temperature and leaf wetness duration. The inoculum levels, leaf wetness, length of interrupted wet periods and humidity during the dry period are important factors in infection and development of the disease. A disease infection model was developed, but information
on factors influencing *S. oleagina* conidium production is required for inclusion in a more comprehensive model. This is described in Chapter 5. After field-testing and modifications, such models can be used for further development of a disease forecasting system, a tool that may then be used in devising a spray schedule that accurately indicates the timing of fungicide applications in response to climatic factors.

4.6 ACKNOWLEDGEMENTS

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4.7 REFERENCES


CHAPTER 5

Factors affecting sporulation and disease development of
Spilocaea oleagina

5.1 ABSTRACT

The effects of temperature and the moisture conditions of continuous wetness, 70 and 100% relative humidity on S. oleagina sporulation and lesion expansion were studied on intact olive plants. Sporulation was observed on olive leaf spot lesions at 5 to 25°C and at all moisture conditions except at 70% relative humidity and 25°C. The optimum condition for sporulation was 15°C and 100% relative humidity. Under continuous wetness and 100% relative humidity, the numbers of conidia per mm² of leaf tissue decreased as temperature increased or decreased from the optimum. Lesion expansion was significantly (P<0.001) affected by temperature, with the highest mean lesion diameter recorded for leaves incubated at 15°C under continuous wetness and very little lesion expansion at 25°C. Lesion age significantly (P=0.032) affected sporulation, with younger lesions producing more conidia than older ones. Sporulation only occurred on olive leaf surfaces where there were no trichomes. The Richards function was used to describe the relationship between temperature and sporulation of S. oleagina over time under various moisture conditions, with $R^2$ values ranging from 0.868 to 0.911. The models explained a large proportion of the variation in the sporulation capacity of lesions of S. oleagina and can therefore be used for predictive purposes.

5.2 INTRODUCTION

Previous reports have shown that S. oleagina conidia can germinate and infect olive leaves over a range of temperatures and leaf wetness conditions and that numbers of OLS lesions increased with increasing inoculum concentration up to about $2.5 \times 10^5$ conidia/mL at all five temperatures tested (5, 10, 15, 20, and 25°C) (Saad and Masri, 1978; Chapters 2 & 4). However, the precise effect of the different environmental factors on S. oleagina conidium production has not been reported. In spring, large numbers of S. oleagina conidia may be produced from the overwintering leaf lesions but there is high variability over time in
numbers and viability of conidia available in the olive groves (Guechi and Girre, 1994; Chapter 7).

Graniti (1993) reported that infection and lesion development was influenced by environmental conditions and the seasonal growth stage of the trees. However, this understanding was largely based on general observations, such as those of Miller (1949) who reported that in California the leaf lesions, which appeared in winter and early spring, continued to enlarge and develop through spring until early summer. These lesions, which appeared as well-defined dark spots in winter and spring, continued to produce abundant conidia as they expanded, often coalescing to cover large areas of the leaf surfaces. In early summer, infected leaves showed dark lesions with faint haloes around the spots, but few conidia were produced from them. During hot, dry summers the lesions stopped expanding and sometimes became dry, hardened, cracked or blistered, with the cuticle breaking away in the centres of the lesions. These lesions did not reactivate in autumn (Miller, 1949). Although lesion survival, growth and sporulation are critical components of OLS development, the effects of environmental factors on lesion development and S. oleagina sporulation have not been investigated. This chapter describes studies into the effect of temperature, leaf wetness and humidity on S. oleagina conidium production and on the development of OLS lesions.

5.3 MATERIALS AND METHODS

Experimental plants
The experiments were conducted at Lincoln University, Canterbury, New Zealand on 1-2 year old olive plants (cv. Barnea), which had been grown in a greenhouse, as previously described.

Humidity tents
Two humidity tents were constructed inside a growth chamber (Conviron PGV36; Controlled Environments Limited, Winnipeg, Manitoba, Canada), as described in Chapter 4.

Plant inoculation
Naturally infected olive leaves (cv. ‘Barnea’) grown in a commercial grove in Canterbury, New Zealand were picked from the trees when needed to provide conidia for inoculation.
Conidial suspensions were obtained from the leaves as previously described (Chapter 4) and the concentration adjusted to $5.0 \times 10^4$ conidia/mL. Conidium viability of all conidial suspensions was determined according to the methods of Saad and Masri (1978), and found to range from 47 to 58%.

Plants were inoculated during autumn and winter in 2004 on olive plants (cv. ‘Bamea’). The new, fully expanded leaves that grew sequentially on the tip of each plant were inoculated on two different dates (4 weeks apart) producing leaves with lesions of two different ages on the same plant. At each inoculation time, three droplets (10 μL) of the conidial suspension ($5.0 \times 10^4$ conidia/mL) were placed separately onto each of the upper leaves and the plants incubated in the dark under high RH (100%) at 20°C for 48 h. Subsequently the plants were transferred to a shadehouse for disease development. The plants were watered in the shadehouse by an automatic overhead sprinkler system that turned on for approximately 10 min per day. The mean daily temperature in the shadehouse ranged from 5 to 20°C. The plants were monitored fortnightly until the lesions on the inoculated leaves were sporulating. These plants were then used for all the experiments.

5.3.1 Temperature and moisture effects on sporulation and lesion expansion of *Spiilocaea oleagina*

The complete experiment was conducted twice between autumn and early summer of 2004. The same growth chamber was used consecutively being reset at the five different incubation temperatures (5, 10, 15, 20, and 25°C) selected at random. For each temperature and moisture treatments, 12 plants with actively sporulating lesions of two ages (12 and 16 weeks old) were used to study the effect of temperature, wetness and humidity (70% and 100% RH) on *S. oleagina* conidium production. At the start of the experiment, all the leaves with sporulating lesions were gently wiped with damped cotton to remove any available conidia on the lesions and the lesion diameters measured using a digital calliper (Mitutoyo Digimatic Caliper, Japan). All plants were initially incubated in the growth chamber set at the designated temperatures, but under continuous wetness for 6 h to initiate sporulation. They were then removed from the chamber and dried by placing the inoculated plants 90 cm from a fan set at slow speed at room temperature (20°C) and 50% RH. Subsequently, the plants were randomly assigned to the moisture regimes of continuous wetness (CW), high RH (100%) or low RH (70%). The plants were incubated under a 12-h photoperiod supplied by

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fluorescent and incandescent fixtures producing a light intensity of 200 μE·m⁻²·s⁻¹ for 3, 7, 10, or 14 days.

At the end of each incubation time, S. oleagina sporulation was quantified as the number of spores produced per lesion area and per plant. For each lesion age, six inoculated leaves were harvested from each of three randomly selected plants. After measuring the diameter of each lesion using a digital calliper, the leaves were placed in Universal tubes containing 3 mL of distilled water. The tubes were vortexed for 2 min and the number of spores in the suspension counted with a haemocytometer. The total number of conidia per lesion area per plant (average of three counts) was calculated as follows: \( S = \frac{\text{number of conidia/mL} \times \text{3 mL of suspension}}{\text{total lesion area (mm}^2\text{)} \text{per plant}} \).

5.3.2 Scanning electron microscopy
Scanning electron microscopy was used to study the morphological characteristics of OLS sporulating lesions on olive leaves. Leaves with sporulating lesions obtained from heavily infected olive plants in the shadehouse, and healthy leaves from plants in the greenhouse were used for this investigation. The leaves were cut into 0.5 × 0.5 cm pieces and were mounted onto a copper stub with double-sided sticky tape, such that upper and lower surfaces of the leaf pieces were faced upward. Mounted specimens were coated with gold in a sputter coater (Polaron 5000), and examined with a LEICA S440 scanning electron microscope operating at 5.0 kV.

5.3.3 Data analysis
All data were analysed using Genstat 7.2 (Lawes Agricultural Trust, Rothamsted Experimental Station). A split-plot analysis of variance (ANOVA) was used to determine the main and interaction effects of temperature and moisture regime on lesion diameters (mm). Residual maximum likelihood (REML) was performed to determine the effect of temperature and moisture regime on sporulation of S. oleagina over time. Temperature, moisture regime, time (day) and their interactions were considered as qualitative factors and fixed effects in the analysis of variance. Fixed effects were evaluated with \( F \) tests based on Wald statistics. Models for estimating sporulation (\( S \)) over time at different temperatures and moisture conditions were developed according to the methods described by Lalancette et al. (1988)
and Carisse and Peyrachon (1999). The models development involved several interrelated steps.

**Step 1**
First, an equation predicting the upper asymptote \( K \) of \( S \) as a function of temperature was derived by regressing \( K \) (the maximum \( S \) observed at each temperature, \( T \)) against the second-order polynomial of temperature:

\[
K = b_0 + b_1 T + b_2 T^2,
\]

where \( b_0, b_1, \) and \( b_2 \) are the regression coefficients.

**Step 2**
Second, the linearized version of the Richards function (Richards, 1959) was fitted to the data for each temperature to obtain estimates for \( r \) and \( B \):

\[
\ln[1 - (S/K)^{1-m}] = -rW + B,
\]

where \( W \) is the duration of moisture regime (3, 7, 10 or 14 days), \( K \) is the estimated asymptote from Equation 1, and \( m, r, \) and \( B \) are the shape, rate, and intercept parameters, respectively. Because the shape parameter has to be set, a range of values of \( m \) were tested to obtain the best possible fit. Values of \( m \) near zero produce a monomolecular-type function. A shape parameter near 1 results in a Gompertz function, whereas a value of 2 results in a logistic function (Richards, 1959).

**Step 3**
The intercept and rate parameter estimates \( (r \) and \( B) \) were then regressed against temperature by fitting a quadratic function similar to Equation 1:

\[
r = b_0 + b_1 T + b_2 T^2
\]

\[
B = b_0 + b_1 T + b_2 T^2
\]

**Step 4**
The final step, modelling \( S \) as a function of \( T \) and \( W \), was achieved by incorporating the
rate and intercept parameter functions (Equations 3 and 4) for $r$ and $B$, respectively, into the Richards function:

$$\ln[1 - (S/K)^{1-m}] = b_0 + b_1W + b_2WT + b_3WT^2 + b_4T + b_5T^2$$ (5)

Regressions were performed separately for each moisture regime using the pooled data of the 12-week old lesions from the two replicate experiments. Some $S$ values were greater than the value of the $K$ parameter predicted by Equation 1, and so a constant (1,000) was added to the predicted $K$ values to avoid having to find the logarithm of a negative number (an undefined quantity).

The selected models (equations predicting $K$, $r$, $B$ and $S$) were evaluated based on significance of the model coefficient, randomness and normality of residuals, coefficient of determination ($R^2$), and coefficient of determination adjusted for degrees of freedom ($R^2_{adj}$). All regression analyses were performed by Genstat 7.2 GLM.

5.4 RESULTS

5.4.1 Effect of temperature, leaf wetness and relative humidity on the sporulation of *Spilocaea oleagina*

*S. oleagina* sporulation

Temperature, type of moisture regime and incubation duration had significant ($P<0.001$) effects on *S. oleagina* conidium production. There was no difference ($P=0.638$) between experimental repeats on the number of conidia produced per lesion area and so data were combined. After 14 days of incubation, mean numbers of conidia (averaged over all moisture regimes) increased from about $4.0 \times 10^2$ conidia/mm$^2$ at 5°C reaching a maximum of $8.6 \times 10^2$ conidia/mm$^2$ at 15°C, with a reduction to $2.0 \times 10^2$ conidia/mm$^2$ at 25°C. Under the high RH treatment, the mean numbers of conidia/lesion area were significantly ($P<0.001$) higher than those from the continuous wetness and low RH treatments, at all temperatures except at 25°C, which gave the same level of conidium production for continuous moisture and 100% RH regimes. No conidium was produced by *S. oleagina* at 25°C when the RH was low (70%). Generally, the number of conidia produced by *S. oleagina* on the lesions increased with increasing incubation period at all temperatures tested. Sporulation was first observed
on the lesions after 3 days when incubated at 5, 10, 15 and 20°C, whereas at 25°C conidium production was first seen after 7 days of incubation (Appendix 5.1). By 14 days, yellow haloes had developed around some of the lesions on plants incubated at 25°C and 70% RH.

**Model parameters for estimating *S. oleagina* sporulation**

When the maximum sporulation, a measure of the upper asymptote $K$ of the Richards model, observed at each temperature was regressed against temperature using Equation 1 (Figure 35), the data from the continuous wetness treatment gave the lowest coefficient of determination ($R^2=0.871$) value (Table 10). The $F$-statistic for this value was only significant at the 0.1 level; however, with only two degrees of freedom, an $F$-test is not very powerful for determining the significance of the relationships calculated here (Neter and Wasserman, 1974). The models predicted a maximum sporulation of 168,651 and 1,711 conidia/mm$^2$ at 12.3, 13.8 and 14.0°C for 70% RH, continuous wetness (CW) and 100% RH, respectively.

**Table 10:** Estimation of the asymptote, rate, and intercept parameters of Richards model by fitting quadratic functions of temperature (T) using the equations $K = b_0 + b_1T + b_2T^2$, $r = b_0 + b_1T + b_2T^2$, and $B = b_0 + b_1T + b_2T^2$

<table>
<thead>
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<th>Model</th>
<th>df</th>
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<th>F</th>
<th>P-value</th>
<th>Estimate/P-value</th>
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</thead>
<tbody>
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<tr>
<td>Continuous wetness (CW)</td>
<td>2</td>
<td>7014</td>
<td>0.871</td>
<td>0.065</td>
<td>14.0</td>
<td>90.9</td>
</tr>
<tr>
<td>100% RH</td>
<td>2</td>
<td>2.75x10^4</td>
<td>0.955</td>
<td>0.023</td>
<td>-325</td>
<td>295.2</td>
</tr>
<tr>
<td>70% RH</td>
<td>2</td>
<td>238</td>
<td>0.973</td>
<td>0.014</td>
<td>11.0</td>
<td>25.6</td>
</tr>
<tr>
<td>Rate parameter, $r$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuous wetness (CW)</td>
<td>2</td>
<td>4.76x10^6</td>
<td>0.966</td>
<td>0.017</td>
<td>0.04826</td>
<td>0.00439 -1.61x10^4</td>
</tr>
<tr>
<td>100% RH</td>
<td>2</td>
<td>5.68x10^5</td>
<td>0.891</td>
<td>0.055</td>
<td>0.0729</td>
<td>0.00822 -3.03x10^4</td>
</tr>
<tr>
<td>70% RH</td>
<td>2</td>
<td>1.22x10^5</td>
<td>0.984</td>
<td>0.008</td>
<td>0.00412</td>
<td>0.00784 -3.16x10^4</td>
</tr>
<tr>
<td>Intercept parameter, $B$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuous wetness (CW)</td>
<td>2</td>
<td>0.00123</td>
<td>0.941</td>
<td>0.029</td>
<td>0.5616</td>
<td>-0.06592 0.00213</td>
</tr>
<tr>
<td>100% RH</td>
<td>2</td>
<td>0.00396</td>
<td>0.924</td>
<td>0.038</td>
<td>-0.0864</td>
<td>-0.0633 0.00255</td>
</tr>
<tr>
<td>70% RH</td>
<td>2</td>
<td>8.86x10^5</td>
<td>0.990</td>
<td>0.005</td>
<td>0.0251</td>
<td>0.0267 0.00107</td>
</tr>
</tbody>
</table>
Figure 35: Relationship between temperature and A, the asymptote ($K$), B, the rate ($r$) and C, the intercept ($B$); all are parameters of the Richards function. The lines represent the predicted values of equations $K = b_0 + b_1 T + b_2 T^2$, $r = b_0 + b_1 T + b_2 T^2$, and $B = b_0 + b_1 T + b_2 T^2$, respectively. Symbols represent the observed values (A) or those generated from the equation $\ln[1 - (S/K)^{1-m}] = -rW + B$ (B and C) for each of the moisture regimes, continuous wetness (CW), 70% RH, and 100% RH.
The fitting of the Richards model (Equation 2) to the data for each temperature and moisture regime to provide the estimates for \(r\) and \(B\) resulted in \(R^2\) values ranging from 0.829 to 0.971. Generally, the models had \(P\)-values of less than 0.10. Several values of the shape parameter \((m)\) were tested for their effect on the regression results but a value of \(m = 0.5\) was chosen because it gave the best overall fit across the different temperature and moisture regimes. The resulting curves were intermediate in shape between the monomolecular and Gompertz models.

In the third step of the model development, the rates and intercepts derived from fitting Equation 2 were regressed against temperature (Figure 35). The quadratic functions (Equations 3 and 4) gave very good fits for the rate and intercept parameters, respectively, with \(R^2\) values ranging from 0.891 to 0.990 and \(P\)-values from 0.005 to 0.055 (Table 10). The rate parameter \(r\) was predicted to reach an optimum of 0.053, 0.078 and 0.128 at 12.4, 13.6, and 13.6°C for 70% RH, CW and 100% RH, respectively. The intercept \((B)\) of the quadratic function (Equation 4) for both CW and 100% RH data predicted minimum values of 0.052 and 0.192 at 15.5 and 12.4°C, respectively, whereas a maximum intercept value of \(-0.479\) at 12.4°C was obtained for the 70% RH.

The final model fitted to the pooled data for each moisture regime accounted for 86.8 to 92.6% of the variation in \(S. oleagina\) sporulation and all \(F\)-statistics were significant at \(P<0.001\) (Table 11). Based on the results of the linear regression for fitting the sporulation model (Table 11), the nonlinear relationship between \(S, W,\) and \(T\) was:

\[
S = K(1 - e^{-rW + B})^{1/(1-m)} \quad (m = 0.5)
\]

where \(K = 14.0 + 90.9T - 3.24T^2 + 1000\) \((CW)\)
\(= -325 + 295T - 10.7T^2 + 1000\) \((100\% \text{ RH})\)
\(= 11.0 + 25.6T - 1.04T^2 + 1000\) \((70\% \text{RH})\)

\(B = 0.526 - 0.0664T + 0.00226T^2\) \((CW)\)
\(= 0.170 - 0.0489T + 0.00195T^2\) \((100\% \text{ RH})\)
\(= 0.108 + 0.00956T + 0.000550T^2\) \((70\% \text{RH})\)

\(-rW = -0.0483W - 0.00438WT + 0.000161WT^2\) \((CW)\)
\(= -0.0422W - 0.01036WT + 0.000388WT^2\) \((100\% \text{ RH})\)
\(= -0.0153W - 0.00545WT + 0.000239WT^2\) \((70\% \text{RH})\)
Table 11: Model parameter estimates for predicting *Spilocaea oleagina* sporulation as a function of temperature ($T$) and duration of moisture regime ($W$) by fitting the Richards function ($\ln[1 - (S/K)^{0.5}] = b_0 + b_1 W + b_2 WT + b_3 WT^2 + b_4 T + b_5 T^2$) to the pooled data for each moisture regime

<table>
<thead>
<tr>
<th>Model</th>
<th>df</th>
<th>F</th>
<th>$R^2$</th>
<th>Estimate/P-value</th>
<th>Error</th>
<th>$b_0$</th>
<th>$b_1$</th>
<th>$b_2$</th>
<th>$b_3$</th>
<th>$b_4$</th>
<th>$b_5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CW$^b$</td>
<td>114</td>
<td>1.17</td>
<td>0.911</td>
<td>&lt;0.001</td>
<td>0.526</td>
<td>-0.0483</td>
<td>-0.00438</td>
<td>1.61x10^-4</td>
<td>-0.0664</td>
<td>0.00226</td>
<td></td>
</tr>
<tr>
<td>HRH</td>
<td>114</td>
<td>1.89</td>
<td>0.926</td>
<td>&lt;0.001</td>
<td>0.170</td>
<td>-0.0422</td>
<td>-0.01036</td>
<td>3.88x10^-4</td>
<td>-0.0489</td>
<td>0.00195</td>
<td></td>
</tr>
<tr>
<td>LRH</td>
<td>114</td>
<td>0.4615</td>
<td>0.868</td>
<td>&lt;0.001</td>
<td>0.108</td>
<td>-0.0153</td>
<td>-0.00545</td>
<td>2.39x10^-4</td>
<td>0.00956</td>
<td>5.50x10^-4</td>
<td></td>
</tr>
</tbody>
</table>

$^{a}$Coefficient of determination adjusted for the number of independent variables.

$^{b}$CW (continuous wetness), HRH (100% RH) and LRH (70% RH).
Figure 36: Numbers of *Spilocaea oleagina* conidia, actual (symbols) and the predicted values of the model (lines) fitted to the pooled data for continuous wetness (CW), 70% RH and 100% RH using the equation \( \ln[1 - (S/K)^{0.5}] = b_0 + b_1 W + b_2 WT + b_3 WT^2 + b_4 T + b_5 T^2 \). The mean numbers of conidia/lesion area were determined on 18 leaves (three plants/replicate and six leaves/plant) averaged over the two experimental repeats. Error bars represent the standard errors of the means.
The model was used to predict sporulation at the different temperatures and moisture regimes. Overall the model fitted the data well, except that at 15°C sporulation was underestimated when the relative humidity was 100% (Figure 36).

Lesion age significantly \( P=0.032 \) affected the number of conidia produced by \textit{S. oleagina}, with older lesions (16 weeks old) producing fewer conidia than young lesions (12 weeks old) (Figure 37). For example, at the optimum conditions of 15°C and 100% RH, the mean numbers of conidia/lesion area recorded for 12 week old lesion was \( 3.5 \times 10^3 \) conidia/mm\(^2\) of lesion area, whereas the mean numbers of conidia produced by 16 week old lesions was \( 1.8 \times 10^3 \) conidia/mm\(^2\) after a further 14 days of incubation.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure37}
\caption{Effect of lesion age on sporulation by \textit{S. oleagina} at different temperatures and wetness regimes, continuous wetness (CW), HRH (100% RH), and LRH (70% RH) after 14 days of incubation (LSD = 285).}
\end{figure}

\textbf{Lesion size}

Temperature significantly \( P<0.001 \) affected OLS lesion expansion and experimental repeats were not significantly \( P<0.362 \) different. After 14 days of incubation, the greatest increase in mean lesion diameter (1.57 mm) occurred at 15°C and the least increase in mean diameter (0.47 mm) occurred at 25°C. The moisture regimes also affected lesion expansion with the mean increases in lesion diameters at all temperatures tested being significantly \( P=0.009 \) higher for plants given continuous wetness than for plants incubated under low RH (70%).
irrespective of the lesion age (Table 12). Overall, there was no difference ($P=0.112$) between continuous wetness and high RH (100%) for mean increases in lesion diameter of the 16 week old lesions but for the 12 week old lesions increases were larger under continuous wetness treatment than with the high RH (100%) ($P=0.027$).

**Table 12:** Effects of temperature, continuous wet periods (CW), high relative humidity (HRH; 100%) and low relative humidity (LRH; 70%) on the increase over the 14-day incubation period in the size of olive leaf spot lesions aged 12 and 16 weeks

<table>
<thead>
<tr>
<th>Temp. ($^\circ$C)</th>
<th>Lesion age 1 (16 wks old)</th>
<th>Lesion age 2 (12 wk old)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CW</td>
<td>HRH</td>
</tr>
<tr>
<td>5</td>
<td>1.10(0.12)$^b$</td>
<td>1.09(0.12)</td>
</tr>
<tr>
<td>10</td>
<td>1.56(0.18)</td>
<td>1.31(0.17)</td>
</tr>
<tr>
<td>15</td>
<td>1.68(0.20)</td>
<td>1.36(0.11)</td>
</tr>
<tr>
<td>20</td>
<td>1.75(0.35)</td>
<td>1.19(0.21)</td>
</tr>
<tr>
<td>25</td>
<td>0.55(0.15)</td>
<td>0.33(0.17)</td>
</tr>
</tbody>
</table>

$^a$Mean lesion diameter (mm) measured on 6 plants, for 3 lesions on each of 6 leaves/plant ($n=36$).

$^b$Standard error of the means

### 5.4.2 Scanning electron microscopic examination of OLS lesions

Scanning electron microscopy was conducted on young sporulating OLS lesions 8 weeks after inoculation when the symptoms of the disease became apparent. On the lesion surfaces, numerous short bulbous, one-celled conidiophores were seen to arise from the hyphae, and emerge through the cuticle to the surface of the leaves. Initially, the conidiophores were roughly spherical (Figure 38A), and then they became oblong (Figure 38B). Conidia usually formed at the apex of the conidiophores, but in some cases, a second conidium formed from the side of conidiophore (Figure 38B). The mature conidia were typically elongate-ovoid to pyriform, with truncate bases (Figure 38C). The tips of the short globose conidiophores were marked by apical conidiation scars (Figure 38B) showing where series of conidia had been produced with the annellide increasing incrementally in length with each successive conidium.
**Figure 38:** Scanning-electron micrograph of *Spilocaea oleagina* sporulation on olive leaves. Newly emerging conidiophores on the adaxial surface of the leaf (A), conidia and globose conidiophore on the adaxial surface (B), conidium partially detached from the globose conidiophore (C), adaxial surface of uninfected olive leaf (D), sporulation between the trichomes of the leaf adaxial surface (E), overlapping trichomes on abaxial leaf surface (F), sparse trichomes on and around midrib of the abaxial leaf surface (G), sporulation on the midrib of the abaxial leaf surface (H). Abbreviations: YCo, young conidiophore; IC, immature conidium; CS, conidiation scars; Co, conidiophore; MC, matured conidium; TR, trichomes; ITR, inter-trichomes spaces; SP, sporulation; LTR, layers of trichomes; M, midrib area; SPM, sporulation on midrib.
The relationship between trichomes (Figure 38D) and disease development was evident from the study of the adaxial and abaxial surfaces of both infected and uninfected olive leaves. Lesions, which occur only on the adaxial surfaces of the leaves, sporulated from those surfaces and not from the abaxial surfaces, except for the midrib area of the abaxial surface (Figure 38E). In no case was sporulation observed on the surface of the trichomes (Figure 38E), although many conidiophores and conidia emerged in the spaces between the trichomes (Figure 38E). On the abaxial surface, there appeared to be several layers of overlapping trichomes with no spaces between them and no sporulation (Figure 38F), while there were some spaces along the midrib (Figure 38G) where conidiophores and conidia arose (Figure 38H).

5.5 DISCUSSION

This is the first report on the effects of temperature and moisture conditions on the sporulation dynamics of OLS lesions on olive leaves. The results of this study clearly showed that temperature, wetness and RH are important environmental factors which influence sporulation of *S. oleagina* on infected olive leaves. Sporulation was favoured by high RH or continuous leaf wetness and cooler temperatures (10-20°C) with a maximum at 15°C. High RH was more favourable than continuous wetness for supporting abundant conidium production. There was also increasing conidium production over time except at 25°C and low RH when conidium production remained at zero throughout. Although relative conidium production at different temperatures and relative humidities has not been previously quantified under controlled conditions, field observations of the sporulation of *S. oleagina* have been reported (Laviola and Scarito 1993; Guechi and Girre 1994; Chapter 7). The results of this study are consistent with the observations described in Chapter 7 that abundant conidia were produced during spring and autumn but conidium production was low during the summer months. Considering the importance of inoculum levels in *S. oleagina* infection and OLS development (Chapter 4), this provides another explanation for low disease severity in hot, dry climates.

From the results of this study, the fundamental environmental requirements for sporulation of *S. oleagina* have been established, but further research is needed to investigate the effect of other factors such as light, wind, cultivar, fluctuating temperature, and interrupted wet period.
on *S. oleagina* conidium production. These factors have been demonstrated to influence spore production in other fungi (Su et al., 2004; Chellemi and Marois, 1991). For example, Chellemi and Marois (1991) found that sporulation of *Uncinula necator* on grape leaves were greater on cv. Carignane than Chardonnay at all temperatures except 19°C.

The use of polynomial theoretical models to describe the effect of temperature and wetness duration on sporulation of other pathogenic fungi has been reported for species such as *Cercospora carotae* (Pass.) Solh. and *Botrytis cinerea* Pers. (Carisse and Kushalappa, 1990; Sosa-Alvarez et al., 1995). The application of polynomial models has the advantage of using simple mathematical calculations that are generally easy to adjust (Steel and Torrie, 1980). However, the Richard’s nonlinear function chosen to describe the data on *S. oleagina* sporulation in this study was considered to have some significant advantages. The parameters estimated for the Richards' model have biological meaning; the asymptote represents the maximum sporulation at a given temperature and r, the rate of sporulation. The Richard’s model also includes a parameter to account for density-dependent regulation and the inclusion of a third parameter (m) allowed the description of a variety of different data sets. The quantitative benefits from use of the Richards function to describe sporulation have been demonstrated for other pathogenic fungi, such as *Plasmopara viticola* on grape leaves (Lalancette et al., 1988) and *Mycosphaerella fragariae* on strawberry leaves (Carisse and Peyrachon, 1999). Overall, the model explained a large proportion of the variation in the sporulation capacity of lesions of *S. oleagina* and can therefore be used for predictive purposes within the temperature range and at various moisture conditions tested.

Olive leaves are susceptible to infection by *S. oleagina* only during their early development (Chapter 4) and, so it is the expansion phase of lesions on mature leaves that is the most damaging. The continuing, prolonged expansion of lesions also provides for large areas of conidium production which may be reactivated quickly under conducive conditions. In this study, temperature and moisture conditions affected OLS lesion expansion, with 15°C and continuous wetness providing for maximum expansion of relatively young lesions after 14 days of incubation. Under 100% RH and continuous wetness for 14 days, mean lesion expansion was 0.65-1.65 mm at 10-20°C and 0.55-0.83 mm at 5°C. However, at 25°C mean lesion expansion after 14 days was only 0.17-0.57 mm and under 70% RH some of the lesions had developed chlorotic haloes by then. The results agree with previous field observations, which stated that disease development was most active during winter and
spring and that during hot, dry summers the lesions stopped expanding (Miller 1949). Although 14 days incubation under 70% at 25°C caused yellow haloes, it may have been too short for lesions to become dry, hardened, cracked or blistered as has been described to occur under hot summer conditions (Miller 1949; Graniti 1993).

Ultrastructural studies showed that on the abaxial leaf surface there were layers of overlapping trichomes with no spaces between them and where no sporulation occurred, except in the spaces between the trichomes on the midrib. However, the adaxial surface had fewer trichomes with abundant sporulation in the spaces between them. As shown in Chapter 3, the trichomes act as a physical barrier to infection and so no mycelium to support sporulation. Clearly, presence of trichomes may contribute to OLS resistance in olive varieties. There is wide variation in the level of susceptibility of olives to OLS (Graniti, 1993; Sutter, 1994); cultivars may be highly susceptible, moderately or highly resistant. The structural differences of the olive leaves could contribute, in part, to the observed levels of susceptibility to OLS. Cultivar variations in disease susceptibility may be associated with similar structural features as in other pathosystems. For example, it was found that trichomes densities were associated with pecan scab resistance (Wetzstein and Sparks, 1983). Further research should be conducted on the relationship between levels of trichomes on olive leaves and susceptibility to OLS for a range of leaf ages and olive cultivars. This information could be useful in breeding for resistance against OLS.

In conclusion, a model for predicting S. oleagina sporulation was developed in this study. The model can be used to predict conditions when sporulation will not occur or identifying periods when risk is high, and its combination with a predictive model developed for infection (Chapter 4) will contribute to the development of an effective disease forecasting system. However, the model needs to be further validated in the field.

5.6 ACKNOWLEDGEMENTS

The author would like to thank Mr Neil Andrews for technical assistance with regards to the scanning electron microscopy. Thanks also to Brent Richards of Lincoln University Nursery for maintaining the olive plants.
5.7 REFERENCES


CHAPTER 6

The genetic structure of *Spilocaea oleagina* populations from
New Zealand olive groves

6.1 ABSTRACT

Sequence analysis of rDNA (ITS1-5.8S-ITS2), restriction fragment length polymorphism (RFLP), and universally primed-polymerase chain reaction (UP-PCR) techniques were used to study the genetic structure of *Spilocaea oleagina* populations. Of the 100 *Spilocaea oleagina* isolates analysed, 98 were collected from twelve known and four unknown cultivars from olive groves in five New Zealand regions. The remaining two isolates originated in Australia and Italy. Alignment of the cloned sequences of four New Zealand isolates with the isolates from Australia and Italy showed great similarity, with similarity indices for ITS1, ITS2 and 5.8S coding regions of the isolates being 96-100, 99.8-100 and 99-100%, respectively. RFLP analysis revealed no differences among the New Zealand isolates. UP-PCR profiles based on 159 markers were used to compute genetic distances between pairs of individuals. Low levels of gene and genotypic diversity were detected in all populations, with 76% of the loci being polymorphic, and with normalized Shannon's diversity indices ranging from 0.0342 to 0.2095. Analysis of molecular variance (AMOVA) showed small but significant ($P = 0.001$) variations among regions, although most of the molecular variability (87%) was found within populations. Clustered analysis showed no evidence of grouping according to geographic origin of the isolates. The low level of genetic diversity found within and among populations agrees with current understanding of this fungus that its reproduction is mainly or entirely by asexual mechanisms. This indicates that any effective control strategies are likely to be useful in all or most New Zealand olive groves.

6.2 INTRODUCTION

Olive leaf spot (OLS) caused by *S. oleagina* has been known in the Mediterranean countries of Italy, Spain, Israel and Greece for over a century. As the olive trees currently grown in New Zealand originated from introduced stock, it is likely that they were the source of this pathogen. Once trees are infected, local spread of the fungus is by conidia being rain-
splashed (Wilson and Miller, 1949; Tenerini, 1964; Laviola, 1968) or carried by insects and wind (De Marzo et al., 1993; Lops et al., 1993; Frisullo et al., 1994). The main source of *S. oleagina* inoculum for primary infection is the sporulating lesions on the leaves that remain on the trees through winter (Guechi and Girre, 1994). It was suggested by Bernès (1923) that fallen diseased leaves also contributed significantly to new infections of OLS, and thus his recommendations for control of leaf spot in France involve the collection and burning of fallen leaves.

The relative contribution of asexual and sexual reproduction to pathogen populations can influence the genetic diversity of a pathogen population. For instance, if asexually derived spores are the main inoculum source, less genotypic diversity is to be expected than if sexual spores are the main source of inoculum. This was demonstrated in a USA study using isozyme markers to compare asexual and sexual reproduction in *Puccinia graminis* populations (Burdon and Roelfs, 1985). The authors reported very high genetic diversity in the sexual populations, whereas there was a restricted array of isozyme genotypes within the asexual population. The existence of a sexual stage of *S. oleagina* has not been reported, and the role of sexual reproduction in the infection process of this pathogen remains unknown.

Pathogen populations constantly adapt to changes in their environment in ways that may improve their ability to cause disease and survive. In agricultural ecosystems, selection pressures that may affect success of pathogens include environmental changes, resistant plant varieties, applications of fungicides and fertilisers, irrigation and crop rotation. Genotypic diversity resulting from recombination during sexual reproduction would allow sexual populations to be selected more rapidly under selection pressures imposed by fungicides or resistant cultivars than would asexual populations (McDonald, 1997). MacHardy (1996) reported that for the closely related apple scab pathogen, *Venturia inaequalis* which reproduces sexually, the repeated use of fungicides resulted in the emergence of new genotypic isolates that were fungicide-resistant. A genetically diverse pathogen population may develop resistance to a particular control measure more quickly than a homogeneous population making it necessary to adopt a more integrated pest management system. The ability to predict how *S. oleagina* will respond to various control strategies, will be enhanced by improved understanding of the genetic variability and epidemiology of the pathogen. An important first step is to study the genetic structure of isolates of *S. oleagina*. 

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Although \textit{S. oleagina} was first described by Boyer in 1891 (Miller, 1949), very little is known about the genetics of the pathogen itself, probably due to its lack of sporulation in culture. Recently, the DNA sequences of the 18S, 5.8S and 28S ribosomal DNA genes (rDNA), and the internal transcribed spacers (ITS) region of this fungus have been determined (González-Lamothe et al., 2002). The non-coding ITS regions (ITS1 and ITS2) evolve faster than the coding regions, and thus may vary among species and populations (McDonald, 1997). Molecular techniques such as random amplified polymorphic DNA (RAPD) markers and polymerase chain reaction-restriction length polymorphism (PCR-RFLP) analysis of rDNA/ITS regions are now routinely employed for examining the genetic diversity and causes of variations in virulence among plant pathogens. Tenzer and Gessler (1997) found high genetic diversity within \textit{V. inaequalis} populations but low differentiation among different populations when analysing ITS regions using RAPD markers and PCR-RFLP.

The universally primed PCR (UP-PCR) technique is similar to the traditional RAPD technique (Williams et al., 1990), but the method employs longer primers (15-20 nucleotides) designed for the fingerprinting of any organism at higher annealing temperatures (Bulat et al., 1998). As the UP-PCR primers selected for fungi primarily target intergenic, more variable areas of the genome, this method is especially suitable for detecting intraspecific variation (Bulat et al., 1998). The main advantage of UP-PCR compared with RAPD is that the higher annealing temperatures (52-56°C) result in a high degree of reproducibility and the generation of more complex banding patterns, which improves the likelihood of identifying isolate-specific and pathotype-specific bands (Bulat et al., 1998; Lübeck et al., 1999). The UP-PCR technique has been successfully used in studying the genetic diversity of other fungi, including \textit{Trichoderma} spp. (Cumagun et al., 2000) and the grapevine pathogen \textit{Phaeomoniella chlamydospora} (Pottinger et al., 2002).

In this study, DNA extracted from New Zealand \textit{S. oleagina} isolates was characterised and compared with two overseas isolates using UP-PCR and rDNA-ITS restriction analysis, an initial step towards developing an understanding of genetic diversity of \textit{S. oleagina} within New Zealand.
6.3 MATERIALS AND METHODS

Production of single spore cultures and DNA extraction

Ninety-eight isolates of *S. oleagina* representing 19 populations (Table 13) were collected from 12 known and four unknown olive cultivars in groves under organic or conventional olive production systems in five locations in New Zealand (Figure 39). A population was defined as a group of *S. oleagina* isolates obtained from a single olive grove and separated from other groves by at least 5 km. Leaves with sporulating lesions were collected from trees chosen at random, but at least 10 m apart, and only one lesion per tree was used to reduce the likelihood of clones. The leaves were dried for 3 days on the bench at room temperature and then stored at 4°C until required for producing the single spore isolates, which were grown on chlortetracycline amended olive leaf extract agar at 15°C for 3 to 6 months. Dried infected olive leaf samples were also obtained from Australia and Italy (Table 13). However, *S. oleagina* could not be cultured from this material, as the conidia were found nonviable.

![Figure 39: Origin of the 19 populations of *Spilocaea oleagina* isolates collected from New Zealand regions.](image-url)
Table 13: Sources and codes of the *Spilocaea oleagina* isolates used in this study

<table>
<thead>
<tr>
<th>Country</th>
<th>Location</th>
<th>Cultivar</th>
<th>No. of Isolates</th>
<th>Source code&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Zealand</td>
<td>Auckland</td>
<td>Barnea</td>
<td>4</td>
<td>AB1, AB2, AB3, AB4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>J5</td>
<td>1</td>
<td>AJ1</td>
</tr>
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<td></td>
<td>Koroneiki</td>
<td>3</td>
<td>AK1, AK2, AK3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leccino</td>
<td>2</td>
<td>AL1, AL2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Picual</td>
<td>5</td>
<td>AP1, AP2, AP3, AP4, AP5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SA Verdele</td>
<td>2</td>
<td>AS1, AS2</td>
</tr>
<tr>
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<td>Southern&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Carolea</td>
<td>1</td>
<td>ISC3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Isolate code includes region of origin, olive cultivar and order of isolation

<sup>b</sup>Infected leaf samples were provided by Dr K.J. Evans, University of Tasmania, Australia

<sup>c</sup>Infected leaf samples were provided by Prof. Salvatore Frisullo, Università di Foggia, Italy

Approximately 0.25 g of mycelium was scrapped from agar culture of each single spore New Zealand isolate and placed in PowerBead tubes (MO BIO Laboratories, Inc, Solana beach, CA, USA). For each of the Australian and Italian isolates, conidia and conidiophores were scraped from a single lesion and placed in PowerBead tubes. Total genomic DNA was
extracted using a PowerSoil DNA kit (MO BIO Laboratories, Inc, Solana beach, CA, USA), according to the manufacturer’s instructions. Total genomic DNA was also extracted from the mycelium of one isolate each of *Cladosporium fulvum* and *V. inaequalis* for comparison.

### 6.3.1 PCR amplification and ITS restriction analysis

PCR amplification of the ITS rDNA region (Figure 40) was performed in a final volume of 25 µL containing 10 mM Tris HCl (pH 8.0), 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM each of dATP, dCTP, dGTP and dTTP (Advanced Biotechnologies Ltd), 5 pmoles of primer ITS1 and ITS4 (Table 14), 1.25 units of Hotmaster Taq DNA polymerase (Eppendorf, Hamburg, Germany), and 10 to 100 ng of total DNA. The amplification conditions using an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) were as follows: DNA denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, primer annealing at 55°C for 30 s, and primer extension at 68°C for 30 s, with a final extension at 68°C for 7 min. The PCR products (4 µL) were separated on 1% agarose gels (Sigma Chemical, St. Louis) in Tris-acetate-EDTA (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), stained with ethidium bromide, visualized using VersalDoc Imaging Systems (Bio-Rad Laboratories, Hercules, CA), and inspected for presence of more than one DNA band. For ITS restriction analysis, the PCR product (10 µL) from each sample was restriction digested with three different enzymes, *Hinfl*, *Taq1* and *Msp1* (Promega, Madison, WI) according to the manufacturer’s instructions. The DNA fragments were separated on 10% polyacrylamide gels in Tris-borate-EDTA (45 mM Tris-borate, 1 mM EDTA, pH 8.0) (TBE) and stained with ethidium bromide as above.

![Figure 40](image-url)

**Figure 40**: Schematic diagram of the nuclear ribosomal DNA gene cluster consisting of the small subunit (18S), 5.8S, the large subunit (28S), and the two internal transcribed spacers (ITS1 and ITS2) based on White et al. (1990). Primers ITS1 and ITS4 were used to amplify ITS1-5.8S-ITS2 region.
Table 14: Nucleotide sequences of primers used in this study

<table>
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<tr>
<th>Primer</th>
<th>Nucleotide sequence 5’→3’</th>
<th>Annealing temp. (°C)</th>
<th>Reference</th>
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<td>ITS4</td>
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<tr>
<td>ITSR</td>
<td>AGGTTTCGGGCGGCGCGG</td>
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</tr>
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<td>50</td>
<td>Lübeck et al., 1999</td>
</tr>
<tr>
<td>AS4</td>
<td>TGTGGGCGCTCGACAC</td>
<td>55</td>
<td>Lübeck et al., 1999</td>
</tr>
<tr>
<td>AS15Inv</td>
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</tr>
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<td>L15</td>
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<td>L15/AS19</td>
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<td>52</td>
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### 6.3.2 Cloning and sequencing

The specific primers ITSF and ITSR (Table 14) were selected from a unique region in the *S. oleagina* rDNA deduced from an alignment of rDNA sequences of the closely related fungi, *Cladosporium caryigenum*, *Cladosporium nigrellum*, *V. inaequalis*, and *Venturia pirina* (Figure 41). Ten nanograms of genomic DNA from each of four New Zealand (AB1, BL1, CB7, and CM1), one Australian (ATP1) and one Italian (ISC3) *S. oleagina* isolates as well as *C. fulvum* and *V. inaequalis* isolates were amplified using primers ITSF and ITSR (Table 14) in 50 μL final reaction volumes. The PCR reaction mixture and conditions were the same as previously described except that an annealing temperature of 65°C was used. These PCR products were separated by electrophoresis on 0.7% agarose gels, visualized by staining with ethidium bromide and the ca 475 bp bands were excised from the gels and purified using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI). The purified DNA samples were ligated into pGEM-T Easy vector (Promega, Madison, WI) according to the manufacturer’s instructions and transformed into *Escherichia coli* strain INVaf’ (Invitrogen Corp., Carlsbad, CA). The transformants were selected using the standard blue-white technique (Sambrook et al., 1989). Plasmid DNA containing the above cloned ITS regions was extracted using Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI) according to the manufacturer’s recommendations. Purified plasmid DNA samples were sequenced with an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA) and the reactions performed with a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) using 100 ng of the plasmid DNA and
5 pmoles of primer SP6 (5'-ATTTAGGTGACACTATAG-3') and T7 (5'-TAATACGACTCTATAGGG-3'). Cycle sequencing was performed as described by Kuhls et al. (1997).

Figure 41: Alignment of rDNA (ITS1-5.8S-ITS2) of Spirocea oleagina, Cladosprium caryigenum (AF065850), Cladosporium nigrellum (AF393719.2) Venturia pirina (AF333438), and Venturia inaequalis (AF531078.1).

6.3.3 UP-PCR amplification

Eleven primers were screened for use in the UP-PCR analysis against six S. oleagina isolates selected at random. Based on banding patterns and intensities, five of the primers were selected for use in this study (Table 14). UP-PCR reactions were performed according to Lübeck et al. (1999) but with modifications. Each reaction volume was 25 μL with the following added to give the final concentration indicated: 2.5 mM MgCl₂, 0.2 mM each of dGTP, dCTP, dATP, and dTTP, 10 mM Tris HCl (pH 8.0), 50 mM KCl, 0.2 mM dNTPs, 5 pmoles of primer (Table 14), 1.25 units of Taq DNA polymerase (Eppendorf, Hamburg, Germany) and 10 to 50 ng of the designated DNA. Amplification was performed using a Mastercycler Gradient apparatus (Eppendorf, Hamburg, Germany). The amplification conditions were as follows: denaturation at 94°C for 2 min, 94°C for 50 s, annealing at the specified temperature (Table 14) for 2 min and primer extension at 72°C for 1 min (4 cycles), then followed by 94°C for 50 s, annealing at the specified temperature for 90 s, and primer extension at 72°C for 1 min (34 cycles), with a final extension at 72°C for 10 min. UP-PCR amplification products were separated on 5% polyacrylamide gels in TBE. The gels were
stained with ethidium bromide and visualized using VersalDoc Imaging Systems (Bio-Rad Laboratories, Hercules, CA).

UP-PCR analysis
Amplification profiles of the 98 S. oleagina isolates that were generated by the five UP-PCR primers were compared with each other, and bands of the DNA fragments were scored manually as present (1) or absent (0). Only the intense and reproducible bands were scored. The resulting data set was entered into a binary matrix and analysed using distance-based methods. PAUP software (PAUP 4.0b3a; Sinauer Associates) was used to construct a dendrogram based on the Jaccard similarity coefficient and the neighbour-joining method (Tegli et al., 2000). Bootstrap analysis was based on 1000 permutations. The Jaccard similarity coefficient was calculated as

$$S_j = \frac{x}{x+y},$$

where $x$ represented the number of 1-1 matches, $y$ the number of 0-0 matches and $n$ the total number of bands compared.

The average gene diversity ($H$; Nei, 1973) was calculated for each UP-PCR cluster, where

$$H = \frac{\sum (1 - \Sigma x_k^2)}{h},$$

$x$ is the allele frequency of the $k$th UP-PCR allele, and $h$ is the number of UP-PCR loci. In addition, genotypic diversity was quantified by a normalized Shannon’s diversity index ($I$) (Sheldon, 1969):

$$I = -\sum P_i \ln P_i / \ln N,$$

where $P_i$ is the frequency of the $i$-th haplotype and $N$ is the number of isolates in each population. Values of $I$ range from 0 (individuals in the sample having a similar genotype) to 1 (each individual in the sample having a different genotype). The presence or absence of a specific band was interpreted as a positive or null allele, respectively. Nei’s measure of genetic differentiation, $G_{ST}$ (Nei, 1973), was also calculated for each region (Auckland, Blenheim, Christchurch, Kapiti, and Masterton), where

$$G_{ST} = (H_T-H_S)/H_T.$$  

The gene diversity (expected heterozygosity; $H_T$) over all loci was calculated as

$$H_T = \frac{\sum (1 - \Sigma x_k^2)}{h},$$

where $x$ is the allele frequency of the $k$th UP-PCR allele in the total population, and $h$ is the number of loci. The subpopulation gene diversity ($H_S$) over all loci was calculated as

$$H_S = \frac{\sum (1 - \Sigma x_k^2)}{nh},$$

where $x$ is the allele frequency of the $k$th allele in the $i$th subpopulation, $n$ is the number of subgroups, and $h$ is the number of loci. A likelihood ratio chi-square test ($G^2$) was conducted with the null hypothesis of no difference in UP-PCR allele frequencies in the five subgroups. An estimate of gene flow ($Nm$) was calculated from $G_{ST}$ values, where

$$Nm = 0.5(1 - G_{ST})/G_{ST}$$

(McDermott and McDonald, 1993). All genetic diversity parameters were calculated using the software POPGENE version 1.32 (Yeh et al., 1999).
Divergence among all sampled populations was assessed by an analysis of molecular variance (AMOVA; Excoffier et al., 1992), based on an Euclidean distance matrix between all pairs of multilocus phenotypes. For this analysis, the total variance in the UP-PCR data set was partitioned into among-region, among-population and within-population components. Paired genetic distances among the 19 populations were estimated using $F_{ST}$ (Wright 1951). The statistical significance of the variance components of the AMOVA and the paired comparisons of $F_{ST}$, were determined by nonparametric procedures using 1023 random permutations. The gametic phase linkage disequilibrium within *S. oleagina* populations was also calculated. The probability test (Fisher's exact test) for each contingency table was performed using the Markov chain method (Raymond and Rousset, 1995), with the number of steps in the Markov chain and number of dememorization steps set at 10,000 and 1,000, respectively. An association between loci was considered significantly different from zero if the exact test gave a probability less than 0.05. AMOVA and linkage disequilibrium analyses were conducted with Arlequin software (Excoffier et al., 1997).

6.4 RESULTS

6.4.1 RFLP analysis

PCR-amplification of the ITS region of *S. oleagina* and *V. inaequalis* rDNA using the primers ITS 1 and ITS 4 generated a single band of ca 550 bp. The band profiles obtained from digested fragments were identical for all 98 *S. oleagina* isolates (Figure 42), with *Hinfl*, *Taq1* and *Msp1* generating 4, 4, and 7 bands, respectively. Several common bands were observed when the *S. oleagina* ITS-RFLP pattern was compared to that of *V. inaequalis*.

6.4.2 ITS sequence analysis

The specific primer pair, ITSF and ITSR, was tested for specificity with six *S. oleagina* isolates and the closely related fungi, *C. fulvum* and *V. inaequalis*. At 65°C, only DNA of *S. oleagina* was amplified while at lower annealing temperatures, DNA from both *C. fulvum* and *V. inaequalis* was also amplified. PCR-amplification produced a single product of 475 bp. The ITS1, 5.8S and the ITS2 regions of four New Zealand, one Australian and one Italian isolates were sequenced. The ITS lengths were similar in all isolates with the exception of one Canterbury isolate (CB7) that was 9 bases longer. The alignment of these sequences showed that the ITS1 regions had a similarity index ranging from 96 to 100%. The similarity
indices of the ITS2 region ranged from 99.8 to 100% while for the 5.8S coding region it ranged from 99 to 100% across sequences. The variations in the sequences were mainly from single base pair changes representing deletions or insertions in the ITS1 region, particularly in isolate CB7 (Figure 43). Six Msp1 restriction sites were found in the sequences, whereas only three were present for each of the enzymes Taq1 and Hinfl. All the restriction sites of Taq1 were found in the coding 5.8S of the rDNA.
Figure 42: RFLP banding profiles for *S. oleagina* using the restriction enzymes (A) *Hinf*1, (B) *Msp*1, and (C) *Taq*1. Lanes 1 to 10: isolates WM1, WL1, KPe7, WU4, WP2, AP5, AB5, WL2, AP6, and *Venturia inaequalis*, respectively. Arrows indicate the fragment sizes. The Invitrogen 25-bp ladder is shown in lane M.
Figure 43: Aligned DNA sequences of ITS1-5.8S-ITS2 regions of *S. oleagina* isolates from New Zealand (BL1, AB1, CM1 and CB7), Australia (ATP1) and Italy (ISC3) after cloning and sequencing. The restriction sites of the endonucleases *Mspl*, *Taql* and *Hinfl* are shaded pink, yellow and violet, respectively. The blue shade indicates site of deletion or insertion of a nucleotide in the sequences.
6.4.3 UP-PCR banding pattern

All the banding patterns for the 98 S. oleagina isolates from the 19 populations in Christchurch, Blenheim, Masterton, Kapiti, and Auckland, were generated using the UP-PCR primers (AA2M2, AS4, AS15inv, L15 and L15/AS19). Figure 44 shows an example of the banding profiles of nine S. oleagina isolates amplified by each UP-PCR primer. Of the five primers, AS4 gave the highest numbers of reproducible bands (40), whereas AS15inv yielded the least number of bands (24). The five primers generated a total of 159 reproducible bands from the 98 S. oleagina isolates of which 121 (76.1%) were polymorphic. Both the total number of loci detected and proportion of the loci that were polymorphic varied from population to population (Table 15). The total number of loci detected in the populations ranged from 79 to 96 and the proportion of the polymorphic loci ranged from 10.2 to 72.8%.

Table 15: The percentage of polymorphic loci, mean genetic diversity ($H$) and Shannon diversity index ($I$) within 19 populations of Spilocaea oleagina

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<th>Sample size</th>
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<th>$I^b$</th>
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*Nei's (1973) gene diversity.

*Shannon's diversity index (Sheldon, 1969).
Figure 44: UP-PCR banding profiles for *S. oleagina* generated with the primers (A) AA2M2, (B) AS4, (C) AS15inv, (D) L15, and (E) L15/AS19. Lanes 1 to 9 represents isolates CL1, CB14, MM1, CS2, CB9, MM4, CM1, CB15 and CB1, respectively. The Invitrogen 100-bp ladder is shown in lane M.
6.4.4 Genetic diversity within and among populations

Results for the population genetic diversity are similar to the polymorphism data (Table 15), with CAL and BED populations having the highest (0.1393) and lowest (0.0234), genetic diversities \( (H; \text{Nei, 1973}) \). The overall mean genetic diversity for all populations was 0.1322, showing that the majority (86.8\%) of the genotypes present within the \( S. \text{oleagina} \) population were similar. The genotypic diversity, quantified by a normalized Shannon’s diversity index \( (J) \) was low in all populations, ranging from 0.0342 (population BED) to 0.2095 (population CAL) with an overall mean value of 0.2078 for all isolates. Nevertheless, the proportion of polymorphic loci, Nei’s and Shannon’s diversity indices found in populations CAL and CST were similar; the CST populations possessed the greatest proportion of unique polymorphisms (15.2\%) compared with CAL populations. Although there were a number of bands that were monomorphic within a population, none of them were exclusive to the population for which the locus was fixed. When the populations were pooled within regions, the genetic structure \( (G_{ST}) \) detected within the regions ranged from 0.1554 to 0.3077 on the average of the 121 polymorphic loci (Table 16). Significant differentiation \( (G_{ST} = 0.3077, P = 0.025; G_{ST} = 0.2604, P = 0.013) \) was seen only among the populations sampled from Blenheim (five) and Christchurch (six). These values indicate that 70\%/74\% of the UP-PCR diversity was within populations and 30\%/26\% was among populations in the region.

Table 16: Nei’s (1973) measures of neutral genetic diversity, structure, and gene flow estimate calculated for all sampled populations of \( Spilocaeeoleagina \). Values were calculated assuming random mating

<table>
<thead>
<tr>
<th>Region</th>
<th>Population size</th>
<th>Sample size</th>
<th>( H_t^a )</th>
<th>( H_s^b )</th>
<th>( G_{ST}^c )</th>
<th>( Nm^d )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Auckland</td>
<td>3</td>
<td>20</td>
<td>0.0764</td>
<td>0.0644</td>
<td>0.1573</td>
<td>2.678</td>
</tr>
<tr>
<td>Christchurch</td>
<td>6</td>
<td>34</td>
<td>0.1518</td>
<td>0.1122</td>
<td>0.2604</td>
<td>1.416</td>
</tr>
<tr>
<td>Kapiti</td>
<td>2</td>
<td>8</td>
<td>0.0584</td>
<td>0.0485</td>
<td>0.1703</td>
<td>2.435</td>
</tr>
<tr>
<td>Blenheim</td>
<td>5</td>
<td>18</td>
<td>0.1178</td>
<td>0.0815</td>
<td>0.3077</td>
<td>1.124</td>
</tr>
<tr>
<td>Masterton</td>
<td>3</td>
<td>18</td>
<td>0.0876</td>
<td>0.0740</td>
<td>0.1554</td>
<td>2.717</td>
</tr>
</tbody>
</table>

\( a \) total genetic diversity
\( b \) genetic diversity within region
\( c \) Nei’s coefficient of genetic differentiation
\( d \) Estimate of gene flow
The AMOVA analysis indicated that approximately 87% of the variation in the data was from genetic variation within populations (Table 17). Only 3% of the variation could be attributed to differences among populations, and 10% was due to regional differences. The proportion of the total variance values was only significant for among region and within population levels.

**Table 17**: Hierarchical analysis of molecular variance (AMOVA) for all *Spilocaea oleagina* populations. The significance value was determined from 1023 permutations.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Sums of squares</th>
<th>Variance component</th>
<th>% Total</th>
<th>F-statistics</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among regions</td>
<td>5</td>
<td>146.64</td>
<td>1.161</td>
<td>10.1</td>
<td>0.1011</td>
<td>0.001</td>
</tr>
<tr>
<td>Among populations</td>
<td>13</td>
<td>152.91</td>
<td>0.355</td>
<td>3.1</td>
<td>0.0344</td>
<td>0.157</td>
</tr>
<tr>
<td>Within populations</td>
<td>79</td>
<td>786.66</td>
<td>9.957</td>
<td>86.8</td>
<td>0.1323</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Pairwise differences varied from -0.279 to 0.516. The differentiation among populations was significant ($P<0.05$) for only 25% (43/171) of all pairwise comparisons (Table 18). When considered as a single population, pairwise comparisons of 123 polymorphic UP-PCR loci gave 2250 (30%) disequilibrium values that were significantly different from zero (Fisher’s exact test, $P<0.05$). The total number of contingency tables (loci combinations) was 123(123-1)/2 = 7503. Population differentiation on the basis of host cultivar was tested with isolates collected from Barnea ($n=4$) and Manzanillo ($n=3$) olive plants grown in the same grove (population CST). Genetic differentiation tests revealed no significant ($P=0.315$) difference in the allele frequencies between the two populations, and a very low level of differentiation was observed with the parameter $F_{ST}$ for this population pair ($F_{ST} = 0.088$).
Table 18: Pairwise differentiation among nineteen populations of *Spilocaea oleagina* isolates in New Zealand. Probability values are based on 1023 permutations.

<table>
<thead>
<tr>
<th>Pop*</th>
<th>ABA</th>
<th>ARO</th>
<th>AST</th>
<th>CAL</th>
<th>CDO</th>
<th>CGC</th>
<th>CGD</th>
<th>CPA</th>
<th>CST</th>
<th>KKA</th>
<th>KAL</th>
<th>BED</th>
<th>BIA</th>
<th>BMA</th>
<th>BMU</th>
<th>NED</th>
<th>MKM</th>
<th>MGR</th>
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<tr>
<td>ABA</td>
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<tr>
<td>AST</td>
<td>0.070</td>
<td>-0.016</td>
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<tr>
<td>CAL</td>
<td>0.382***</td>
<td>0.335***</td>
<td>0.298**</td>
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<tr>
<td>CDO</td>
<td>0.246**</td>
<td>0.213***</td>
<td>0.121</td>
<td>0.121</td>
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<tr>
<td>CGC</td>
<td>0.298*</td>
<td>0.237***</td>
<td>0.157</td>
<td>-0.042</td>
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<tr>
<td>CGD</td>
<td>0.516**</td>
<td>0.404*</td>
<td>0.472</td>
<td>0.149</td>
<td>0.207*</td>
<td>-0.015</td>
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<tr>
<td>CPA</td>
<td>0.190***</td>
<td>0.155***</td>
<td>0.152</td>
<td>0.074</td>
<td>0.060</td>
<td>-0.008</td>
<td>0.056</td>
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<tr>
<td>CST</td>
<td>0.201*</td>
<td>0.162**</td>
<td>0.112</td>
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<td>0.040</td>
<td>-0.046</td>
<td>0.034</td>
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<tr>
<td>KKA</td>
<td>0.043</td>
<td>-0.035</td>
<td>0.021</td>
<td>0.267***</td>
<td>0.195**</td>
<td>0.171*</td>
<td>0.393*</td>
<td>0.097</td>
<td>0.107*</td>
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<td>KAL</td>
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<td>0.062</td>
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<td>BED</td>
<td>0.280</td>
<td>0.122</td>
<td>0.261</td>
<td>0.150</td>
<td>-0.007</td>
<td>-0.026</td>
<td>0.232</td>
<td>-0.117</td>
<td>-0.047</td>
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<td>0.099</td>
<td>0.037</td>
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<td>0.078</td>
<td>-0.007</td>
<td>-0.095</td>
<td>0.139</td>
<td>0.020</td>
<td>0.018</td>
<td>-0.031</td>
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<td>BMA</td>
<td>0.270**</td>
<td>0.217***</td>
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<td>-0.001</td>
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<tr>
<td>BMU</td>
<td>0.210***</td>
<td>0.134*</td>
<td>0.230</td>
<td>0.301*</td>
<td>0.063</td>
<td>0.206</td>
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<td>0.007</td>
<td>0.181***</td>
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<td>NED</td>
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<td>0.230</td>
<td>0.289*</td>
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<td>0.143</td>
<td>0.465</td>
<td>0.056</td>
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<td>0.229</td>
<td>0.029</td>
<td>0.297</td>
<td>0.021</td>
<td>-0.010</td>
<td>-0.041</td>
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<tr>
<td>MKM</td>
<td>0.081</td>
<td>-0.010</td>
<td>0.125</td>
<td>0.298***</td>
<td>0.237*</td>
<td>0.202</td>
<td>0.482*</td>
<td>0.135*</td>
<td>0.135*</td>
<td>-0.121</td>
<td>0.113</td>
<td>0.222</td>
<td>0.029</td>
<td>0.219*</td>
<td>0.290*</td>
<td>0.376*</td>
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</tr>
<tr>
<td>MGR</td>
<td>0.053</td>
<td>0.024</td>
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<td>0.130*</td>
<td>0.068</td>
<td>-0.010</td>
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<td>-0.062</td>
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<tr>
<td>MLO</td>
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<td>0.071</td>
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<td>0.225***</td>
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<td>0.355*</td>
<td>0.118*</td>
<td>0.139*</td>
<td>-0.047</td>
<td>-0.009</td>
<td>0.108</td>
<td>-0.012</td>
<td>0.210*</td>
<td>0.284***</td>
<td>0.334*</td>
<td>-0.038</td>
<td>-0.013</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01, ***P < 0.001.

*Populations of *Spilocaea oleagina*
Figure 45: Phylogenetic tree indicating the relationships among *Spilocaea oleagina* isolates based on UP-PCR analysis. Unrooted trees were created using the neighbour-joining method (PAUP 4.0b3a; Sinauer Associates) with genetic distance values generated from the Jaccard-transformed similarity matrix. The isolate codes indicate region of origin, olive cultivar and order of isolation.
Individual isolates of *S. oleagina* did not cluster into distinct groups corresponding to geographic origin in the unrooted neighbour-joining dendrogram generated from the Jaccard-transformed similarity matrix (Figure 45). However, isolates collected from Christchurch olive groves appeared to have greater genotypic diversity than the other populations denoted by the longer branch lengths. Two small genotypically distinct clusters, A and B, were identified (Figure 45). Five of the isolates in cluster A were from the Christchurch region while the other isolates were from Blenheim and Masterton. All the individuals in this cluster were isolated from the cultivar Barnea. In group B, all the isolates were evenly distributed between two regions (Christchurch and Blenheim) with no cultivar or population differentiation within the group.

### 6.5 DISCUSSION

The nucleotide sequences of the ITS1-5.8S-ITS2 region of rDNA were obtained from four New Zealand, one Australian and one Italian isolates. The comparative analysis of these sequences showed a high degree (96 to 100%) of similarity among the isolates. When these ITS sequences were compared with those of other pathogenic fungi from GenBank, they were found to be closely related to that of *Venturia pirina* with 96% homology. This result is similar to the report of González-Lamothe et al., (2002) who showed that the ITS1–5.8S rDNA–ITS2 sequence with the highest identity was from *V. hantliniana* (97.8%), and that *S. oleagina* is an anamorph of a yet unidentified *Venturia* sp. The results suggest that analysis using only the ITS region is not enough for establishing variations among species and among pathogen populations.

Molecular techniques such as PCR-RFLP analysis of rDNA/ITS regions and UP-PCR markers are now increasingly being used as means of studying genetic diversity and population structure of plant pathogens (Tenzer and Gessler 1997; Bulat et al., 1998; Cumagun et al., 2000; Rekab et al., 2004). PCR-RFLP of ITS and 5.8S rDNA regions targets a single locus, whereas UP-PCR analysis targets a large number of loci from across the genome and thus is more likely to accurately reflect overall population structure. In this study, the PCR-RFLP analysis showed no polymorphism among all the 98 *S. oleagina* isolates from New Zealand, indicating that they belong to the same species and their ITS-5.8S-ITS2 region is conserved.
The very high similarity in the pathogen populations revealed by PCR-RFLP analysis was supported by molecular analysis using UP-PCR markers. Overall, this study found low levels ($H = 0.1322$) of genetic variation among New Zealand *S. oleagina* isolates, which had not been reported previously. However, similar results have been reported for other pathogenic fungi such as *Hemileia vastatrix* (Gouveia et al., 2005), *Discula destructive* (Zhang and Blackwell, 2002), and *Trichoderma* spp. (Cumagun et al., 2000) using UP-PCR markers. It is known that the basic mechanisms that generate variation in pathogen populations are mutation, migration and recombination (asexual or sexual). This variation is shaped by the forces of selection and genetic drift (McDonald and McDermott, 1993; Zeigler et al., 1995). It is possible that because *S. oleagina* was only recently introduced into olive groves in New Zealand, these forces have not acted sufficiently strongly to produce substantial genotypic variation in the pathogen population. Fungal pathogen populations that are mainly asexual will display a high degree of clonality, with few genotypes present at relatively high frequencies. In contrast, random mating (sexual recombination) populations are expected to show a high degree of genotypic diversity (Burdon and Roelfs, 1985; Kohn 1995). Thus, the low genetic diversity suggests that an asexual mode of reproduction predominates among *S. oleagina* population in New Zealand, although the occurrence of a low level of sexual reproduction cannot be excluded.

Molecular analysis revealed that most of the genetic variation (87%) found was within the pathogen population. The variations among populations and between regions accounted for 3% and 10% of the total genetic variation, respectively. The high proportion of the genetic diversity attributed to within *S. oleagina* populations supports the hypothesis that there were multiple introductions of the pathogen into New Zealand or that local mutation occurred over time. It is also possible that other factors such as parasexual recombination may be occurring in the pathogen populations, as was reported to have had a major impact on the population structure of *Rhynchosporium secalis* (Newman and Owen, 1985) and *Magnaporthe grisea* (Crawford et al., 1986; Zeigler et al., 1997). Low to moderate genetic differentiation ($G_{ST} = 0.155$ to 0.307) was found for *S. oleagina* populations within the regions. The low geographical population structure and overall low genetic diversity is expected for a disease that has emerged due to recent introduction or translocation. Such human-related gene flow resulting from frequent movement of infected plant materials has also been demonstrated for *Phytophthora ramorum* (Ivors et al., 2004).
In this study, no evidence of population differentiation was found among the *S. oleagina* subpopulations analyzed ($G_{ST} = 0.034$, $P = 0.157$). Tenzer and Gessler (1997) also found low differentiation ($G_{ST} = 0.040$) in Switzerland among different populations of the closely related fungus, *V. inaequalis*, when analysing ITS regions and using RAPD markers. This was explained by the history of apple growing in that country. In this study, the lack of differentiation found could be explained by subpopulations of *S. oleagina* being founded by a common source population after being introduced to New Zealand, with limited migration or mutation among subpopulations to cause genetic differentiation from genetic drift (Goodwin et al., 1993).

Olive production in New Zealand is relatively new. The disease on present day cultivated olive trees originated from stock imported from overseas in the 19th century for ornamental purposes. Initially only a single nursery was involved in the propagation and distribution of the olive plants (D. Cross, pers. comm.). These plant materials were the initial sources of primary inoculum for new infection. Of these plant materials imported, ‘Barnea’, which is highly susceptible to OLS, was most commonly propagated and grown in olive groves. The lack of adequate knowledge of disease control strategies and management practices, such as the use of overhead irrigation systems, which provided conducive conditions for the development of the pathogen led to disease spread in the nursery. Eventually, inoculum was spread over large distances through the distribution of OLS-infected plant materials by human activity (D. Cross, pers. comm.).

An alternative explanation for the lack of differentiation among the pathogen populations among the regions would be that enough migration and gene flow among populations is occurring to homogenize the allele frequencies (Slatkin, 1987), which could be the result of long-distance conidial dispersal. However, this hypothesis is unlikely to be true because *S. oleagina* conidial dispersal is likely to be limited to within groves and between neighbouring groves, since it is known that conidia are dispersed mainly by rain splash (Graniti, 1993; Guechi and Girre, 1994).

Cluster analysis exhibits unstructured variability of this pathogen with regard to geographical origin or host. Significance testing of the $F_{ST}$ estimates demonstrated differences between pairwise comparison of all *S. oleagina* populations from Christchurch and two from Auckland. The reasons for these differences are unclear. A possible explanation for the
observed differences could be that the planting materials were sourced from different nurseries and, thus different sources of inoculum for new infection. The differences could also be due to the selection of pathogen genotypes by the environmental conditions and disease management in the different groves.

The gametic phase disequilibrium analysis (test of deviation from random mating) was used to test the hypothesis that the *S. oleagina* isolates used in this study originated from a random mating population. The analysis of loci associations showed significant gamete phase disequilibrium in the majority (80%) of all possible combinations among the 123 polymorphic UP-PCR loci in the 98 isolates. The high levels of linkage disequilibrium in the populations indicate that *S. oleagina* maintains a genetic structure that is consistent with asexual (nonrandom mating) reproduction (McDonald et al., 1995), which usually generates a limited series of clonal lineages where many of the alleles are linked. If this pathogen had reproduced predominantly by sexual means, the independent assortment of alleles at unlinked loci at each sexual generation would have resulted in random association among alleles at different loci and so linkage equilibrium (McDonald et al., 1994).

Gametic phase disequilibrium may arise in a population from founder effects, chromosomal linkage, selection pressures or nonrandom mating, including asexual reproduction (McDonald et al., 1994). The known biology of *S. oleagina* indicates that asexual reproduction is the main means of reproduction for this fungus since no sexual stage of this pathogen has been found in olive groves worldwide (Graniti, 1993), which is consistent with the observed linkage disequilibrium. It is also possible that selection pressures may have contributed to the observed linkage disequilibrium, since the olive groves in New Zealand are now planted with genetically uniform hosts that originated from relatively few imported plants. This agricultural practice increases the strength of the pathogen selection ability. In this situation, particular combinations of virulence genes in the pathogen are expected to increase in frequency as a consequence of strong selection to match the corresponding resistance genes in the host. This type of selection can result in high levels of disequilibrium among particular combinations of virulence genes, as was reported for barley powdery mildew (Hovmøller and Østergård, 1991).

Host specificity within *S. oleagina* was investigated by comparing population differentiation between Barnea and Manzanillo subpopulations sampled from the same olive grove. The 9%
difference found between the limited numbers of cultivar subpopulations tested could not show strong evidence of host specificity. The use of a larger number of isolates obtained from more plants of a greater number of cultivars would give a more accurate assessment of genetic differentiation based on host cultivar within a grove.

In conclusion, this study provided understanding of the population dynamics of *S. oleagina* in New Zealand. The low level of genetic diversity found within and among populations shows that reproduction of this fungus must be largely through asexual mechanisms. The low genetic diversity in *S. oleagina* populations suggests that any effective control strategies are likely to be useful in all or most New Zealand olive groves.

6.6 ACKNOWLEDGEMENT

Funding for this research was provided by the New Zealand Foundation for Research, Science and Technology and the New Zealand Olive Association. The author would like to thank Dr K.J. Evans of the University of Tasmania, Australia and Prof. Salvatore Frisullo of the Università di Foggia, Italy for supplying the OLS infected leaf samples. I would also like to thank Margaret Auger of Lincoln University for assisting in the scoring of the UP-PCR bands.

6.7 REFERENCES


Chapter 7

Sources of variation in a field evaluation of the incidence and severity of olive leaf spot

7.1 ABSTRACT

Incidence (% infected leaves) and severity (number of lesions/leaf) of olive leaf spot disease, caused by *Spilocaea oleagina*, were assessed every 2 weeks on 20 trees in a Canterbury olive grove for 12 weeks during summer 2003/04. All the trees were infected by olive leaf spot disease (OLS) and although disease incidence and severity varied between trees \( (P<0.001) \), it did not vary between branches over time \( (P=0.088) \). There was a strong correlation \( (R^2=0.869) \) between disease incidence and severity. It was estimated that at least five trees and 50 leaves/tree were required to correctly estimate the mean values of the parameters measured. Throughout the duration of the experiment, no new leaf lesions formed and although old lesions increased in size \( (P<0.001) \), spore numbers decreased from \( 5 \times 10^4 \) to \( 1 \times 10^3 \) conidia/cm\(^2\) of lesion and viability of conidia declined from 55 to 10%.

Slightly modified publication by

F.O. Obanor\(^1,2\), M. Walter\(^2\), E.E. Jones\(^1\) and M.V. Jaspers\(^1\)
\(^1\)Bio-Protection and Ecology Division, Lincoln University, Canterbury, New Zealand;
\(^2\)HortResearch, P O Box 51, Lincoln, Canterbury, New Zealand.
Corresponding author: fobanor@hortresearch.co.nz

Olive leaf spot (OLS), also called peacock spot disease, is caused by the fungus, *Spilocaea oleagina*, Castagne (Hughes) (syn. *Cycloconium oleagina*). It is widespread in all olive growing regions of the world, and has been known in the Mediterranean areas for over a century (Bernès, 1923). OLS usually occurs on the upper surface of the olive leaf. As the spots expand and coalesce to cover a large proportion of leaf area, leaves often senesce and are shed from the tree prematurely. Leaf spots are usually more abundant on the lower parts of olive trees, and many shoots in these parts become completely defoliated. Recurrent infections often cause poor growth and dieback of defoliated twigs (Miller, 1949; López-Doncel et al., 2000).

The influence of leaf cardinal point location on the distribution of OLS disease has been well documented. In the region of Setif, Algeria, OLS infection was reported to be more severe on north facing leaves as a result of their prolonged surface moisture retention and lower temperature (Guechi and Girre, 1994). In New Zealand, OLS disease was found to be more abundant on south facing leaves, in trees with large dense canopies and in some cultivars, although all cultivars were affected (MacDonald et al., 2000).

The assessment of disease levels is usually by incidence or severity. “Disease incidence is the proportion (0 to 1) or percentage (0 to 100) of diseased entities within a sampling unit. Severity is the quantity of disease affecting entities within a sampling unit” (Seem 1984). For many plant diseases, only disease severity estimates are considered to give an accurate indication of their effects on the plants or of the efficacy of control treatments. Many different methods of estimating disease severity have been developed by various researchers although visual estimates of severity have been used almost exclusively. Estimates of severity are frequently based on lesion area but may also be based on lesion number. Seem (1984) noted that mean lesion counts per entity can be used to provide a true measure of severity, even though they cannot be expressed as a proportion or percent. However, visual estimates of disease severity can vary substantially between assessors whereas assessment of disease incidence is faster and more objective (Nutter, 1995).
The purpose of this study was to develop a robust sampling and disease evaluation technique that tracked OLS development in further field trials. Since a study by MacDonald et al. (2000) found a high correlation ($P<0.001$) between numbers of OLS-infected leaves per tree, numbers of lesions per leaf and diseased leaf areas, and OLS lesions are known to expand very slowly, assessment of disease severity as the number of lesions per leaf was considered to be valid for this study. The relationship between disease incidence (% leaves infected) and severity was determined, and the rates of lesion development and spore production were also monitored.

7.3 MATERIALS AND METHODS

Field assessment was conducted during the summer of 2003/04 on 20 olive trees (6 years old, cv. Barnea) in the centre two rows of an olive grove in Canterbury, New Zealand. On each tree, seven branches were randomly selected on the south side at eye level, and labelled. At each fortnightly assessment, all the fully-expanded matured leaves on one branch for each tree were removed and taken to the laboratory for evaluation of disease incidence, severity and latent infection. Incidence was assessed by determining the percentage of infected leaves and severity by counting the number of lesions on each leaf (Teviotdale and Sibbett, 1995). Lesion size was measured using a digital calliper (Mitutoyo Digimatic Caliper, Japan). To determine latent infection, 10 leaves randomly selected from each branch sample were dipped in 5% NaOH for 30 min (Shabi et al., 1994) and examined for characteristic black spots.

Conidium numbers and their viability were determined on a randomly selected sub-sample (10 leaves with visible lesions) by washing the leaf lesions cut from each leaf vigorously for 1 min in 1 mL of distilled water to dislodge the conidia. Conidia were counted (haemocytometer) in four sub-samples from each suspension, and their number/cm$^2$ leaf lesion calculated. To determine conidium viability, the 10 spore suspensions were pooled, the concentration adjusted to $5 \times 10^4$ conidia/mL and 100 µL aliquots plated (three replicates) onto olive leaf extract agar (Saad and Masri, 1978). After incubation at 20°C for 48 h, 100 conidia/plate were examined using a light microscope at $\times 200$ magnification, and the percentage germination recorded. A conidium was considered germinated if the length of the germ tube exceeded half the length of the conidium.
Analysis of variance (ANOVA) and model fitting were conducted using GenStat 7.2.

### 7.4 RESULTS AND DISCUSSION

All the trees were infected by olive leaf spot disease, but disease levels varied between trees ($P<0.001$), ranging from 1 to 52% for number of infected leaves and 0.01 to 1.7 for numbers of lesions/leaf. The variation in the levels of OLS between the trees was not due to a row effect ($P=0.236$) and may have been due to canopy size and density. It is known that the denser the olive tree canopy the greater the humidity and moisture retention capacity within and between the trees. This makes the trees more favourable for OLS infection (Teviotdale and Sibbett, 1995; MacDonald et al., 2000). Therefore in future trials, olive trees of similar age/size and canopy density should be selected in order to reduce the between-tree variability of OLS disease.

The time of assessment had no significant ($P=0.088$) effect on OLS disease levels. The mean number of infected leaves ranged from 8 to 16% and the number of lesions/leaf ranged from 0.11 to 0.22, with no differences between branches over time for the 2003/04 summer. Assessing the mature leaves on one branch on the south side of the tree was considered an adequate strategy for estimating incidence and severity and this should be used in future trials. This work validates earlier research by McDonald et al. (2000). From the variance figures generated by analysis of variance, it was estimated that at least 5 trees and 50 leaves/tree were required to accurately estimate the mean values of these parameters.

The relationship between disease incidence (% infected leaves) and disease severity (number of lesions/leaf) for OLS was best described by a second order polynomial regression (Figure 46; $R^2=0.869$). Incidence data (% infected leaves) are faster to collect with greater accuracy than severity data (number of lesions/leaf), particularly when leaves are wet, since the lesions are not clearly visible. Therefore the % infected leaves should be used for assessing the efficacy of any control measures tested in the future. If required, the number of lesions/leaf can be derived from Figure 46; however, under high disease pressure (>50%) the level of accuracy may be reduced.
Figure 46: Relationship between OLS incidence (% infected leaves) and severity (number of lesions/leaf) across all trees and assessment times determined from a 12-week field assessment.

Lesion sizes did not differ significantly ($P=0.095$) between trees during the assessment period (data not shown), indicating that OLS lesion expansion was uniform between the trees. Although no new lesions on the leaves developed, latent infections were observed throughout the duration of the experiment. There was a significant ($P<0.001$) increase in the mean size of old lesions on the leaves for the first 8 weeks of disease monitoring (Figure 47). Lesion sizes did not increase significantly from mid-December 2003 to 26 January 2004. This may have been due to the unusually hot and dry weather conditions recorded in Canterbury during the assessment period (Figure 48). The results agree with previous overseas reports, which stated that during hot, dry summers the lesions stopped expanding and became dry, hardened, cracked or blistered (Miller, 1949; Graniti, 1993).
The production of conidia by *S. oleagina* decreased over the period of assessment with the initial mean conidium numbers being $5 \times 10^4$ conidia/cm$^2$ of leaf lesion, whereas at the end of the experiment, the mean number of conidia was $1 \times 10^2$ conidia/cm$^2$. Conidium viability also followed a similar trend with spore germination declining from 55% to 10%. Conidium production and viability seemed to be related to environmental conditions, particularly rain and temperature, although lesion and conidium age could also be contributing factors as was found in Chapter 5. For example, in November 2003 the mean daily temperature ranged from 7 to 17°C and mean monthly rainfall was 36.7 mm. However, in January 2004 the mean temperature ranged from 12 to 25°C and mean rainfall was only 3.6 mm (Figure 48). This result is consistent with Mediterranean research trials, which reported abundant conidium production during spring and autumn, but limited conidium production during the summer months (Laviola and Scarito 1993; Guechi and Girre, 1994).
**Figure 48:** Conidium production by *Spilocaea oleagina* in relation to temperature and rain events during the months of October 2003 through to January 2004. Percent values are germination of conidia at 3 Nov 2003 and 26 January 2004.

### 7.5 CONCLUSION

This study has shown that the main source of variation in OLS disease evaluation in the field in summer was between individual olive trees. There was a strong correlation between OLS incidence (% infected leaves) and severity (number of lesions/leaf). The % infected leaves measurement of disease incidence is less time-consuming than the number of lesions/leaf, and will be conducted on at least five trees and 50 leaves per tree in subsequent field trials evaluating fungicides for disease control.

### 7.6 ACKNOWLEDGEMENT

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7.7 REFERENCES


Chapter 8

In vitro effects of fungicides on conidium germination of Spilocaea oleagina, the cause of olive leaf spot

8.1 ABSTRACT

Twenty fungicides were tested in vitro for their effects on the germination of conidia of Spilocaea oleagina, the fungus that causes olive leaf spot. Conidia used in this evaluation were obtained from naturally infected olive leaves in Canterbury. Of the fungicides tested, kresoxim-methyl and captan were the most effective in preventing conidium germination at low concentrations, with EC50 values of 0.002 and 0.003 μg/mL, respectively. The newer fungicides, boscalid and boscalid/pyraclostrobin, were also effective (EC50=0.031 and 0.006 μg/mL, respectively). Of the benzimidazole fungicides tested, carbendazim was effective (EC50=0.005 μg/mL), but thiophanate-methyl was not (EC50=2.6 μg/mL). None of the demethylation inhibitor fungicides tested were very effective (EC50 values > 1 μg/mL), except flusilazol (EC50=0.075 μg/mL). Two copper-containing fungicides, copper hydroxide and copper sulphate, were ineffective for preventing conidium germination (EC50=3.0 and 44.3 μg/mL, respectively). This study has identified candidate fungicides for further evaluation as tools for management of olive leaf spot.

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F.O. Obanor1,2, M. Walter2, E.E. Jones1 and M.V. Jaspers1
1Bio-Protection and Ecology Division, Lincoln University, Canterbury, New Zealand;
2HortResearch, P O Box 51, Lincoln, Canterbury, New Zealand.
Corresponding author: fobanor@hortresearch.co.nz

In vitro effects of fungicides on conidium germination of Spilocaea oleagina, the cause of olive leaf spot
8.2 INTRODUCTION

The New Zealand olive industry is a new, fast-growing industry with potential for high returns from premium oil. One of the major problems threatening this industry is the disease olive leaf spot (OLS), also called peacock spot. This disease, caused by the fungus *Spilocaea oleagina* Castagne (Hughes) (syn. *Cycloconium oleagina*), is widespread in all olive growing regions of the world, and has been recognised in Mediterranean areas for over a century (Bernès, 1923). In warm, dry climates the disease is not usually a significant problem because cool, moist weather is required for epidemic development.

A preliminary survey of the prevalence of OLS during the summer of 1999/2000 revealed that the disease is widespread throughout New Zealand, with all regions and cultivars affected (MacDonald et al., 2000). Forty percent of all olive trees assessed were infected with OLS, suggesting that it is a serious disease in New Zealand olive groves and may play a major role in the low productivity of olives. Losses in olive yield due to OLS have been estimated to be as high as 20% in California (Wilson and Ogawa, 1979).

The principal method used to control OLS throughout olive-growing regions of the world is chemical fungicides (Teviotdale et al., 1989; Graniti, 1993). The most commonly used fungicides contain copper, and these have included Bordeaux mixture, copper hydroxide, copper oxide and copper oxychlorides, although some long-persisting preventative fungicides (e.g. chlorothalonil and dodine) have also been used to control the disease. These fungicides are usually applied before or at the beginning of the main infection periods, which often coincide with the main shoot-growth seasons (spring and/or autumn) (Prota, 1995).

Teviotdale et al. (1989) reported that in Californian olive groves, one annual application of a copper-containing fungicide, in autumn before rain began, effectively controlled OLS under low disease pressure, irrespective of the rate or type of fungicide. However, there have been no reports on the control of OLS using copper-containing fungicides under high disease pressure, as may occur in wet or humid conditions. In New Zealand, the cool, moist climate favours OLS development causing higher levels of disease, which are unlikely to be controlled with single applications of copper-containing fungicides. In addition, new fungicides have been developed during the last few decades with very effective and
sustainable control of many other diseases, and these may provide more effective control of OLS.

Systemic fungicides, such as difenoconazole, myclobutanil, fenarimol and tebuconazole, have the potential to replace copper-containing fungicides to effectively control OLS under high disease risk. Apple scab, which is caused by the closely related pathogen *Venturia inaequalis*, and other fungal diseases of tree crops have been successfully controlled with systemic fungicides (Jones, 1981). Because the modes of infection of *S. oleagina* and *V. inaequalis* are similar (Graniti, 1993), it is likely that these fungicides could be effective in controlling OLS.

In the present study, 20 fungicides were screened for their effects on germination of *S. oleagina* conidia, to identify chemicals that have potential for control of OLS and that may be suitable for further evaluation in growth chamber and field studies.

**8.3 MATERIALS AND METHODS**

The fungicides selected for the study were from several chemical classes, representing different modes of action, and included those with contact and systemic activity as well as some known to be effective against *V. inaequalis*. The formulations of the 20 fungicides tested, their chemical classes and concentrations used are listed in Table 19. The selected fungicide concentrations were based on the range of activity for each product. For all fungicides, 10-fold dilutions of stock solutions were prepared in water with at least five different concentrations of each chemical.

Olive leaf extract (OLE) was prepared according to Saad & Masri (1978) and was enriched with 40 g/litre of potato dextrose broth (PDB; Difco Laboratories, USA) (OLE+PDB). Conidia of *S. oleagina* used for the evaluation were from naturally infected olive leaves (cv. Barnea) picked from a commercial olive grove in Canterbury. Conidium suspensions were obtained from the leaves by agitating them in distilled water and filtering the suspension through a double layer of cheesecloth to remove leaf debris. The conidium concentrations were adjusted to $5 \times 10^5$ conidia/mL using a microscope and haemacytometer to determine numbers of conidia in the suspension. The conidium suspension (100 μL) was then mixed
with 100 μL of each fungicide stock solution and 800 μL of OLE+PDB in Eppendorf tubes. Three 20 μL droplets of the suspension were placed separately onto three replicate glass slides. The slides were then placed on the lid of a Petri dish (90 mm) containing approximately 30 mL water agar to provide high humidity (>95%) and the dish was then incubated upside down at 20°C for 24 h. Germination of 100 randomly selected conidia in each droplet was evaluated with a compound microscope at ×200 magnification, and the mean percent germination, relative to nil fungicide controls, was calculated for each fungicide. A conidium was considered germinated if the length of the germ tube exceeded half the length of the conidium. The experiment was repeated three times.

All data were analysed using the command GLM Probit analysis (Genstat 7.2) to determine the EC50 values (effective concentrations of the fungicides that reduced conidium germination by 50%) and their confidence intervals.

8.4 RESULTS AND DISCUSSION

In this study, five of the fungicides tested markedly reduced the germination of S. oleagina conidia (Table 19). The cyclic imide and strobilurin chemicals, captan and kresoxim-methyl, respectively, were the most effective in preventing conidium germination with EC50 values of less than 0.003 μg/mL. A similar effect by kresoxim-methyl on spore germination has also been reported for V. inaequalis (Ypema and Gold, 1999). Kresoxim-methyl is characterized by a novel mode of action (inhibition of electron transport at the bc1-complex in mitochondria) and by having a broad spectrum of activity. It has been used for many scab diseases of fruit trees as a protectant fungicide, and acts by building a stable deposit on the leaf surface and in the epicuticular wax layers. Besides having a direct preventative effect on spore germination, kresoxim-methyl prevents the formation of conidiophores and conidia from active scab lesions 7 days after application as an antisporeulant (Politi, 1997).

The contact fungicide, captan, was reported to be consistently effective in inhibiting spore germination in Rhizopus oryzae, which causes storage rot of potato (Amadioha, 1996). However, a high EC50 value of 34.1 μg/mL for this chemical was reported for conidial germination of Phaeomoniella chlamydospora (Jaspers 2001). In New Zealand, the fungicide is widely used to control many crop diseases such as apple scab and OLS.
### Table 19: EC$_{50}$ values for different fungicides tested at different concentrations for inhibition of germination of *Spilocaea oleagina* conidia. Values are the mean from the three experiments

<table>
<thead>
<tr>
<th>Chemical class/ fungicide common name</th>
<th>Trade name</th>
<th>Conc. range (µg ai$^2$/mL)</th>
<th>EC$_{50}$ (µg ai/mL)</th>
<th>95% CI ($\pm$)</th>
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<tbody>
<tr>
<td><strong>Anilopyrimidine</strong></td>
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<td>Cyprodinil/fludioxonil</td>
<td>Switch</td>
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<td>0.011</td>
<td>0.005</td>
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<td>Pyrimethanil</td>
<td>Scala</td>
<td>10–300</td>
<td>15.16</td>
<td>3.098</td>
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<tr>
<td><strong>Benzimidazole</strong></td>
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<tr>
<td>Carbendazim</td>
<td>Bavistin</td>
<td>0.005–0.5</td>
<td>0.005</td>
<td>0.001</td>
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<td>Thiophanate-methyl</td>
<td>Topsin M-4A</td>
<td>1–100</td>
<td>2.599</td>
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<td><strong>Copper</strong></td>
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<tr>
<td>Copper hydroxide</td>
<td>Kocide DF</td>
<td>10–2000</td>
<td>2.991</td>
<td>4.980</td>
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<td>Bordeaux mixture</td>
<td>Cuprofix Disperss</td>
<td>10–2000</td>
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<td>1.550</td>
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<td><strong>Carboxamide</strong></td>
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<td>Endura</td>
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<td>0.013</td>
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<td>Captan</td>
<td>CropCare Captan WG</td>
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<td><strong>Dithiocarbamate</strong></td>
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<td>Mancozeb</td>
<td>Penncozeb DF</td>
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<td><strong>DMI$^1$-piperazine</strong></td>
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<td>Triforine</td>
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<td>Rubigan Flo</td>
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<td>Bravo 720SC</td>
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<td><strong>Strobilurin</strong></td>
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<tr>
<td>Kresoxim-methyl</td>
<td>Stroby WG</td>
<td>0.001–0.05</td>
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</table>

$^1$Demethylation inhibitor.
$^2$ai=active ingredient.
$^3$CI=Confidence interval.

The new-generation fungicides, boscalid and boscalid/pyraclostrobin, were effective in preventing conidium germination, with EC$_{50}$ values of 0.031 and 0.006 µg/mL, respectively. These fungicides have been registered in the USA for use on apples and grapes in the control
of scab and botrytis rot (Babadoost, 2004), respectively. However, they are not yet registered in New Zealand for use to protect any crop from fungal diseases. Of the anilopyrimidine fungicides, only cyprodinil/fludioxonil was effective at low concentrations, with an EC$_{50}$ value of 0.011 µg/mL.

Two benzimidazole fungicides were tested, but only carbendazim was effective in inhibiting conidium germination with an EC$_{50}$ value of 0.005 µg/mL. A similar inhibitory effect has also been reported for the chickpea blight pathogen, *Ascochyta rabiei* (Demirci et al., 2003). Carbendazim is a systemic fungicide with both protective and curative action. The fungicide is absorbed through roots and green tissues of treated plants and acts by inhibiting development of the fungal germ tubes, formation of appressoria and mycelial growth. This fungicide has been used both in New Zealand and overseas in the control of fungal diseases of a range of crops, including stone and pome fruits, vines and vegetables. However, the chemical is not currently being used for the control of OLS in New Zealand.

All the DMI fungicides tested were ineffective in inhibiting conidium germination of *S. oleaginosa*, except flusilazol, for which the EC$_{50}$ value was 0.075 µg/mL. Although inhibition of spore germination by triazole fungicides has been reported for other fungal species (Clarkson et al., 1997), their ineffectiveness in preventing germination of *S. oleaginosa* conidia was not surprising because they are generally known to inhibit hyphal growth rather than spore germination (Sisler and Ragsdale, 1984).

The two copper-containing fungicides, copper hydroxide and Bordeaux mixture, were ineffective at reducing germination of *S. oleaginosa* conidia, having EC$_{50}$ values of 3.0 and 4.43 µg/mL, respectively. However, the inhibitory effect of copper on conidium germination has been demonstrated for other plant pathogenic fungi. For example, Franich (1988) reported that exposure of *Dothistroma pini* conidia to 20 µg/mL Cu$^{2+}$ for 1.5 h was sufficient to kill the spores. In addition, at lower concentrations the rate of conidium germination was not greatly reduced but the germ tube length was significantly reduced. Copper fungicides are the chemicals most commonly used to control OLS in New Zealand olive groves and have been showed to be effective in controlling OLS in California olive groves (Teviotdale et al., 1989). Their ineffectiveness in inhibiting *S. oleaginosa* conidium germination at low concentrations suggests that their mode of action may be through means other than conidium
mortality. Although several copper-containing fungicides are used on olives in New Zealand, only Bordeaux mixture (Cuprofix; recommended field rate of 500 g/100 L) is registered for this use, and its efficacy in controlling OLS has not been established. The advantages of systemic fungicides over contact fungicides are well known, but contact fungicides are generally less expensive than systemics and are suitable as rotation chemicals in fungicide resistance management programmes (Fry, 1982).

8.5 CONCLUSIONS

This work has identified several fungicides as potent inhibitors of *S. oleagina* conidium germination and these may have potential to protect olive trees from OLS. They include kresoxim-methyl, captan, boscalid, cyprodinil/fludioxonil, and boscalid/pyraclostrobin. *In vivo* screening with whole plants is considered to be the most accurate approach for predicting fungicide performance in the field (Knight et al., 1997). The selected fungicides are currently being tested with potted plants and in the field, to further validate their efficacy in controlling OLS.

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8.7 REFERENCES


Chapter 9

Greenhouse and field evaluation of fungicides for activity against *Spilocaea oleagina*, the cause of olive leaf spot

9.1 ABSTRACT

Olive leaf spot or peacock spot is a major disease of olive worldwide, particularly in areas with cool and moist weather conditions. During 2004-2005 in New Zealand, the fungicides, boscalid, captan, carbendazim, copper hydroxide, copper sulphate, difenoconazole, dodine, kresoxim-methyl, and a kresoxim-methyl/copper hydroxide mixture, were tested in the greenhouse and in the field for control efficacy. Greenhouse studies showed that all fungicides tested significantly \((P<0.001)\) reduced disease severity of the leaves but this was also affected by the time interval between fungicide application and pathogen inoculation. For the field study, trees in commercial olive groves in three regions received two fungicide sprays in each of three consecutive seasons, winter, spring and autumn. Fungicide and time of application affected the disease levels on the trees \((P<0.001)\). In winter, none of the fungicides, except copper sulphate, reduced disease incidence on the trees compared to unsprayed controls. Most of the fungicides reduced the number of infected leaves on the trees after spring and autumn applications, with the fungicides being most effective when applied in autumn. Of the fungicides tested, copper sulphate and a mixture of kresoxim-methyl/copper hydroxide were the most effective giving control of 85-96% and 63-93%, respectively. The results of this research have enabled a standard fungicide spray programme to be developed for control of olive leaf spot in New Zealand.

9.2 INTRODUCTION

In New Zealand, olive leaf spot (OLS) infection occurs during autumn through to early spring but the pathogen is dormant during hot, dry summers (Chapter 7). *S. oleagina* survives during summer as mycelium in the lesions on leaves which remain on the tree. In autumn, the margins of these lesions expand into adjacent healthy tissues where conidia are produced. These conidia, which are the primary source of inoculum for new infection, are
dispersed mainly by downward movement of water (Miller, 1949). Wet weather conditions favour \textit{S. oleagina} sporulation, conidium germination and infection, and young olive leaves are more susceptible than older ones. Conidium production was found to be optimum at 15°C and under high humidity (100%), whereas continuous free moisture for 12-24 h and temperatures ranging from 5 to 25°C were required for conidium germination and infection (Chapter 2 & 4). These conditions are common in New Zealand olive groves, particularly in autumn and early spring when new leaves are formed on the trees.

Application of chemical fungicides is the main method of OLS control throughout olive-growing regions of the world (Teviotdale et al. 1989; Graniti 1993), with the most commonly used ones containing copper. Timing of the fungicide applications is vital for effective control of OLS (Graniti, 1993), and in the Mediterranean they are usually applied before the onset of the main infection periods which often coincide with the main shoot-growth seasons (spring and/or autumn) (Prota, 1995). In Californian olive groves, Teviotdale et al. (1989) reported that one annual application of copper-containing fungicides in late fall, before rain began, effectively controlled OLS under low disease pressure.

Systemic fungicides, such as difenoconazole and kresoxim-methyl have the potential to effectively control OLS under high disease pressure. These fungicides are more selective than copper-containing fungicides and may also have a curative effect when applied within 96 hours after infection (Shabi et al., 1994; Viruega and Trapero, 2002). In Israel, Shabi et al. (1994) reported that 89-95% of the leaves on olive trees treated in autumn with a mixture of difenoconazole (Score EC) and oil (Texaco Spraytex CT774) were free from OLS when assessed the following spring, whereas only 66-82% of the leaves from trees treated with Bordeaux mixture were free of the disease. Apple scab, caused by the closely related pathogen, \textit{Venturia inaequalis}, and other fungal diseases of tree crops have also been successfully controlled by systemic fungicides (Jones, 1981). Because they are closely related and the modes of infection of \textit{S. oleagina} and \textit{V. inaequalis} are similar (Graniti 1993), it is likely that these fungicides could be effective in controlling OLS. In New Zealand olive groves, several applications of copper-containing and/or systemic fungicides are commonly used annually, but often fail to give effective control of the disease possibly because of the high disease levels frequently found. However, at the time of this study only copper sulphate (Cuprofix® disperss) was registered for use on olives in New Zealand. There
is a need, therefore, to investigate the rate, number, and timing of applications as well as the efficacy of different fungicides in controlling OLS under New Zealand field conditions.

In New Zealand, an *in vitro* study initially designed to screen candidate fungicides for their ability to prevent conidial germination revealed that some fungicides have the potential to control OLS (Chapter 8). Some of these fungicides were selected for this study, which investigated their efficacy at different application times in controlling OLS in greenhouse and field conditions.

### 9.3 MATERIALS AND METHODS

#### 9.3.1 Greenhouse experiment

The experiment was conducted on 1-2 year old olives plants cv. ‘Barnea’, a cultivar which is highly susceptible to OLS. Plants were grown individually in plastic pots (13 cm diameter) containing a mixture of composted bark and pumice (4:1, v/v) with slow release fertilizer (N:P:K = 15:4:7.5) in a greenhouse maintained at 22 ± 5°C and 30 - 60% RH. The top fully expanded leaves were marked at the beginning of the trial.

The fungicides used for the trial were the three that had demonstrated high efficacy in controlling OLS in the *in vitro* assays (kresoxim-methyl, boscalid and captan) (Chapter 8) and those fungicides that are commonly used for OLS control in New Zealand (copper sulphate, difenoconazole, copper hydroxide, dodine and carbendazim) as well as a mixture of copper hydroxide/kresoxim-methyl. The fungicide products, formulations, sources and rates used are listed in Table 20. Distilled water was used as the control. To evaluate the protective and curative activity of the fungicides, they were applied to the plants 0, 1, 3 or 7 days prior to and 1, 3 or 7 days after *S. oleagina* conidium inoculation. Fungicides were applied with a hand-held spray bottle until run-off, using a volume of 10-15 mL per plant. For the day-0 treatment, sprayed plants were allowed to dry for 2 h prior to inoculation.
Table 20: Fungicide treatments used for the greenhouse (G) and field trial (F)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Region¹</th>
<th>Fungicide</th>
<th>Product name and formulation</th>
<th>A.i. conc.² (g/kg)</th>
<th>Product rate (per litre water)</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>Water</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F, G</td>
<td>B</td>
<td>Boscalid</td>
<td>Endura 50 WG</td>
<td>700</td>
<td>0.1 g</td>
<td>BASF NZ Ltd</td>
</tr>
<tr>
<td>F, G</td>
<td>A, B, C</td>
<td>Captan</td>
<td>Crop Care Captan WG</td>
<td>800</td>
<td>1.25 g</td>
<td>Nufarm Ltd</td>
</tr>
<tr>
<td>G</td>
<td>Carbendazim</td>
<td>Bavistin® DF</td>
<td>500</td>
<td>0.16 g</td>
<td>BASF NZ Ltd</td>
<td></td>
</tr>
<tr>
<td>F, G</td>
<td>C</td>
<td>Copper hydroxide</td>
<td>Champ DP</td>
<td>375</td>
<td>1.75 g</td>
<td>Nufarm Ltd</td>
</tr>
<tr>
<td>F</td>
<td>C</td>
<td>Copper hydroxide</td>
<td>Champ DP</td>
<td>375</td>
<td>2.5 g</td>
<td>Nufarm Ltd</td>
</tr>
<tr>
<td>F</td>
<td>C</td>
<td>Copper hydroxide</td>
<td>Kocide 2000</td>
<td>350</td>
<td>1.9 g</td>
<td>Du Pont and Elliott Chemicals</td>
</tr>
<tr>
<td>F, G</td>
<td>A, B, C</td>
<td>Copper sulphate</td>
<td>Cuprofix® Disperss</td>
<td>200</td>
<td>5 g</td>
<td>Nufarm Ltd</td>
</tr>
<tr>
<td>F, G</td>
<td>A, B, C</td>
<td>Difenconazole</td>
<td>Score® 10 WG</td>
<td>100</td>
<td>0.25 g</td>
<td>Syngenta Crop Protection Ltd</td>
</tr>
<tr>
<td>G</td>
<td>Doline 400</td>
<td>-</td>
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</tr>
<tr>
<td>F, G</td>
<td>A, B, C</td>
<td>Kresoxim-methyl</td>
<td>Stroby® WG</td>
<td>500</td>
<td>0.1 g</td>
<td>BASF NZ Ltd</td>
</tr>
<tr>
<td>F, G</td>
<td>B, C</td>
<td>Kresoxim-methyl</td>
<td>Stroby® WG</td>
<td>500</td>
<td>0.05 g</td>
<td>BASF NZ Ltd</td>
</tr>
<tr>
<td>F, G</td>
<td>Copper hydroxide</td>
<td>Kocide 2000</td>
<td>350</td>
<td>0.95 g</td>
<td>Du Pont and Elliott Chemicals</td>
<td></td>
</tr>
</tbody>
</table>

¹Groves in Auckland (A), Blenheim (B) and Canterbury (C).
²A.i. = active ingredient concentration.
On the day of inoculation (day 0), a conidium suspension \(5.0 \times 10^4\) conidia/mL was made by washing off conidia from naturally infected olive leaves (cv. Barnea) as described previously in Chapter 2. The suspension was sprayed onto plants using an atomizer to just before run-off. Plants were placed in a humid tent (18°C), constructed inside the greenhouse to maintain sufficient moisture for infection, where they remained for 7 days.

For the 1 and 3-day post inoculation fungicide applications, the plants were treated and returned to the tent. However, the 7-day treatment was applied 30 mins before all plants were transferred to the shadehouse, where an overhead misting system operated for 10 min per day, for disease symptoms to develop. The mean daily temperature in the shadehouse ranged from 10 to 15°C. There were four replicate plants for each treatment combination, a total of 280 plants, which were arranged in a completely randomized design. After 12 weeks, disease severity, defined as the mean number of lesions per leaf (Chapter 4), was assessed for the top six fully expanded leaves marked at the time of inoculation. The experiment was conducted twice.

9.3.2 Field trials

The New Zealand field trials were conducted in 2004 and 2005 in commercial olive groves in three regions, Canterbury, Blenheim and Auckland. From each region two groves (A and B) were selected, each of which had a history of olive leaf spot and had trees that were 6-8 years old and spaced 6 m \(\times\) 6 m. The Canterbury and Blenheim groves contained ‘Barnea’ trees, whereas in Auckland, the groves contained ‘Picual’. Methods of fertilization, irrigation and other cultural practices were similar for all groves and followed the standard procedures described in the Olive New Zealand growers’ manual (Olive New Zealand, 2004). Each fungicide treatment was applied to four-tree plots, all plots arranged in a completely randomized block design replicated in five blocks for all groves. Each plot was separated from the next one in the row by one to five buffer trees and from the plots in adjacent rows by one buffer row. Unsprayed trees were used as controls. Each fungicide was applied twice in winter 2004, spring 2004 and autumn 2005 (Table 21), using growers’ spray equipment (hydraulic boom sprayer fitted with hollow-cone nozzles).
Table 21: Field trial sites, cultivars, application and assessment dates for field trials in New Zealand regions

<table>
<thead>
<tr>
<th>Region</th>
<th>Grove</th>
<th>Cultivar</th>
<th>Trial</th>
<th>Application dates</th>
<th>Assessment dates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canterbury</td>
<td>A</td>
<td>Barnea</td>
<td>Winter</td>
<td>09.07.2004</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>04.11.2004</td>
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<td>18.11.2004</td>
<td>20.04.2005</td>
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<td>05.04.2005</td>
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<td></td>
<td></td>
<td>19.05.2005</td>
<td>10.09.2005</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Barnea</td>
<td>Winter</td>
<td>11.07.2004</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td>21.07.2004</td>
<td>11.10.2004</td>
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<td>07.11.2004</td>
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<td>17.11.2004</td>
<td>20.04.2005</td>
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<td>10.04.2005</td>
<td>10.09.2005</td>
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<td></td>
<td>20.05.2005</td>
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</tr>
<tr>
<td>Blenheim</td>
<td>A</td>
<td>Barnea</td>
<td>Winter</td>
<td>20.07.2004</td>
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<td>03.08.2004</td>
<td>19.10.2004</td>
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<td>02.11.2004</td>
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<td>12.11.2004</td>
<td>16.04.2005</td>
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<td>29.04.2005</td>
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<td>13.05.2005</td>
<td>02.09.2005</td>
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<tr>
<td></td>
<td>B</td>
<td>Barnea</td>
<td>Winter</td>
<td>20.07.2004</td>
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<td>03.08.2004</td>
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<td>02.11.2004</td>
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<td>12.11.2004</td>
<td>16.04.2005</td>
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<td>29.04.2005</td>
<td>13.05.2005</td>
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<tr>
<td>Auckland</td>
<td>A</td>
<td>Picual</td>
<td>Winter</td>
<td>31.07.2004</td>
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<td></td>
<td></td>
<td>13.08.2004</td>
<td>10.10.2004</td>
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<td>01.11.2004</td>
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<td>10.11.2004</td>
<td>31.03.2005</td>
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<td>03.04.2005</td>
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<td>12.04.2005</td>
<td>15.09.2005</td>
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<tr>
<td></td>
<td>B</td>
<td>Picual</td>
<td>Winter</td>
<td>01.08.2004</td>
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<td>14.08.2004</td>
<td>10.10.2004</td>
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<td>17.10.2004</td>
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<td>27.10.2004</td>
<td>31.03.2005</td>
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<td>30.04.2005</td>
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<td></td>
<td></td>
<td></td>
<td>14.05.2005</td>
<td>15.09.2005</td>
</tr>
</tbody>
</table>

*No data was collected from the trees because they had been removed*

Treatments were applied at a rate ranging from 1000 to 1200 L/ha, depending on size of trees. No other fungicides were sprayed on the trial trees, but all other normal grower practices were performed throughout the duration of the trials. In all groves, the application time and efficacy of the following fungicides was tested, captan, kresoxim-methyl,
difenoconazole and copper sulphate. However, in Canterbury and Blenheim the following fungicide treatments, copper hydroxide, a kresoxim-methyl/copper hydroxide mixture and boscalid were also included in the trial (Table 20).

The necessity for additional treatments in autumn was determined by adding sprays in autumn 2005 to a subset of trees within experimental plots. The same groves and trial plots used for both winter and spring 2004 were again used for the autumn applications. Each trial plot consisting of four trees was halved resulting in a split-plot design, with half of the trees (randomly selected) being sprayed. To further test the efficacy of kresoxim-methyl/copper hydroxide mixture applied in autumn in Canterbury (grove A) and both groves in Blenheim, the control plots were also halved, with two randomly selected trees from each control plot being sprayed with the mixture. Disease was evaluated at the beginning of the trial (winter 2004) and 2-6 months following treatments, as shown in Table 21. On each occasion, disease incidence (percent) was assessed as the number of infected leaves from the 100 randomly sampled leaves per tree.

9.3.3 Statistical analysis
Greenhouse and field trials data were subjected to analysis of variance (ANOVA) to evaluate the effect of treatment, time of application and their interaction. For the greenhouse data, there was homogeneity of error variances and so the data for the two experimental repeats were combined. However, due to heterogeneity of error variances in the field trials, data for each region and grove were analysed separately. Mean comparisons for greenhouse and field data were conducted with Fisher's unprotected least significant difference (LSD) test at the 5% probability level.

9.4 RESULTS

9.4.1 Greenhouse experiments
The main effects of treatment, time and the treatment × time interaction were highly significant ($P<0.001$), and the experiment repeats did not differ ($P=0.326$). Compared to the control, all fungicides significantly ($P<0.001$) reduced numbers of olive leaf spot lesions by 12 weeks after inoculation, for all application times except for dodine 7 days before pathogen inoculation (Table 22). For fungicides applied before pathogen inoculation the time interval
did not affect numbers of leaf lesions per plant except for carbendazim, difenoconazole and kresoxim-methyl whose efficacy varied depending on the interval before inoculation. However, fungicide application after inoculation was of reduced efficacy by 3 days for captan, difenoconazole, copper hydroxide and copper sulphate and by 7 days for the remaining fungicides. The most efficacious fungicides were copper sulphate, kresoxim-methyl and the combination of kresoxim-methyl and copper hydroxide, which reduced disease severity by 74 to 99% when applied up to 7 days before inoculation and 1 day after inoculation (Table 22). The least effective fungicide was dodine, which reduced disease severity by 13 to 57% over the same period.

Table 22: Effect of fungicides and interval between application and pathogen inoculation on olive leaf spot severity (number of leaf lesions per plant) 12 weeks after inoculation

<table>
<thead>
<tr>
<th>Fungicide</th>
<th>Days before inoculation</th>
<th>Days after inoculation</th>
<th>LSD&lt;sup&gt;d&lt;/sup&gt;(P=0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>81.8</td>
<td>82.2</td>
<td>88.0</td>
</tr>
<tr>
<td>Boscalid</td>
<td>31.5</td>
<td>19.2</td>
<td>24.5</td>
</tr>
<tr>
<td>Captan</td>
<td>36.2</td>
<td>29.0</td>
<td>24.2</td>
</tr>
<tr>
<td>Carbendazim</td>
<td>62.5</td>
<td>44.8</td>
<td>51.0</td>
</tr>
<tr>
<td>Copper hydroxide&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.2</td>
<td>22.2</td>
<td>25.0</td>
</tr>
<tr>
<td>Copper sulphate</td>
<td>5.2</td>
<td>3.2</td>
<td>4.5</td>
</tr>
<tr>
<td>Difenoconazole</td>
<td>54.5</td>
<td>49.0</td>
<td>34.0</td>
</tr>
<tr>
<td>Dodine</td>
<td>70.8</td>
<td>65.0</td>
<td>61.2</td>
</tr>
<tr>
<td>Kresoxim-methyl</td>
<td>21.0</td>
<td>15.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Kresoxim-methyl/copper hydroxide&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.5</td>
<td>2.2</td>
<td>3.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean numbers of leaf lesions per plant (6 leaves per plant and 4 plants per treatment) averaged over the two experimental repeats.

<sup>b</sup>Fungicide application rates are shown in Table 1. Plants were sprayed with the fungicides 7, 3, 1 and 0 days prior to and following inoculation with <i>Spirocar Lisa oleagina</i> conidia.

<sup>c</sup>Keocide 2000 formulation.

<sup>d</sup>Fisher's unprotected least significant difference (LSD) test.

9.4.2 Field trials

Overall, OLS incidence on the unsprayed trees in Auckland, Canterbury and Blenheim olive groves during 2004 and 2005 increased gradually with increasing assessment time except for Grove B in Auckland (Figure 49). There was a more rapid increase in disease incidence in all groves between April and September 2005. OLS incidence was found to range from 5% leaf infection to 100% leaf infection where total defoliation, 100% fruit infection, and zero yields...
have been observed. Complete defoliation with no cropping was most frequently observed in Canterbury and Auckland regions.

The main effects of treatment, time and the treatment \times time interaction were highly significant ($P<0.001$) under field conditions but differed between groves and regions (Table 23). The relative performance of individual fungicides differed between winter, spring and autumn as indicated by the significant treatment and application time interaction. In winter 2004, the two applications of each fungicide caused a small and non-significant reduction in disease incidence when compared to the unsprayed control in all the groves and regions, except in Canterbury groves where copper sulphate (Cuprofix® disperss) and copper hydroxide (Champ DF: 2.5 g/L) caused a small but significant ($P=0.05$) reduction. There were high disease levels on all trees which resulted in severe defoliation of the trees in all groves irrespective of the treatment. In addition, leaf tissue damage was observed on trees treated with copper-containing fungicides, indicating copper phytoxicity on olive leaves. In spring 2004 and autumn 2005, a tank mix of kresoxim-methyl (Stroby® WG) and copper hydroxide (Kocide 2000), which were used in only one Canterbury grove (2004) and one
Blenheim (2005) gave the highest level of OLS control. Copper sulphate was the most effective of the fungicides used in all other groves.

Table 23: Olive leaf spot incidence on olive trees sprayed with different fungicides and at different times in 2004 and 2005 relative to the unsprayed control treatment. Actual disease incidence values for the unsprayed controls are shown in parentheses

<table>
<thead>
<tr>
<th>Application time</th>
<th>Fungicide</th>
<th>Disease incidence (%)</th>
<th>Canterbury</th>
<th>Blenheim</th>
<th>Auckland</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td><strong>July 2004</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Winter)</td>
<td>control</td>
<td>100 a (25)</td>
<td>100 a (39)</td>
<td>100 a (12)</td>
<td>100 a (19)</td>
</tr>
<tr>
<td></td>
<td>copper sulphate</td>
<td>80 bc</td>
<td>81 bcd</td>
<td>87 ab</td>
<td>84 ab</td>
</tr>
<tr>
<td></td>
<td>Captan</td>
<td>95 ab</td>
<td>90 ab</td>
<td>91 a</td>
<td>82 ab</td>
</tr>
<tr>
<td></td>
<td>difenoconazole</td>
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\(^2\)Mean numbers of infected leaves per tree (100 leaves per tree and 4 trees per treatment). Numbers in column followed by different letters are significantly different (P<0.05) (Fisher's unprotected least significant difference (LSD) test).

\(^3\)Kocide 2000 formulation.

\(^4\)Champ DF: 175 g/100 L formulation.

\(^5\)Champ DF: 250 g/100 L formulation.

\(^6\)Mixture of Kocide 2000 (0.95 g/L) and Stroby® WG (0.05 g/L) formulations.

\(^7\)No data were collected for Blenheim Grove B because the trees were removed.
The efficacy of the fungicides was dependent on the regions, with the fungicides being more effective in Blenheim, where the disease pressure was lowest, compared to the other two regions. For the autumn 2005 applications, all the fungicides were highly effective in Blenheim Grove A, with the disease incidence being reduced by 64 to 96%. Overall, the most consistent and efficacious fungicides in controlling OLS were the copper hydroxide/kresoxim-methyl mixture and copper sulphate. The two treatments reduced disease incidence by 85 to 96% and 63 to 93%, respectively, in all the groves except in Auckland (Grove B) in which copper sulphate failed to control the disease.

Figure 50: Extended effects of consecutive applications of copper sulphate (Cs), and kresoxim-methyl/copper hydroxide (Kocide 2000) mixture (KK) on olive leaf spot incidence. Control = unsprayed; KK (2 appli.) = two applications in September 2005 only with a mixture of kresoxim-methyl (Stroby® WG) and copper hydroxide (Kocide 2000); Cs (4 appli.) = sprayed with copper sulphate twice in winter and spring 2004 only; Cs (6 appli.) = the same as Cs (4 appli.) plus two further sprays in autumn the following year. Bars represent the standard errors of the means.
For those fungicides that were effective in spring and in all groves, two additional autumn sprays in the subsequent year significantly ($P=0.001$) reduced disease incidence on the trees. For example, disease incidence was about 25% on trees in Canterbury (Grove A) sprayed twice with copper sulphate in winter and spring 2004, and in autumn 2005, compared to about 65% for those sprayed only in winter and spring 2004 (Figure 50).

**9.5 DISCUSSION**

The systemic and contact fungicides, representing seven chemical classes, were evaluated for their potential to control OLS in the greenhouse and in the field. In the greenhouse trial, all fungicide formulations tested were able to reduce OLS severity on olive foliage for up to 3 days after inoculation. In a previous study (Chapter 8), kresoxim-methyl and captan were effective in inhibiting conidium germination but the two copper-containing fungicides, copper hydroxide and copper sulphate, were ineffective. In the present study, kresoxim-methyl alone or mixed with copper hydroxide were the most effective in protecting young olive leaves from OLS infection. Copper sulphate was also effective in protecting olive leaves, suggesting that this fungicide may act through means other than by causing spore mortality.

Generally, all the fungicides, including the contact fungicides, had both protective and curative activity against OLS up to 3 days after inoculation possibly because of the slow growth of the pathogen. When they were applied later than 3 days after inoculation, disease severity increased. However, the curative effect of kresoxim-methyl alone or in a mixture persisted even to 7 days after inoculation. This result is consistent with that of Viruega and Trapero (2002) who showed that mixing kresoxim-methyl with cupric or organocupric fungicides significantly reduced OLS severity up to 10 days after inoculation. A similar result was also reported for the closely related pathogen, *V. inaequalis* (Ypema et al., 1999) although it was not the case for dodine which has been reported to show high curative activity against the apple scab fungus, *V. inaequalis*, when applied 48 or 144 h after inoculation (Gupta and Kumar, 1985; Thakur et al., 1992). The eradicant activity of a fungicide is related to the efficacy of its absorption and distribution within the leaf, and the position and mass of the invading pathogen. Therefore, the low efficacy of the demethylation inhibitor (difenoconazole) and guanidine (dodine) fungicides, which are known to have...
eradicant, systemic activity could be due to the low amount of these compounds absorbed by the olive leaves, which was insufficient to inhibit the pathogen growth when applied 7 days after inoculation.

*In vivo* screening is considered to be a robust and relevant strategy for determining fungicide performance in the field, particularly with systemic fungicides (Knight et al., 1997). The results of this study showed that the greenhouse study was a reliable means for predicting fungicide performance for disease control in the field and the copper-containing fungicides (some with kresoxim-methyl) were the most effective. In other olive-growing regions, such as California, Greece and Italy several copper-containing fungicides, including copper sulphate, copper oxychloride, copper hydroxide and copper oxide were also found to be effective in controlling OLS (Wilson and Miller, 1949; Bourbos and Skoudridakis, 1993; Teviotdale and Sibbett, 1995; Iannotta et al., 2002). Captan and difenoconazole have also shown both protective and curative activity against *V. inaequalis* (Jones et al., 1993). In this study, the efficacy of these fungicides in controlling OLS was not consistent except in Blenheim groves where there was low disease pressure. In Israel, however, Shabi et al. (1994) reported that difenoconazole was more effective than copper-containing fungicides, particularly when it was mixed with petroleum oil. They found that when olive trees were treated with difenoconazole (Score EC) mixed with oil in autumn, 89-95% of the leaves were free from OLS, whereas 66-82% of the leaves from trees treated with copper sulphate were free of the disease. With another DMI fungicide, tebuconazole, Iannotta et al. (2002) reported that in Italy two applications in spring did not reduce *S. oleagina* infections compared to the controls. The inconsistencies in the reports may be attributed to differences in the environmental conditions and time of applications in these regions.

The efficacy of the fungicides tested in this study was greater in Canterbury and Blenheim than in Auckland. The poor performance of the fungicides in Auckland olive groves may be attributed to the environmental conditions being more favourable for OLS development (Graniti, 1993). The total amount of rainfall recorded in Canterbury and Blenheim during the period of the trial (April 2004-September 2005) were 811 and 1320 mm, respectively, whereas in Auckland a total of 1580 mm was recorded. This indicates that olive growers in the Auckland region may have to apply sprays more frequently than in drier regions.
In these field trials, OLS incidence on the trees was generally high, with the groves in Auckland having the greatest disease incidence. There was a significant increase in OLS incidence in untreated olive trees in all the regions in the period between April-September 2005 compared to the same period of the previous year. Although the weather conditions around these times were slightly different it is unlikely to account for the differences in OLS incidence. In Auckland during April-September 2004, the minimum and maximum temperatures ranged from 1 to 15°C and 9 to 22°C, respectively, with 20.7 mm rainfall, while in the period April-September 2005 the maximum and minimum temperatures ranged from 2 to 18°C and 5 to 24°C, respectively, with 21.8 mm rainfall. Thus, the rapid increased in the OLS severity is unlikely to be due to the more favourable environmental conditions from April-September 2005. The most likely reason for the rapid increased in disease levels can be attributed to the inoculum build-up on the unsprayed trees since no fungicide applications were applied to the trees over a long period of about 15 months during the trial, but were sprayed in the previous years.

Usually, olive growers in New Zealand apply sprays in winter immediately after harvest to avoid possible residues in the oil. The results of this study clearly showed that none of the fungicides gave satisfactory control of the disease when applied in winter. In addition, the application of copper-containing fungicides was detrimental to olive leaves. This could explain the fungicide failure and severe defoliation of trees often observed in New Zealand groves. However, two applications of the more effective fungicides in spring gave better protection when applied again in autumn the following year to coincide with new growth of the trees. This study also demonstrated the remarkable efficacy of a kresoxim-methyl/copper hydroxide mixture when applied to coincide with new growth in autumn. Two applications of the mixture were able to reduce OLS levels by 96%.

In the Mediterranean regions, such as Italy and Spain, three spray applications (winter end, summer end and late autumn) are recommended (Graniti, 1993). In Californian olive groves, Wilson and Miller (1949) observed that a single spray of copper sulphate (Bordeaux mixture) gave satisfactory control of OLS if it was applied prior to protracted rains in autumn or early winter. This was supported by Teviotdale et al. (1989) who reported that one annual application of a copper-containing fungicide in autumn before rain began was sufficient to effectively control the disease in Californian olive groves. However, the results presented here demonstrated that two applications each in spring and autumn effectively control the
disease under New Zealand field conditions. In California olive groves, OLS incidence rarely exceeds 10% (Teviotdale and Sibbett, 1995), whereas disease incidence of up to 100% can be found in New Zealand groves. Thus, the differences in the results are probably due to the high disease pressure resulting from New Zealand environmental conditions (cool and wet) favouring OLS development compared to the hot and dry climatic conditions in California.

The use of autumn fungicide applications will need to be further investigated to determine the minimum period before harvest that results in low or no spray residues on the fruit and in the oil. This is relevant for both the kresoxim-methyl and copper-containing fungicides. Teviotdale et al. (1989) who studied their persistence on olive leaves in California were able to detect substantial copper residues on olive leaves treated once annually in autumn, even 115 days after fungicide applications. Corda et al. (1993) who studied the degradability of five systemic fungicides used to control OLS in Italy reported that there was a rapid decrease in benomyl, thiophanate-methyl, and bitertanol in the oil from treated olives sampled at 0, 10, and 20 days after treatment, but the degradation of fenarimol and penconazole was slower. Maximum residue levels of these fungicides in olive oil have not been established. However, oil from the samples of Corda et al. (1993) taken 20 days after treatment with benomyl, thiophanate-methyl and bitertanol, applied at the normal rate, was below the safety limits established for stone fruits (0.5 ppm, 0.5 ppm and 1 ppm, respectively). In contrast, the levels of fenarimol and penconazole found in the oil was slightly and much higher than allowed (0.1 ppm), respectively. These results suggest that before fungicide recommendations can be made residue tests must be done on oils from olives sprayed in autumn with the fungicides found to be effective in this study.

Treatment history may also have cumulative effects on OLS severity in any given season. Spraying olives in one season might influence the amount of disease development the following season, either by causing a reduction in the inoculum levels in the trees or through the effect of fungicide residues which remain on the leaves until the next season. In this study, OLS levels increased rapidly by autumn 2005 on the trees treated with the most effective fungicide, copper sulphate, in spring 2004 but left unsprayed in autumn 2005. This is consistent with reports of Wilson and Miller (1949) and Teviotdale and Sibbett (1995), which showed that in California groves, OLS severity gradually decreased in trees treated annually. In addition, trees treated in one year had less OLS than non-treated trees in the subsequent year when all the trees were left untreated.
In conclusion, this study has shown that OLS levels in New Zealand groves are much greater than those found in California and Mediterranean regions. Two applications each of either copper sulphate (Cuprofix® disperss) or a mixture of kresoxim-methyl (Stroby® WG) and copper hydroxide (Kocide 2000) in spring to coincide with new growth and two additional sprays in autumn can control the disease. However, further research should investigate the toxicological aspects and residue degradation of these fungicides.

9.6 ACKNOWLEDGEMENT

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9.7 REFERENCES


CHAPTER 10

Concluding discussion

Olive leaf spot is a particularly serious problem in New Zealand because the climate provides the cool, moist conditions needed for its development. The disease can be devastating in some groves, with almost all the leaves on olive trees dropping prematurely, particularly during winter months. The disease has been recognized in New Zealand since the late 1990s and was thought to be the biggest single issue facing NZ olive growers (MacDonald et al. 2000) but at the time, influence of environmental variables, such as temperature and duration of leaf wetness, were not understood. Knowledge of these effects on the epidemiology of the pathogen, *S. oleagina*, is necessary prior to development of reliable and effective disease management strategies. Therefore, the overall goal of this PhD project was to improve the understanding of the factors affecting development of OLS in New Zealand olive groves and to develop strategies for the disease management.

10.1 INFECTION AND DISEASE DEVELOPMENT

As described in the literature review (Chapter 1), relatively few studies have demonstrated an association between temperature and OLS disease development under various leaf wetness conditions. These studies (Chapter 2) showed that the rate of germination and appressorium formation was determined by temperature as well as leaf wetness duration. In general, percentage germination on detached leaves increased with increasing wetness duration at temperatures ranging from 5 to 25°C, but at 30°C no conidium germinated at any of the wetness durations tested. At 5°C, a minimum of 24 h of leaf wetness was necessary for germination to occur, whereas at 20°C conidium germination required at least 9 hours of continuous leaf wetness. The leaf wetness durations found in this study are shorter than those previously reported for this disease (Saad and Masri, 1978). The germination frequency of *S. oleagina* conidia was highest (58%) at 20°C as was the rate of penetration, which occurred 24 h post-inoculation (Chapter 3). Prior to penetration, the growing germ tubes formed appressoria at 5-20°C but not at 25°C, where limited penetration was observed. The histological observations indicate that penetration and infection of olive leaves by *S. oleagina*
most likely involves both enzymatic and mechanical processes, which was also suggested by Graniti (1993). During these PhD studies the trichomes on olive leaf surfaces were also observed to inhibit germ tube penetration, which has not been reported previously for the olive-S. oleagina pathosystem.

Results from detached leaf assays were validated in planta (Chapter 4). The minimum leaf wetness periods required for infection and subsequent development of leaf lesions at 5, 10, 15, 20, and 25°C were 18, 12, 12, 12, and 24 h, respectively, with disease severity being highest at 15°C and lowest at 25°C. The results of this study went some way to explaining the very high OLS levels found in New Zealand groves, since these conditions are common, particularly in autumn, winter and spring. While young olive leaves (2 weeks old) were more susceptible to olive leaf spot than older leaves (10 weeks old), the overall significance of leaf age was moderated by the temperature and leaf wetness duration. The implication of this is that control measures must target new growth of the trees if they are to be effective.

It is well understood, that conidia produced by lesions on overwintering and oversummering leaves are the primary source of inoculum for new infections in spring and autumn, respectively (Guechi and Girre, 1994). In this study, it was demonstrated that lower temperatures (<20°C) and free moisture favour conidium production by S. oleagina, with the optimum temperature for sporulation being 15°C. However, OLS lesions produced no conidia at 25°C under low RH (70%), even after 14 days incubation. This is the first report on the effect of environmental conditions on S. oleagina conidium production under controlled conditions (Chapter 5).

The studies on the effect of climatic parameters on disease development facilitated the development of three preliminary predictions models. These models now could be incorporated into a disease forecasting system for further testing in the field. They may then be used in the development of a spray schedule that indicates the timing of fungicide application in response to climatic factors and olive phenology. This may improve disease control for New Zealand growers by optimising the number and timing of fungicide applications.
10.2 DISEASE CYCLE AND EPIDEMIOLOGY

The research conducted here allowed for the more detailed description of the *S. oleagina* disease cycle (Figure 51) by providing the environmental conditions necessary for spore production, germination and infection in New Zealand. This low-temperature adapted pathogen is usually active in autumn, winter and spring rather than in summer. In summer, the fungus is inactive in quiescent lesions on twigs, leaves, pedicels, fruit and buds. Once maximum daily temperatures fall below 25°C and rain events occur, lesions are likely to resume growth with conidium production and dispersal via rain. However, when conidia are detached from conidiophores, they lose their viability within 7 days. Conidia are readily washed from sporulating lesions to be deposited onto young leaves and other susceptible tissues, often resulting in increased disease levels in the lower canopy. The leaves and fruit in the shaded side of the trees are also more likely to be affected than are sun-exposed leaves and fruit. Young leaves are more susceptible than older leaves. Mature leaves from the previous season are resistant to new infections but existing infections continue to expand and produce conidia.

![Diagram of olive leaf spot disease cycle](image)

**Figure 51:** Disease cycle of olive leaf spot (art by Catherine Snelling).
For the initiation of germination free moisture is required for 6 h, however germination then proceeds as long as the relative humidity is about 100% with 20°C and free moisture providing optimal conditions. The time required for infection depends on temperature and the leaf wetness period with penetration occurring within 24-48 h. Hyphae penetrate the leaf surface and become established between the epidermis and cuticle, but epidermal cells do not appear to be invaded, with the main source of nutrients for hyphae being leakage from the epidermal cells. Subcuticular growth remains symptomless until formation of conidiophores, with leaf wetness, high relative humidity (100%) and cooler temperatures (5-20°C) favouring sporulation.

Most of the infected leaves are short-lived, with the rate of leaf abscission depending on severity of infection. The estimated life span of an olive leaf is normally 2-3 years (Martin, 1994) but infected leaves may last much less, thereby reducing the photosynthetic potential of the tree and consequently yield (Verona and Gambogi, 1964; Graniti, 1993). Infected leaves drop onto the ground, where saprophytic fungi quickly colonise the leaves (Miller, 1949; Prota, 1958) and by 15 days the OLS lesions on fallen leaves will have ceased production of conidia (Guechi and Girre, 1994). Thus, it is the conidia produced by lesions on leaves remaining on the trees that initiate the onset of the disease cycle.

10.3 DIVERSITY IN SPILOCAEA OLEAGINA POPULATIONS

Studies on the DNA profiles and genetic variability of S. oleagina populations in New Zealand and overseas were addressed using RFLP, UP-PCR and sequence analysis (Chapter 6). The ITS1-5.8S-ITS2 sequences of all the isolates, including two from Italy and Australia were found to be similar. The low level of genetic diversity found within and among olive groves suggests that chemical control strategies are likely to be universally effective in New Zealand olive groves. The genetic uniformity also indicates that this fungus is likely to reproduce only by asexual method. This research is the first study on the population genetic structure of this pathogen.
10.4 DISEASE MANAGEMENT

Overseas researchers have shown that chemicals, such as difenoconazole, captan, dodine, tebuconazole and copper-containing fungicides, can be used to effectively control OLS in the field. Based on overseas work and in-vitro dose-response curves (Chapter 7), fungicides were selected and tested in the laboratory and field for efficacy in controlling OLS. Most of the fungicides tested were ineffective in reducing OLS leaf incidence in New Zealand groves. However, copper sulphate (Cuprofix disperss®) and a combination of kresoxim-methyl (Stroby®) and copper hydroxide (Kocide 2000) reduced the disease levels by up to 90 and 96%, respectively (Chapter 9). Timing of fungicide applications is known to be essential for effective control since fungicide applications must coincide with the flushes of new leaves which are highly susceptible to the disease (Wilson and Miller, 1949; Chapter 4). Under New Zealand conditions, four fungicide applications (two in autumn and two in spring) gave effective control of the disease in olive groves, in contrast to California where copper fungicides applied once in autumn before winter rains gave effective control (Teviotdale and Sibbett, 1995). This PhD research showed that spray applications in both autumn and spring are likely to be necessary for the effective management of the disease. However, further field studies are needed to investigate minimum withholding periods for fungicide applications so that fruits and oils do not contain excessive residues.

The OLS survey conducted by MacDonald et al. (2000) gave New Zealand olive producers an indication of the problem of the disease. However, until now, no report has shown the extent of the damage that may be caused by OLS in commercial New Zealand groves. OLS incidence was found to range from 5% leaf infection (one grove) to 100% leaf infection (several groves) where total defoliation, 100% fruit infection, and zero yields have been observed. Complete defoliation with no cropping was most frequently observed in Canterbury and Auckland regions. This is in sharp contrast to disease levels reported in other olive-growing regions of the world, which have warmer and dryer climates. In Spain, Italy and California, USA, OLS severity rarely exceeds 10% (Prota, 1995; Teviotdale and Sibbett, 1995). Understandably, control measures adopted in those countries are likely to be insufficient in New Zealand. However, New Zealand olive growers, to their detriment, have relied on control recommendations obtained from overseas countries. Therefore, it is not
surprising that, based on the combination of highly favourable disease conditions and inadequate control, OLS epidemics in New Zealand olive groves have been so severe.

Based on the results of this research, the following guidelines have been developed for the effective control of olive leaf spot in New Zealand:

1. It is difficult, if not impossible, to eradicate OLS once established on the trees. Therefore, it is essential to plant new groves with disease-free stocks. In order to achieve this, nurseries should ensure disease-free plant materials are used for propagation.

2. Growers should consider using less susceptible OLS cultivars e.g. ‘Leccino’ and ‘Frantoio’.

3. Olive leaf spot requires free moisture and high humidity for its development. Growers should, therefore, select regions with dry climates for their groves. They should also prune their olive trees regularly, at least once every two years, to produce narrow, airy trees that allow access to heat and light, which will reduce canopy moisture retention. In addition, open trees allow good penetration and coverage of fungicides, which also improves control. Growers should not prune in spring as this will encourage vegetative growth rather than flower development.

4. Olive growers should regularly (2-4 times/year) monitor olive groves for disease incidence so that they might assess disease progress and the need to apply fungicides. For easy identification of the disease and to detect latent infections, leaf samples should be dipped in 5% NaOH for 15-20 mins to reveal the characteristic dark spots on the leaves.

5. Cuprofix® disperss is the only registered fungicide for use on olives in New Zealand. It can be used by itself to control the disease effectively when incidence is low. When incidence is high (>10% of leaves infected), then it is more efficacious to use a combination of Stroby® WG and Cuprofix® disperss OR Stroby® WG and Kocide 2000. The application rate for Cuprofix® disperss is 500 g/100 L, Kocide 2000 is 190 g/100L and Stroby® WG is 10-15 g/100 L.

6. Spraying is most effective if done in early spring and in late autumn (apply not less than 4 weeks before harvest). At each of these spraying times, a second application of fungicide should ideally be applied 10-14 days later, but not once trees are in flower as copper may affect flower buds and fruit set. Residue tests should be conducted to ensure the safety and quality of the fruit and the oil.
10.5 FUTURE WORK

Much of the work presented in this thesis has already been discussed in terms of future research that could be done to further improve the understanding of OLS. The four key research areas that are most likely to improve olive productivity in New Zealand are:

- Development of a rapid screening method for disease resistance in olive varieties, based on the detached leaf assay (Chapter 2) and the potential correlation between leaf trichome density and susceptibility to OLS.
- Validation of predictive models for targeted disease management in the four major olive growing regions for the three most commonly used cultivars.
- Cultivar selection for resistance to OLS and frost tolerance. This may include marker selection for breeding for OLS resistance genes (Mekuria et al. 2001).
- Registration of fungicides which will include additional efficacy and residue tests.
- Development of economic disease thresholds and corresponding cost benefit analysis for disease control.

If these objectives are met they may significantly improve disease management and economic production of premium olives in New Zealand.

10.6 PROSPECT OF OLIVE PRODUCTION IN NEW ZEALAND

Olive oil is not an important part of the world’s diet. Between 1995 and 1999, olive oil accounted for only 3.7% of all the vegetable oils consumed annually and a mere 1.5% of all oils and fats (FAO, 2006). The world average consumption of olive oil per capita per year is only one third of a litre. In the late 1990s only thirty countries had an intake above the world average and eighteen of these were in the Mediterranean region, which accounted for 86% of all consumption. Little olive oil is currently consumed in Australasia, and in New Zealand it is increasing steadily with the intake in 2003 estimated at 2.9 g per capita per day, most of which was imported from Spain and Italy. The increased consumption could be attributed to a greater awareness of the health and culinary benefits of olive oil (FAO, 2006).

The major olive producing countries in 2004 were Spain and Italy, followed by Greece, Turkey and Tunisia with about 4.9, 4.5, 2.1, 1.6 and 0.65 million tonnes, respectively (FAO, 2006). Newly emerging producers of olives include Egypt, the USA, Libya and Argentina.
Although the first New Zealand olive trees arrived with the first European settlers, over 150 years ago, the trees and their fruit were considered to be obscure and exotic (Lysar, 2000). In New Zealand, widespread cultivation of olives for commercial purpose began in the 1990s but by 2005 had increased to 2,350 hectares of olives, comprising approximately 850,000 trees (Statistics New Zealand, 2006). Most olives grown are processed for oil, an estimated 49,500 litres being produced in New Zealand in 2002.

Olive production thrives in climates with warm and dry summers, but very high summer temperatures adversely affect yield, as does high rainfall (Grigg, 2001). The trees can tolerate some frost, but not sustained low temperatures. These climatic requirements for olive cultivation contrast sharply with New Zealand’s temperate climate dominated by cool, wet springs and summers, and frosty winters. Olive production in the New Zealand climate is at best ‘marginal’ and so it is not surprising that olive productivity is low in New Zealand. In Italy, for example, a mature irrigated olive tree (9 –12 years) produces 50 kg of fruit to yield 20% oil (Tosi and Zazzerini, 2000), whereas in New Zealand, 9-year-old irrigated olive trees produce about 10 kg of fruit, yielding 13% of oil (Lysar, 2000).

Presently, there are over 50 different olive varieties available in New Zealand. The choice of a cultivar for a particular region is important, especially under marginal production conditions. Unfortunately, most of the early commercial groves were planted with olive cultivars whose environmental requirements for optimum productivity and disease susceptibility were unknown. The most commonly cultivated olive variety in New Zealand, particularly in the Canterbury region, is ‘Barnea’, which was imported from Israel. Ironically, this cultivar is highly susceptible to OLS and frost. Other varieties found in New Zealand groves include, Frantoio and Leccino, which originated from Italy. The poor cultivar and site selections have impacted significantly on the productivity of olives in New Zealand and the high OLS levels.

In spite of low productivity, olive oil produced in New Zealand is of very high quality, with most oil meeting the “extra virgin” criteria. It is worth noting that only 5% of the worldwide olive oil can be classified as “extra virgin”, compared to 95% of the New Zealand olive oils. In May 2005, New Zealand olive growers won nine gold medals and 11 silver medals at the biggest and one of the most prestigious international competitions at the Los Angeles County Fair, California. New Zealand growers submitted 26 of the 366 entries, which came mainly
from more traditional olive growing countries such as Italy, Greece and Spain (Anonymous, 2006). The outstanding olive oil quality and the resulting premiums achieved by selling to niche markets make olive oil production in New Zealand attractive and counteracts the low yields due to disease, frost and other production limitations associated with restraints of the New Zealand climate and geography.

10.7 REFERENCES


CHAPTER 11

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PUBLICATIONS/PRESENTATIONS FROM THIS THESIS

PAPERS:


POSTERS:


PRESENTATIONS:

Appendices

Appendix 1.1: Olive leaf extract (OLE) agar protocol
- Place 20 g of fresh olive leaves in 500 mL of dH₂O in a beaker
- Boil for 20 min and filter through cheesecloth
- To the filtrate add 10 g of glucose, 20 g granulated agar and make up to 1 L
- Autoclave and cool the agar to about 50°C and then add antibiotic (chlortetracycline, 10 mg/L of agar) before pouring the agar

Appendix 2.1: Germination of *Spilocaea oleagina* conidia on olive leaves

Appendix 2.2: Percentage germination of *Spilocaea oleagina* conidia on three different media and at different temperatures after 48 h of incubation

<table>
<thead>
<tr>
<th>Medium</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Water agar</td>
<td>5^a</td>
</tr>
<tr>
<td>Potato dextrose agar (PDA)</td>
<td>15</td>
</tr>
<tr>
<td>Olive leaf extract agar</td>
<td>22</td>
</tr>
</tbody>
</table>

^aValues are means of three experimental repeats with three replicate plates for each inoculum concentration and 100 conidia were counted from each plate.
Appendix 3.1: Germination of *Spilocaea oleagina* conidia on OLE agar at different inoculum concentrations at 20°C after 48 h of incubation

<table>
<thead>
<tr>
<th>Concentration (×10⁴ conidia/mL)</th>
<th>Germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>62ᵃ</td>
</tr>
<tr>
<td>0.10</td>
<td>58</td>
</tr>
<tr>
<td>5.0</td>
<td>55</td>
</tr>
<tr>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>100</td>
<td>52</td>
</tr>
<tr>
<td>500</td>
<td>41</td>
</tr>
<tr>
<td>1000</td>
<td>33</td>
</tr>
</tbody>
</table>

ᵃValues are means of three experimental repeats with three replicate plates for each inoculum concentration and 100 conidia were counted from each plate.

Appendix 4.1: *Spilocaea oleagina* conidium production at various temperatures and under continuous wetness (CW), 70% RH and 100% RH

<table>
<thead>
<tr>
<th>Incubation period (days)</th>
<th>Moisture regimeᵃ</th>
<th>Mean number of conidia/mm²ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Temperature (°C)</td>
</tr>
<tr>
<td>3</td>
<td>CON</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>HRH</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>LRH</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>CON</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>HRH</td>
<td>177</td>
</tr>
<tr>
<td></td>
<td>LRH</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>CON</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>HRH</td>
<td>709</td>
</tr>
<tr>
<td></td>
<td>LRH</td>
<td>69</td>
</tr>
<tr>
<td>14</td>
<td>CON</td>
<td>369</td>
</tr>
<tr>
<td></td>
<td>HRH</td>
<td>904</td>
</tr>
<tr>
<td></td>
<td>LRH</td>
<td>109</td>
</tr>
</tbody>
</table>

ᵃCON = continuous wetness, HRH = high relative humidity (100%) and LRH = low relative humidity (70%).

ᵇValues are mean numbers of conidia/lesion area determined on 18 leaves (3 plants/replicate and 6 leaves/plant) averaged over the two experimental repeats.