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Temperature, wetness period and inoculum concentration influence infection of canola (*Brassica napus*) by pycnidiospores of *Leptosphaeria maculans*

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Abstract. Infection of canola (*Brassica napus*) by pycnidiospores of the blackleg fungus (*Leptosphaeria maculans*) and subsequent development of leaf and stem infection were influenced by temperature, wetness period and inoculum concentration. On cv. Hyola 42, as temperature decreased the latent period for leaf infection increased exponentially. The greatest number of leaf lesions developed on plants exposed to a day/night temperature of 18/15°C with a 96 h wetness period. Incidence of stem infection was greatest at 23/20°C with a 48–72 h wetness period, whereas no stem infection occurred 42 days after inoculation when plants were incubated at 8/6°C. This is the first report of the effect of temperature and wetness period on infection by pycnidiospores. The number of leaf lesions and incidence of stem cankers were greatest when plants were inoculated with 10^6 or 10^7 pycnidiospores/mL, respectively, the highest concentrations used in the experiments. The susceptible cv. Q2 developed significantly more leaf and stem lesions than the less susceptible cvv. Monty and Dunkeld when inoculated with 10^7 pycnidiospores/mL, but not at the lower concentrations. The moderately resistant cv. Dunkeld developed significantly fewer stem cankers than the other cultivars when inoculated with pycnidiospores at concentrations greater than 10^4/mL.

Additional keywords: epidemiology, oilseed rape, *Phoma lingam*, phoma stem canker, conidia.

Introduction

The most important disease of canola (*Brassica napus* var. *oleifera*) in Australia is blackleg, caused by the fungus *Leptosphaeria maculans* (anamorph *Phoma lingam*), and it has the potential to devastate crops in an epidemic (Bokor *et al*. 1975). Blackleg occurs worldwide and is of major economic importance in the main oilseed growing areas of Europe and Canada (West *et al*. 2001). Ascospores, the primary inoculum, are spread predominantly by wind, whereas pycnidiospores (conidia), the secondary inoculum, are spread mainly by rain-splash (Hall 1992). Ascospores are more efficient at inducing disease than pycnidiospores (Wood and Barbetti 1977). However, Barbetti (1976) demonstrated that pycnidiospores could play a significant role in establishment of leaf, crown and stem infections in Australia. Hammond *et al*. (1985) inoculated leaves of oilseed rape plants with both pycnidiospores and ascospores, and reported that hyphal growth on the leaf surface, penetration of the stomata, and systemic colonisation of the leaf, petiole and stem were similar irrespective of the type of spore. According to the review by West *et al*. (2001), infection caused by pycnidiospores is more common in Australia than in Europe and Canada.

The incidence and severity of blackleg can vary from season to season and between regions (West *et al*. 2001; Sosnowski *et al*. 2004), reflecting the influence of environment on disease development. Temperature and wetness period affect the germination of ascospores and pycnidiospores, as well as the colonisation of canola leaves and stems. Hall (1992) reported that optimal conditions for germination of ascospores were 28°C with at least 4 h of continuous wetness, and 24°C with at least 16 h of continuous wetness for germination of pycnidiospores. Maximum leaf infection required 20°C and 48 h of leaf wetness (Biddulph *et al*. 1999; Toscano-Underwood *et al*. 2001), whereas above or below this temperature, the number of leaf lesions decreased. Stem cankers were most severe when temperatures ranged between 20 and 24°C (Barbetti 1975; McGee and Petrie 1979; Gladders and Musa 1980).

Pycnidiospores have been used as inoculum in previous studies (Sosnowski *et al*. 2001) as they are readily produced

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in vitro and enable individual isolates to be investigated. Wood and Barbetti (1977) found that the concentration of pycnidiospores had little effect on disease incidence, whereas Hammond and Lewis (1987) showed that increasing the concentration of pycnidiospores could increase disease incidence and severity. This contradiction may be due to differences in methodology and highlights the need for further investigation. Pycnidiospores are able to infect unwounded leaves reliably when applied at a concentration of 10^6 pycnidiospores/mL, but infection is variable at lower concentrations (Wood and Barbetti 1977; Hammond *et al*. 1985; Vanniasingham and Gilligan 1989; Sosnowski *et al*. 2001).

Temperature, moisture and inoculum concentration are important in the development of an epidemic of blackleg. This paper describes the response of the moderately susceptible cv. Hyola 42 to infection by *L. maculans* under a range of controlled conditions and the effect of pycnidiospore concentration on three canola cultivars differing in blackleg resistance.

Methods

Preparation of inoculum and plant material

L. maculans isolate 66/97 was obtained from a leaf lesion on canola in South Australia (Sosnowski *et al*. 2001). This aggressive A-group (virulent, Tox+) isolate was chosen to represent the population of *L. maculans* in Australia. Agar plugs containing mycelia of *L. maculans* were retrieved from storage at 3–4°C in sterile distilled water and cultured on quarter-strength potato-dextrose agar (1/4PDA; Oxoid). The cultures were incubated at room temperature (~22°C) for 2 weeks under blacklight (Hitachi 8W/BL350) and fluorescent light (Phillips 18W/W43) for 12 h each day. Suspensions of pycnidiospores were prepared by flooding plates with ~10 mL of sterile distilled water plus 0.05% Tween 20 (BDH Laboratory Supplies) as a surfactant, followed by scraping of culture surfaces with a bent glass rod; spores were then counted using a haemocytometer.

All canola plants were initially grown in pasteurised potting mixture (Etebarian *et al*. 2000) in containers on benches in a glasshouse (18–29°C, in natural light) and watered by hand with tap water until plants reached GS 2.3 (Harper and Berkenkamp 1975).

Effects of temperature and wetness period

The trial was conducted in an Environ Air controlled environment chamber (100 × 120 × 145 cm, with fan-forced refrigeration) to test the effect of temperature and wetness period on infection of cv. Hyola 42 by *L. maculans*. Light was provided by four metal halide lights (Venture HIE 150W/C/U) and two cool white fluorescent tubes (VHO Sylvania 115W-F48T12) which were set at a 12-h photoperiod and controlled by a Theben (WF 64CA7) timer. Temperature was kept constant using an RKC REX-C100 temperature controller. Temperature and relative humidity were monitored using a data logger (Tinytag plus, Hasting Data Loggers). Only one controlled environment chamber was available for this study and, while efforts were made to ensure that plants were uniform, it was accepted that some variation may have occurred due to seasonal variation as a result of growth in natural light prior to inoculation.

The experiment was arranged as a split plot design with two replications, using plants at GS2.3 which were inoculated with a suspension of 10^6 pycnidiospores/mL by spraying plants with a hand-held sprayer until run-off occurred. Four temperature regimes, 8/6°C day/night, 13/10°C, 18/15°C or 23/20°C, formed the main plots and were imposed consecutively, in random order, in the environment chamber. For each temperature regime, plants were exposed to seven wetness periods, forming the sub-plots, produced by covering plants with plastic bags immediately after inoculation then removing them 3, 9, 24, 48, 72 or 96 h later, with the control left uncovered (0 h). Twelve plants, three in each of four pots (140-mm-diameter) were exposed to each of the wetness periods. The pots were arranged randomly, in white trays and tap water was poured into the trays every few days for 6 weeks to keep the potting mixture moist.

Plants were briefly checked daily and the number of days to the appearance of the first leaf lesions was recorded only in the second replication. In both replications, plants were assessed in detail at 7-day intervals after inoculation by counting the total number of leaf lesions at the weekly assessment time which followed the first observation of lesions (1–7 days after the first lesion was observed), after which time lesions began to coalesce and leaves to senesce. The number of plants with external stem infection (lesions occurring on any part of the stem) was counted 42 days after inoculation. The relationship between the mean number of leaf lesions and wetness periods was analysed by linear regression separately for each temperature regime, and the mean number of plants with stem infection was compared among treatments by analysis of variance using Statistix for Windows (Anonymous 1994).

Effect of inoculum concentration

A series of pycnidiospore suspensions was prepared in order to determine the effect of concentration on the incidence of leaf and stem disease. By means of a series of dilutions in sterile distilled water, concentrations of 10^6, 10^5, 10^4 and 10^3 pycnidiospores/mL were achieved.

The experiment was arranged as a split plot design with plants of three cvv.; Q2 (susceptible, from Canada), Monty (moderately susceptible) and Dunkeld (moderately resistant) (Potter and Stanley 2002) forming the main plots and three replications. Nine punnets (900 mL), each containing two plants at GS 2.3 in the potting mixture, were randomly arranged in trays so that each tray contained three punnets of each cultivar. Each tray of plants was sprayed evenly with 25 mL of pycnidiospore suspension at one of the five concentrations which formed the sub-plots, and then moved into humidity tents in the glasshouse for 4 days, after which the tents were opened to reduce humidity.

The number of leaf lesions on each plant was recorded 14 days after inoculation, after which time lesions began to coalesce and leaves to senesce. The number of plants with stem canker (stem completely girdled at the crown with necrotic tissue) was recorded 37 days after inoculation. Data were subjected to analysis of variance and the mean number of leaf lesions and percentage of plants with stem canker were compared among inoculum concentrations and cultivar using Statistix for Windows (Anonymous 1994).

Results

Effects of temperature and wetness period

The latent period declined as temperatures increased. Leaf lesions were first detected in the second replication on plants of cv. Hyola 42 25 days after inoculation at the day/night temperature of 8/6°C, 16 days at 13/10°C, 9 days at 18/15°C and 6 days at 23/20°C. The relationship between mean number of leaf lesions and wetness period for each of the four temperature regimes at selected times after inoculation, *viz* before lesions began to coalesce, is shown

in Fig. 1. At the higher temperatures, 18/15°C and 23/20°C, the slope was greater (0.24 and 0.17, respectively) than at the lower temperatures of 8/6°C and 13/10°C (0.05 and 0.03, respectively). The greatest number of lesions (33) was recorded at the temperature regime of 18/15°C with a wetness period of 96 h.

Although there was no interaction between temperature regime and wetness period, there were significant

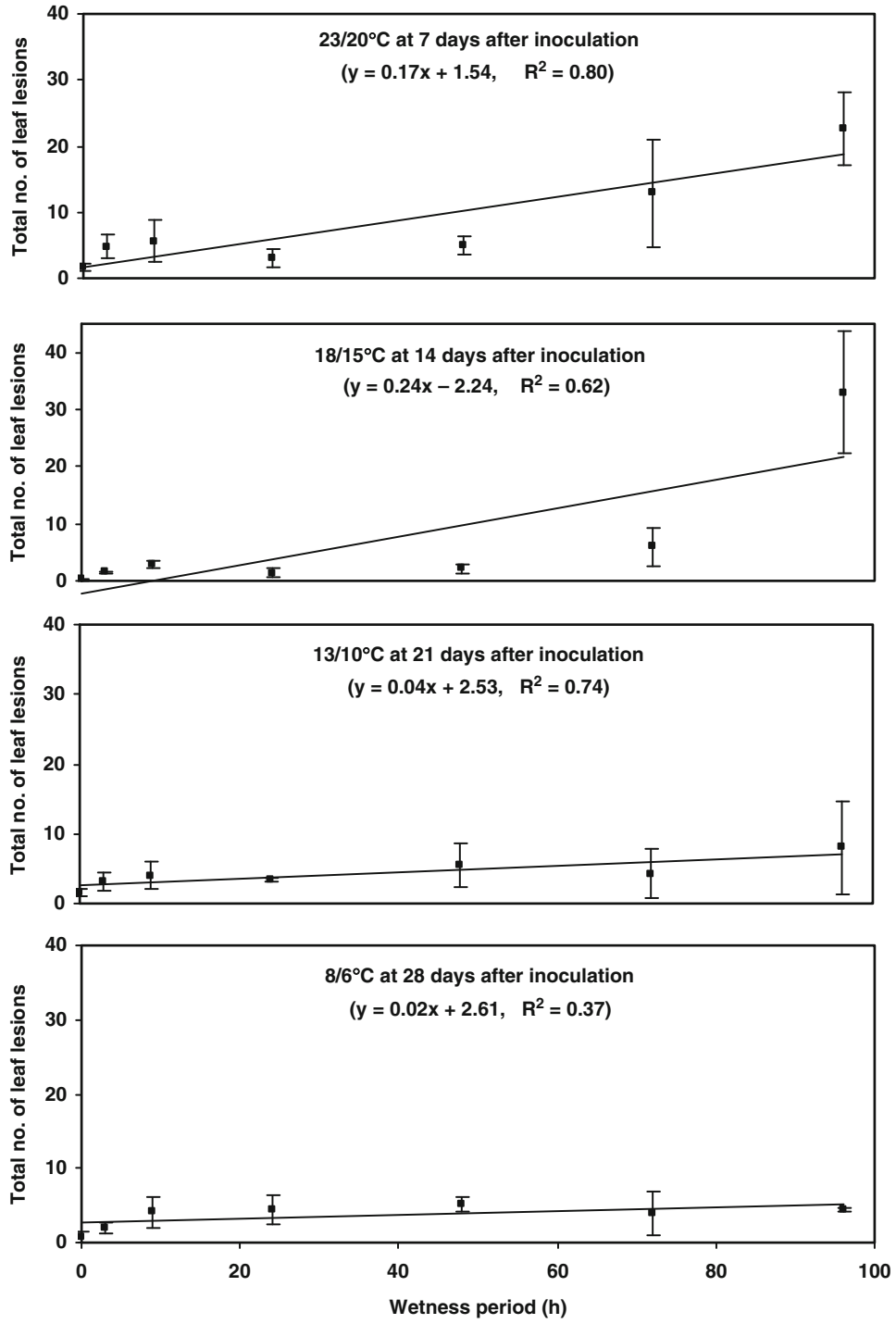

Fig. 1. Relationship between the total number of leaf lesions which developed on 12 canola plants (cv. Hyola 42) and wetness period for four temperature regimes (day/night) at certain times after inoculation with pycnidiospores of *Leptosphaeria maculans* isolate 66/97. Bars represent standard error of the mean.

differences ($P < 0.05$) in the number of plants with stem infection among both temperatures and wetness periods 42 days after inoculation (Fig. 2). At 8/6°C, stem infection did not occur. At 13/10°C, stem lesions were observed only for the 96 h wetness period, and on 10% of the plants. At the two higher temperatures, as the wetness period increased from 0 to 96 h, the incidence of stem infection increased from 10 to 60% for 18/15°C and from 20% to over 80% for 23/20°C.

Effect of inoculum concentration

The number of leaf lesions per plant 14 days after inoculation differed significantly ($P < 0.05$) among the five concentrations of pycnidiospores as well as among the three cultivars (Fig. 3). Very few leaf lesions developed at the two lowest concentrations (10^3 and 10^4 pycnidiospores/mL water) on all cultivars and then for each 10-fold increase in concentration, the mean number of leaf lesions on all cultivars increased. At the highest concentration, 10^7 pycnidiospores/mL, there was a significant difference between cv. Q2 and cvv. Monty and Dunkeld, with ~12 and six leaf lesions per plant, respectively; however, these differences were not observed at lower inoculum concentrations.

There was a significant interaction ($P < 0.05$) between pycnidiospore concentration and the incidence of stem canker for the different cultivars 37 days after inoculation. At the two lowest concentrations (10^3 and 10^4 pycnidiospores/mL), stem canker developed on fewer than 20% of the plants, and there was no infection on cv. Dunkeld (Fig. 4). As the concentration of pycnidiospores/mL increased from 10^5 to 10^6, the incidence of stem canker for cvv. Monty and Q2 increased from ~40–50% to 70–80% but there was little or no further increase when 10^7 pycnidiospores/mL were used. For cv. Dunkeld, stem canker developed on 10% of plants at 10^5 to 10^6 pycnidiospores/mL and then increased to 20% at 10^7.

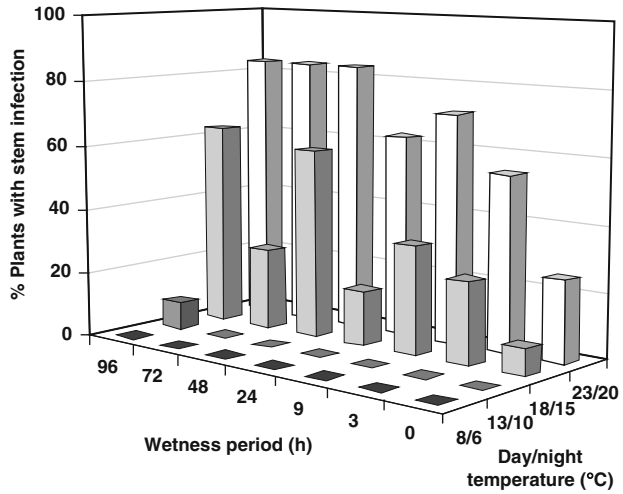

Fig. 2. Effect of temperature (day/night) and wetness period on the percentage of plants (cv. Hyola 42) with stem infection, 42 days after inoculation with pycnidiospores of *Leptosphaeria maculans* isolate 66/97. $LSD_{0.05}$ (temperature) = 10, $LSD_{0.05}$ (wetness period) = 14.

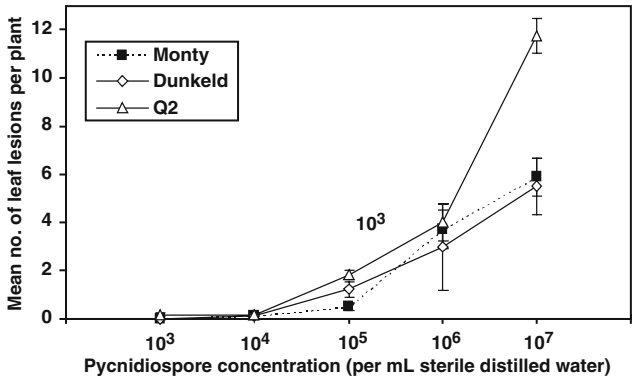

Fig. 3. Effect of pycnidiospore concentration and canola cultivar on the mean number of leaf lesions per plant, 14 days after inoculation with *Leptosphaeria maculans* isolate 66/97, with six plants per treatment. Bars represent standard error of the mean.

Discussion

The decrease of latent period as temperature increased suggested that temperature is important in the infection of canola by pycnidiospores of *L. maculans*. Biddulph *et al*. (1999) found that ascospore-derived leaf lesions occurred 5 days after inoculation at 20°C and after 14 days at 8°C; in this experiment, the predicted latent period was similar at 20°C, but was 25 days at 8°C. This difference may reflect the type of inoculum (ascospores *v.* pycnidiospores) or isolate used in each study, differences in blackleg susceptibility of cv. Nickel, used in the previous study, compared with cv. Hyola 42 or the effect of the night temperature of 6°C in this experiment *v.* 8°C in the experiment by Biddulph *et al*. (1999).

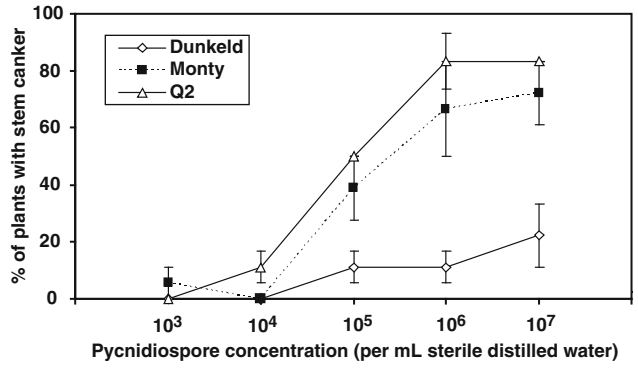

Fig. 4. Effect of pycnidiospore concentration and canola cultivar on the incidence of stem canker, 37 days after inoculation with *Leptosphaeria maculans* isolate 66/97, with six plants per treatment. Bars represent standard error of the mean.

This study revealed that, at temperatures below 13/10°C, wetness period had little effect on the development of leaf lesions. The optimal day/night temperature and wetness period regime for leaf infection in the present study was 18/15°C and 96 h. In comparison, Biddulph *et al*. (1999) showed that the optimal conditions were 20°C with a 48 h wetness period and Toscano-Underwood *et al*. (2001) reported optimal conditions of 15 to 20°C with a 48 h wetness period; both studies involved ascospore-derived infection. In a previous field study, Sosnowski *et al*. (2004) showed that increased rainfall during the season, which is related to wetness period, could increase the incidence of stem canker. The optimal temperature in this study corresponded with that found in past studies, but wetness period did not. There is little information on the effects of temperature and wetness period on development of pycnidiospore-derived infection highlighting the value of this study. As 96 h was the longest wetness period tested in this experiment, it is not known if the amount of infection would have increased further with a longer wetness period. The difference in optimal wetness periods could be due to the type of inoculum, isolate, cultivar resistance to blackleg or timing of disease assessment, which differed for each study. A small amount of leaf infection was detected on plants subjected to no wetness period (0 h) and a 3-h wetness period. This was expected, as the controlled environment chamber ranged from 60 to 100% relative humidity, depending on the amount of water present in the trays underneath the pots. The inoculum suspension would have contributed to a short wetness period for the control as well. As plants were watered immediately after inoculation, humidity would have ranged from 80 to 100% over the following 24 to 48 h, especially in darkness.

In this study, it was assumed that stem infection originated from leaf infection, via the petioles, since direct infection via the stem requires wounding (Hammond *et al*. 1985). The optimal conditions for development of stem infection were 23/20°C with a 48–72 h wetness period. Likewise, previous studies revealed that the optimal temperature for stem infection ranged between 20 and 24°C (Barbetti 1975; McGee and Petrie 1979). At 18/15°C, there was a reduced incidence of stem infection for the 24 h and 72 h wetness periods, compared with 9 h and 48 h, which may be due to experimental error, such as the plastic bags being incompletely sealed and hence not maintaining 100% relative humidity following inoculation. No stem infection occurred at 8/6°C in this study, although a previous study showed that infection developed at 8°C, albeit less severely than at 12 and 15°C (McGee 1977). It is possible that, had a longer incubation period been imposed in this study, stem infection may have developed. Even at 13/10°C, only a few plants subjected to the 96 h wetness period developed stem infection. These results highlight the importance of temperature in the development of stem infection. One possible reason for the greater prevalence and severity of stem canker in Australia than in Europe and Canada is that average temperatures are greater in Australia (West *et al*. 2001; Purwantara *et al*. 2000).

The concentration of pycnidiospores had a significant effect on both leaf infection and stem canker, which confirmed the findings of Hammond and Lewis (1987). However, an assessment of the viability of pycnidiospores at inoculation would be needed to confirm this, in case germination percentage varied. The greatest inoculum concentration used in this study (10^7 pycnidiospores/mL) resulted in the most leaf infection and this appears to be a suitable concentration of pycnidiospores to maximise leaf infection. In a previous study (Sosnowski *et al*. 2004), it was concluded that there was no resistance in the cultivars tested to leaf infection by pycnidiospores in the glasshouse. In that study, inoculum was applied at 10^6 pycnidiospores/mL and the results correspond with the present study at the same concentration. The differences in leaf infection on the various cultivars when using the highest inoculum concentration indicated that differences in leaf resistance may become apparent under very high inoculum pressure. In this study, leaves were not wounded before inoculation, which may have resulted in less leaf infection than if they had been wounded because hyphae penetrate through stomata and wounds (Chen and Howlett 1996).

According to this study, a concentration of 10^6 pycnidiospores/mL will result in 60 to 80% incidence of stem canker in susceptible cultivars. The resistant and susceptible cultivars could be distinguished on the basis of the incidence of stem canker resulting from inoculation with pycnidiospores. However, in the current study, cv. Q2 had increased levels of leaf infection and stem canker only at the higher concentrations. Thus, for growing regions in which there is exposure to smaller amounts of inoculum, such as lower rainfall regions, it would be possible to grow less resistant cultivars, which may have advantages such as early maturity or increased yield. In regions where the potential for inoculum production is increased, it would be important that resistant cultivars be used to minimise yield loss due to blackleg.

This study has revealed the importance of temperature, wetness period and inoculum concentration in the development of infection of canola by pycnidiospores of *L. maculans*. Further experiments to compare cultivars with a range of blackleg resistance would be necessary to determine if there is any interaction between cultivar and temperature or wetness period. Furthermore, a range of isolates could be tested to determine if any interaction exists between isolate and temperature or wetness period.

The ability of pycnidiospores to infect unwounded canola leaves and eventually cause stem canker in this study highlights the importance of secondary infection by *L. maculans* in Australia. The application of Impact

(flutriafol) fungicide at sowing has been shown to reduce infection of canola seedlings by *L. maculans* (Khangura and Barbetti 2002), which, in turn, would lead to an increase in yield (Sosnowski *et al*. 2004). Foliar fungicides also could be used to manage secondary infection by pycnidiospores, but this would require field evaluation in Australian conditions.

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