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Maturation of pseudothecia and discharge of ascospores of *Leptosphaeria maculans* on oilseed rape stubble

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Abstract The effects of temperature, wetness and darkness on formation of pseudothecia and the effect of temperature on the release of ascospores of *L. maculans* on oilseed rape stubble were studied in a controlled environment in South Australia. Pseudothecia of *L. maculans* developed at 5–20°C and the time taken to reach maturity and discharge ascospores decreased from 58 days at 5°C to 22.2 days at 15°C. The optimum temperature of those tested for pseudothecium maturation was between 15°C and 20°C but fewer pseudothecia were observed at 20°C than at 15°C. Exposure to a 12 h photoperiod enhanced pseudothecium formation on the stubble compared with continuous darkness. No pseudothecia formed on stubble moistened once a day at 15°C, whereas three sprays of water per day decreased maturation time in comparison with two sprays per day. More ascospores were released for a longer duration at 20°C than at 5–15°C, although peak sporulation occurred earlier at 5–10°C than at 20°C. These findings highlight the importance of moisture, temperature and light for production and release of inoculum from stubble. This information, combined with field data, may help to predict the onset of inoculum release.

Keywords Blackleg · Canola · Darkness · Phoma stem canker · Temperature · Wetness

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Introduction

Phoma stem canker (blackleg), caused by *Leptosphaeria maculans,* is an important disease of oilseed rape (*Brassica napus* ssp. *oleifera*) in Australia, Canada and Europe. Inoculum of *L. maculans* develops on infested oilseed rape stubble left in the field after harvest. Ascospore discharge occurs after wetting of stubble by rain and dew at appropriate temperatures (Guo and Fernando 2005; McGee 1977; Pérès and Poisson 1997). In Australia, harvest is generally in spring or early summer (November–December), and ascospore release begins in May in response to winter rainfall, which also stimulates the emergence of oilseed rape seedlings (McGee 1977). McGee and Petrie (1979) determined patterns of ascospore discharge in Victoria, Australia, using a Burkard spore trap, continuously sampling air at the rate of 10 l min^{-1} onto slides coated with ovalbumin. Ascospore capture often peaked in June—August

when temperature was 8–12°C and rainfall >1 mm (McGee and Petrie 1979). Salam et al. (2003) reported that ascospore release in Western Australia began at an hourly rainfall of >2 mm. The optimum temperature for ascospore production by *L. maculans* was 15°C and peak sporulation occurred at 20°C in Canada (Petrie 1994) and, in France, a mean temperature of 14°C and 2.5 mm rainfall every 3–4 days in the field, or 100% relative humidity (RH) in a controlled environment, were the most suitable conditions for the appearance of pseudothecia on oilseed rape stubble (Pérès et al. 1999). At 10°C, mature pseudothecia were not observed, whereas at 14, 16, 18 and 20°C pseudothecia matured after 14, 17, 21 and 34 days, respectively, in 95–100% RH in a controlled environment (Pérès et al. 1999). The time taken for pseudothecia of *L. maculans* to mature on naturally infested stubble decreased nearly linearly when temperature increased from 5 to 20°C in continuous wetness in a controlled environment but was longer in natural conditions, particularly in dry weather in the UK (Toscano-Underwood et al. 2003). In south-eastern Australia (Marcroft et al. 2003) and Western Australia (Salam et al. 2003), more ascospores were discharged earlier in regions with higher rainfall than those with less.

Although forecasting models have been developed to predict the onset of maturity of pseudothecia in France (Bernard et al. 1999) and Western Australia (Salam et al. 2003), collection of climatic and biological data is required in South Australia to develop or adapt a forecasting model for this state. Furthermore, although the potential of host resistance to reduce production of pseudothecia and ascospores on stubble has been demonstrated (Marcroft et al. 2004), the effects of interrupted wetness and darkness on inoculum production on stubble are little understood. Although burial of stubble in soil reduces production of pseudothecia of *L. maculans* (Huang et al. 2003; Kharbanda and Ostashewski 1997; Naseri et al. 2008b), the influence of darkness has not been distinguished from other physical, chemical and biological aspects of burial.

We examined the influence of temperature, darkness, continuous and discontinuous wetness on pseudothecium maturation on stubble, collected in South Australia, in a controlled environment. The effect of temperature on ascospore discharge was also studied.

Materials and methods

Effect of temperature on pseudothecium maturation in continuous wetness

Stubble of oilseed rape was obtained from a phoma stem canker disease nursery at Struan, in the south-east of South Australia in January 2004, shortly after harvest, as described by Naseri et al. (2008b). The stubble comprised stem and root pieces from a mixture of commercial cultivars and breeding lines with phoma stem canker-resistance rating ranging from 1 to 9, where 1 = greatest susceptibility and 9 = immunity (Potter and Stanley 2002). The stubble was dried and stored at room temperature (approx. 24°C). Pieces of tap root and stem base (*ca* 20–30 cm in length) with phoma stem cankers (without pseudothecia) were soaked in sterile distilled water (SDW) for 12 h then placed in plastic trays lined with moist, sterile sand, as described by Naseri et al. (2008b). Ten replicate stubble pieces were placed in a tray for each temperature treatment. Each tray was placed in a plastic bag that had been sprayed inside with distilled water to maintain 100% RH. Trays were placed in one of two incubators (Glyson Germination Incubator, Crown Scientific Pty Ltd) adjusted, sequentially, to 5, 10, 15 or 20°C with a 12 h photoperiod (fluorescent NEC T8 FL20SSBR/18-HG, Japan; black light NEC T5 8W/FL8BL, Japan).

Development of pseudothecia was assessed every 5 days until most had matured, as follows. Five pseudothecia were cut randomly from each of the 10 stubble pieces and placed, separately, in a drop of water on a glass slide. Gentle pressure was applied to force asci from the pseudothecia. Each pseudothecium was then grouped into one of five maturity classes (A, B, C, D or E) using the simplified scale described by Naseri et al. (2008b), adapted from those of Bernard et al. (1999) and Toscano-Underwood et al. (2003). In brief, A = pseudothecia and asci immature, ascospores absent; B = pseudothecia immature, asci <8 spores and ascospores <5 septa; C = pseudothecia immature, asci with 8 spores and ascospores with 5 septa; D = pseudothecia and asci mature, ascospores 5-septate, constricted at first septum and yellow; and E = ascospores discharged. At the last assessment, the density of pseudothecia on each piece was assessed as follows. A segment of 0.5×1 cm was cut from each piece and pseudothecia

were counted using a dissecting microscope at 25× magnification. The average density of pseudothecia in each tray was calculated as the mean density on the 10 stem pieces. The experiment was conducted three times.

Effect of darkness on pseudothecium maturation in continuous wetness

Ten stubble pieces were placed on sand in one tray inside a moistened plastic bag, as above, then covered with foil. The tray was incubated at 15ºC in darkness, so that the stubble received no light except when assessed for pseudothecia. Development of pseudothecia, the number of pseudothecia at each stage of maturity and the density of pseudothecia on each piece of stubble were assessed as above. The experiment was conducted three times.

Effect of discontinuous wetness on pseudothecium maturation

Ten stubble pieces were placed on sand in each of three trays as above, but without plastic bags, and incubated at 15°C with a 12 h photoperiod. One tray was sprayed with distilled water from a hand-held sprayer, to produce a thin film of water on the stubble, once a day (at 09.00 h), the second was sprayed twice (at 09.00 h and 17.00 h), and the third three times a day (at 09.00 h, 13.00 h and 17.00 h). Development of pseudothecia, maturity and density were assessed as above. The experiment was conducted three times.

Effect of temperature on ascospore release in a controlled environment

Stubble incubated at 15°C in the first experiment described above was air-dried for 24–48 h and stored in polypropylene bags at approximately 22°C until use. Six stubble pieces (3 cm long, with mature pseudothecia) were attached to the underside of each of 36 Petri dish lids using petroleum jelly. These were flooded with SDW for 15 s, the water poured off and the lid placed over the base (Naseri et al. 2008a). Nine replicate Petri dishes were incubated at each of 5, 10, 15 and 20°C. Ascospore discharge was assessed as follows, until no more ascospores were detected. Every 2 h, the base of each Petri dish was replaced with a fresh base, so that the lids and stubble pieces remained inside the incubator for the duration of experiment. Then 1 ml SDW was added to each Petri dish base, and the ascospores suspended using an artist's brush. Ascospores in three replicate 25-µl aliquots of the resulting suspensions were counted using a light microscope. In addition, the duration of ascospore discharge at each temperature was recorded.

Statistical analysis

The incubation time (days) required to reach each stage of differentiation of pseudothecia was recorded as the day when the number of pseudothecia at each stage of maturity reached its maximum at each temperature. ANOVA using GenStat version 6.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, 2002) was used to examine differences in the time taken to reach each stage of maturity and the differences in density of pseudothecia on stubble incubated in a 12 h photoperiod, in darkness under continuous wetness at 15°C, and in discontinuous wetness (K. Dowling, pers. com., 2005). Data for the time taken for pseudothecia to mature at different temperatures in continuous wetness in a 12 h photoperiod were used to develop a polynomial regression curve.

Data for the number of ascospores released into Petri dishes were $\log_e$-transformed before analysis to improve the homogeneity of the data. The effect of temperature on ascospore discharge was analysed by linear mixed models (REML) using GenStat version 8.2 (Lawes Agricultural Trust, 2005). REML was used due to the unbalanced experimental structure (unequal number of sampling times at different temperatures), and Wald tests (GenStat version 8.2) were used to examine temperature and time effects. Mean values for $\log_e$-transformed data were calculated and back-transformed.

Results

Effect of temperature on pseudothecium maturation in continuous wetness

Pseudothecia of *L. maculans* differentiated and matured at 5–20°C in continuous wetness and a 12 h photoperiod. Temperature significantly affected

(LSD=6.8; $P<0.05$) the mean incubation time required for pseudothecia to reach each stage of maturity (Fig. 1). The mean time required to reach each stage of maturity was longer ($P < 0.05$) at lower than higher temperatures over 5 to 15°C and was similar ($P>0.05$) at 15 and 20°C. The mean time required for immature pseudothecia to appear on stubble (stage A) decreased ($P<0.05$) from 29.3 days at 5°C to 8.5 days at 15°C and was similar for 15 and 20°C. The incubation time for pseudothecia to reach stage D decreased ($P<0.05$) from 58.3 days at 5°C to 22.2 days at 15°C and was 25.7 days at 20°C. The polynomial regression curve of mean incubation time ($R^2=1$) until the number of pseudothecia was maximal at stage D is shown in Fig. 2. The mean time required for pseudothecia to release ascospores (stage E) decreased ($P<0.05$) from 64 days at 5°C to 28.5 days at 15°C and was 31.7 days at 20°C (Fig. 1). At each stage of maturity, the difference in the incubation time was more marked when comparing 5°C with 10°C than 10°C with 15°C or 10°C with 20°C.

At 5°C, the mean incubation time required for transition from stage A to B and stage B to C increased ($P<0.05$) from 29.3 to 45.3 days and from 45.3 to 54 days at stage C, respectively, and was similar from C to E (Fig. 1). At 10–20°C, the mean incubation time required for transition from each stage of maturity to the next was similar, except for transition from stage B (reached at 24.7 days) to stage C (reached at 35.7 days) at 10°C and from stage A (8.7 days) to stage B (15.7 days) at 20°C.

Pseudothecial development proceeded through stages A, B, C, D and E in continuous wetness at 5–20°C (Fig. 3). The average maximum number of pseudothecia at stages A, B and C observed in the three repetitions of the experiment was greater at 5°C than at 10–20°C. More pseudothecia at stages D and E were observed at 15°C than at the other temperatures tested. The rates of maturation of pseudothecia (i.e. time taken to reach stage D) were similar at 5 and 10°C, and were less than those at 15–20°C (Fig. 4). Of the temperatures tested, pseudothecia matured most rapidly at 15°C.

Temperature had little effect on the density of pseudothecia of *L. maculans* and only the mean density of pseudothecia at 15°C was significantly greater (LSD=9.1; $P<0.05$) than that at 20°C (Table 1).

Effect of darkness on pseudothecium maturation in continuous wetness

Pseudothecia of *L. maculans* differentiated, matured and released ascospores at 15°C in darkness and continuous wetness. There was no significant differ-

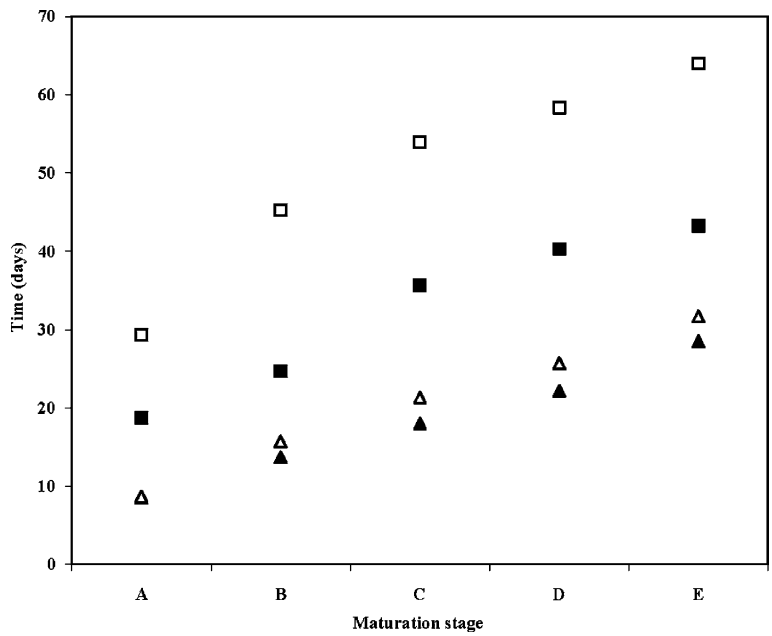

Fig. 1 Time required for pseudothecia of *Leptosphaeria maculans* to reach each stage of maturity (such that the number of pseudothecia at that stage was maximal) at 5 (□), 10 (■), 15 (▲) and 20°C (Δ) in a controlled environment; LSD=6.8; $P<0.05$

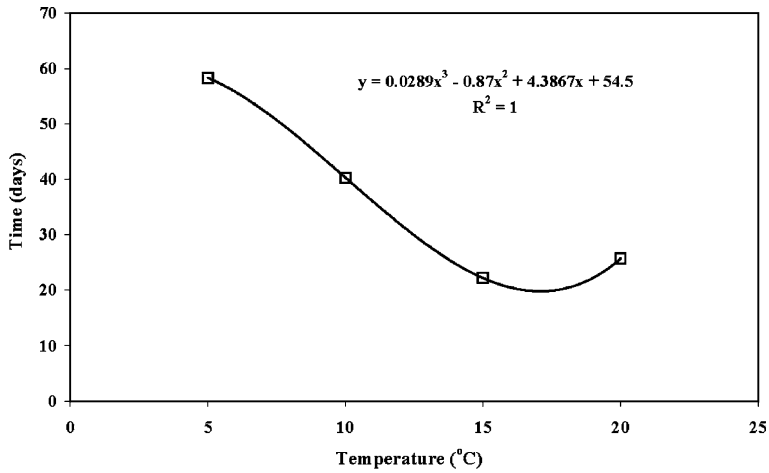

Fig. 2 Relationship between temperature and maturation of pseudothecia of *Leptosphaeria maculans* at 5–20°C in a controlled environment; the time taken for pseudothecia to mature was the day when the number of pseudothecia at D stage was maximal over the assessments at each temperature

ence in the mean time required for pseudothecia to differentiate and mature at 15°C in the 12 h photoperiod and in darkness, except that stage A was reached later in darkness (8.5 and 13.3 days, respectively, Table 2). In darkness, the transition from stage A to B was shorter (LSD=3.1; $P<0.05$) than subsequent transitions. The mean density of pseudothecia formed in continuous darkness (17.5) was significantly less (LSD=9.3; $P<0.05$) than that with a 12 h photoperiod (53.4) at 15°C (Table 1).

Effect of discontinuous wetness on pseudothecium maturation

Pseudothecia of *L. maculans* differentiated, matured and released ascospores at 15°C in discontinuous wetness (2–3 sprays of water per day) and a 12 h photoperiod, but were not observed on stubble that received one spray of water per day (Tables 1 and 2). Discontinuous wetness increased (LSD=5.3; $P<0.05$) the mean time taken for pseudothecia to mature (to reach stage D) to 53.3 days and 46.7 days, for two and three sprays per day respectively, in comparison with 22.2 days in continuous wetness (Table 2). Furthermore, the time taken for pseudothecia to reach each stage of maturity (stages A to E) in discontinuous wetness (two or three sprays per day) was longer ($P<0.05$) than that in continuous wetness. Differentiation of pseudothecia on stubble receiving two sprays per day took longer (LSD=6.1; $P<0.05$) than that with three sprays per day, although the time to release ascospores (stage E) was similar.

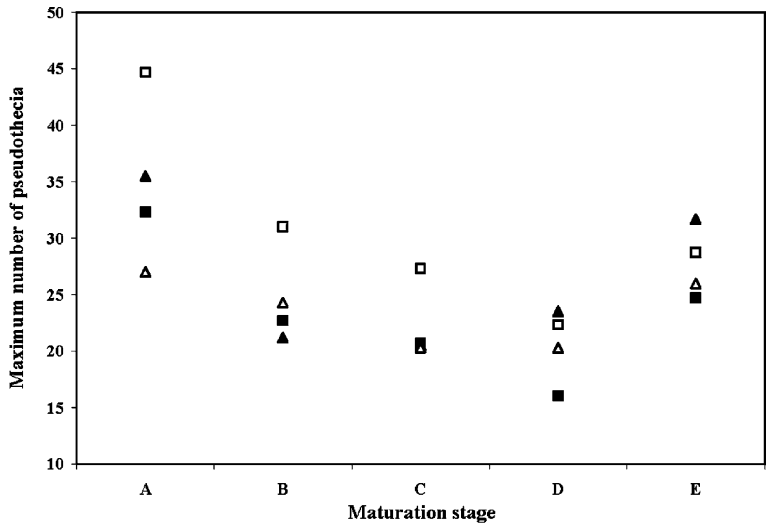

Fig. 3 Average maximum number of pseudothecia observed at each stage of maturity from three repetitions of the experiment at 5 (□), 10 (■), 15 (▲) and 20°C (Δ) in a controlled environment

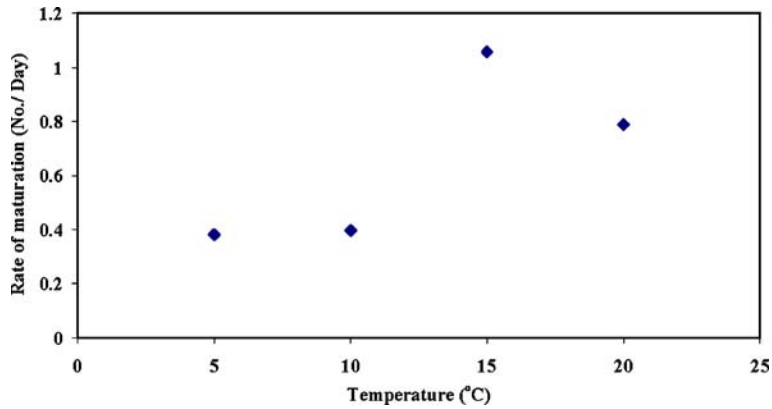

Fig. 4 The rate of maturation of pseudothecia of *Leptosphaeria maculans* at 5–20°C in a controlled environment; rate of maturation was calculated as average maximum number of pseudothecia observed at stage D from three repetitions of the experiment at each temperature divided by average time (day) for pseudothecia to reach stage D

Effect of temperature on ascospore release in a controlled environment

The interaction between temperature and time affected the number of ascospores released from the stubble into Petri dishes (Table 3). The mean number of ascospores released was greater (LSD=0.34; $P<0.05$) at 20°C than the other temperatures tested at each time. Ascospores were not discharged after 10 h at 5–10°C, but were discharged until 10 h and 12 h at 15°C and 20°C°C, respectively. At 5°C, the mean number of ascospores collected decreased (LSD=0.48; $P<0.05$) over time. At 10°C, ascospore discharge did not differ over 2 h to 4 h, then decreased ($P < 0.05$) at 6 h and 8 h. At 15°C, ascospore release was greatest ($P < 0.05$) at 4 h and then decreased over 6–10 h of incubation. At 20°C, the mean number of ascospores increased ($P < 0.05$) from 2 h to 4 h of incubation and then decreased from 6 h to 8 h and from 10 h to 12 h of incubation.

Discussion

Pseudothecia of *L. maculans* developed at 5–20°C and the time taken to reach maturity and discharge ascospores decreased from 5°C to 15°C. The optimum temperature, of those tested, for pseudothecium maturation was between 15°C and 20°C. Exposure to a 12 h photoperiod enhanced the production of inoculum by increasing the density of pseudothecia on the stubble compared with continuous darkness.

Table 1 Effect of temperature, darkness and discontinuous wetness on density of pseudothecia of *Leptosphaeria maculans* on oilseed rape stubble in a controlled environment

Parameter		Density of pseudothecia
Temperature in 12 h photoperiod & continuous wetness	5°C	47.7[a]
	10°C	49.4
	15°C	53.4
	20°C	42
Continuous wetness & darkness	15°C	17.5[b]
Discontinuous wetness & 12 h photoperiod	1 spray per day	0
	2 sprays per day	22.8[c, d]
	3 sprays per day	25.2

[a] $P<0.05$; LSD=9.1 between rows 1–4; [b] LSD = 9.3 between rows 1–4 and row 5; [c] LSD=5.1 between rows 6–8; [d] LSD=8.4 between row 1 and rows 6–8.

Table 2 The incubation time (the day when the number of pseudothecia at each stage of maturity reached its maximum) required for pseudothecia of *Leptosphaeria maculans* to reach each stage of maturity at 15°C in a controlled environment

Conditions	Stage of maturity					
	A	B	C	D	E	
Continuous wetness & 12 h photoperiod	8.5[a]	13.7	18	22.2	28.5	
Continuous wetness & darkness	13.3[b]	15	20	25	30	
Discontinuous wetness & 12 h photoperiod	1 spray	0[c]	0	0	0	0
	2 sprays	26.7[d, e]	36.7	46.7	53.3	56.7
	3 sprays	20	25	36.7	46.7	51.7

[a] LSD=6.8 within row 1; [b] LSD=3.1 within row 2 and between rows 1 and 2; [c] LSD=5.3 between rows 1 and 4–5; [d] LSD=6.1 within and between rows 4–5.

Table 3 The effects of temperature and incubation time on ascospore discharge from pseudothecia of *Leptosphaeria maculans* on oilseed rape stubble in a controlled environment

Temperature (°C)	Incubation time (h)					
	2	4	6	8	10	12
5	2.53[a]	2.1	1.51	0.96	0	0
10	2.35	2.34	1.62	1.18	0	0
15	2.2	2.43	2.15	1.86	1.56	0
20	2.81	3.23	3.31	2.94	2.85	1.91
5	11.58[b]	7.17	3.51	1.62		
10	9.51	9.36	4.06	2.27		
15	8.04	10.35	7.58	5.43	3.75	
20	15.59	24.18	26.3	17.93	16.34	5.75

[a] Log_e-transformed means of number of ascospores collected in three replicate 25-µl aliquots of suspension from nine replicate Petri dishes; LSD between log_e-transformed means within time and within temperature were 0.48 and 0.34, respectively; [b] Back-transformed means of ascospore collected.

Interrupted wetness prolonged the period to inoculum production, highlighting the importance of wetness.

The time to reach maturity was significantly longer at lower than higher temperatures, although only slightly longer at 20°C than 15°C. The polynomial regression identified that the optimum lay between these two temperatures. This is close to the findings of Pérès et al. (1999) that the optimum temperature was 14°C for pseudothecium maturation in a controlled environment and in the field in France. However, in Britain, Toscano-Underwood et al. (2003) reported that the time required for pseudothecia to mature decreased almost linearly with temperature from 5 to 20°C under continuous wetness, both in a controlled environment and in natural conditions. Therefore, the optimum temperature for maturation reported in the British study differs somewhat from that in the present research and in France. In addition, the time to reach stage D at the near-optimum temperature in this study (22.2 days at 15°C, 25.7 days at 20°C) differed from that in France (14 days at 14°C), but was similar to that in the UK (23.1 days at 20°C). In France, no pseudothecia developed at 10°C (Pérès et al. 1999), whereas in the present study and in the British study (Toscano-Underwood et al. 2003) pseudothecia matured after 40.3 and 40.7 days at this temperature, respectively. In the British study, temperature did not affect the density of pseudothecia, whereas the density was significantly greater at 15°C than 20°C in this research. Differences in methodology, including the means of estimating maturity, age of stubble used, environmental conditions, and the pathogen and host genotypes used may have influenced pseudothecium development in the three studies. Furthermore, there was no difference between the timing of pseudothecium maturation on stubble of susceptible, slightly susceptible and tolerant cultivars in France (Poisson and Pérès 1999); however, stubble of resistant cultivars discharged fewer ascospores than stubble of susceptible cultivars in Australia (Marcroft et al. 2004). Using stubble from a mixture of cultivars may have increased experimental error in this study. Maturation of pseudothecia on stubble of cultivars differing in resistance to phoma stem canker could be compared in future studies.

The results support previous findings (Pérès et al. 1999) that continuous 95–100% RH is suitable for the development of pseudothecia in a controlled environment. Here, moistening of stubble once a day provided insufficient moisture for the development of pseudothecia at 15°C. Maturation took longer in discontinuous wetness than continuous wetness. As the frequency of moistening increased from two to three times a day, the time taken for pseudothecia to mature (stage D) and discharge ascospores (stage E) decreased. Further research, in which the duration of wetness after each spray is estimated, would provide an indication of whether the total duration of wetness or the distribution of wetness periods influenced the rate of pseudothecium maturation. Nevertheless, the findings in the current study that interrupted wetness reduced the production of pseudothecia on stubble agree with earlier findings that the quantity of rainfall affected the quantity of ascospores released from stubble in Australia (Marcroft et al. 2003). Marcroft et al. (2003) reported that large numbers of ascospores were released from 6 month-old stubble collected from high rainfall areas whereas stubble from medium to low rainfall areas continued to release fewer ascospores for longer periods. Similarly, in Saskatchewan, Canada, sporulation occurred earlier in the field as the frequency of moistening infested stubble between April and June increased (Petrie 1994). The amount of stubble discharging ascospores, the number of ascospores collected per trapping date and maximum number of ascospores caught were also positively correlated with the number of days with measurable

rainfall in April to July (Petrie 1995). In the UK, this relationship with rainfall has been used to predict the timing of ascospore release in the field (Huang et al. 2007).

Although pseudothecia matured most rapidly between 15°C and 20°C, the mean density of pseudothecia was greater at 15°C than 20°C. The latter temperature is favourable for growth of most fungi and it is possible that the growth of stubble-associated fungi other than *L. maculans* might have contributed to the restricted pseudothecium development on stubble observed here (Naseri et al. 2008b). Although darkness did not affect the time taken for pseudothecia to mature at 15°C, it decreased the density of pseudothecia on stubble, suggesting that burying phoma stem canker-affected stubble in soil may reduce pseudothecium formation compared with surface-placed stubble exposed to a day/ night photoperiod. Likewise, Naseri et al. (2008b) reported that pseudothecium production of *L. maculans* decreased with increasing duration of burial in field soil over 10 months. In contrast, Kharbanda and Ostashewski (1997) found that burying infested stubble in soil shortened the time taken for pseudothecia to develop compared with surface-placed stubble. This may reflect differences in methods, soil characteristics and climatic conditions.

The optimum temperature for ascospore discharge in terms of the amount of ascospores released was 20°C, although peak sporulation occurred earlier at 5–10°C than at 20°C. The longest duration of sporulation occurred at 20°C. Using a similar method, Huang et al. (2005) reported that temperature did not affect ascospore release from stubble incubated for 4 h at 5–20°C, in contrast to the findings of the present research, and they did not examine the duration of ascospore release. That temperature affected ascospore release in the present research supports earlier findings on ascospore discharge and the inclusion of temperature as a crucial factor in the development of predictive models (Guo and Fernando 2005; Huang et al. 2007; McGee 1977; Pérès et al. 1999).

Pérès et al. (1999) reported that the first capture of ascospores was usually observed after rainfall as temperature decreased at the end of summer in France. The optimum temperature for ascospore production on naturally infected stubble was 15°C, although peak sporulation occurred earlier at 20°C and sporulation continued longer at 10°C in Canada (Petrie 1994), results which differed from those in the present study. These differences may reflect different methods used or the age of the stubble in the different regions. The dry and hot summer in South Australia is expected to delay maturation and release of ascospores until conditions are conducive, which coincides with the growing season. Cold winter temperatures in the Northern Hemisphere are likely to have a similar effect.

Salam et al. (2003) considered the difference in the timing of pseudothecium maturity to be the main cause of variation in the initiation of ascospore release between locations within a season and between seasons within a location. Hence the emphasis of their 'Sporacle' model is to predict the timing of release but not the absolute numbers of ascospores released from crop residue or the onset of maturity. Further research on the effects of temperature on maturation of ascospores may allow forecasting of the time of ascospore maturation at regional and farm scale (Salam et al. 2003).

In summary, the optimum conditions for production of pseudothecia and ascospores were continuous wetness between 15°C and 20°C in a 12 h photoperiod and the optimum temperature for ascospore discharge over time was 20°C. Such information could be combined with future fieldwork to predict the onset of inoculum release.

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