Temperature Drives Contrasting *Alternaria* Leaf Spot Epidemic Development in Canola and Mustard Rape from *Alternaria japonica* and *A. brassicae*

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Abstract

Recent surveys of canola (*Brassica napus*) crops across southern Australia highlighted that *Alternaria* leaf spot on canola is not solely caused by *Alternaria brassicae* but that other *Alternaria* spp. are also involved, including *A. japonica*. Studies were undertaken into the effects of different temperatures (14 and 10°C [day and night] or 22 and 17°C [day and night]) on development of *Alternaria* leaf spot caused by *A. japonica* as compared with *A. brassicae* in cotyledons (embryonic leaves) and true leaves (first leaves) of canola (*B. napus* 'Thunder TT') and mustard rape (*B. juncea* 'Dune'). Both pathogens expressed less disease at lower temperatures of 14 and 10°C with percent disease index (%DI) of 19.1 for *A. japonica* and 41.8 for *A. brassicae*, but expressed significantly more disease at higher temperatures of 22 and 17°C with %DI of 80.8 and 88.2 for the same pathogens, respectively. At 14 and 10°C, mustard rape cotyledons showed less disease (percent cotyledons disease index [%CDI] = 18.1) from *A. japonica* but showed more disease (%CDI = 75.0) from *A. brassicae*. However, at 22 and 17°C, cotyledons and true leaves of both canola and mustard rape showed significantly more disease and varied in expressing the disease severity to the two pathogens; true leaves

of mustard rape showed less disease (percent true leaf disease index [%TDI] = 48.4) from *A. japonica* but showed more disease (%TDI = 92.0) from *A. brassicae*. At 22 and 17°C, cotyledons of canola expressed more disease from *A. japonica* (%CDI = 99.1) than from *A. brassicae* (%CDI = 70.7). At the lower temperature, both host species showed the least disease, with mean %DI of 27.3 and 33.5 for canola and mustard rape, respectively, as compared with the higher temperatures, where there was a greater DI, with %DI values of 87.9 and 81.2 for these same host species, respectively. We believe that these are the first studies to highlight the critical role played by temperature for *A. japonica* as compared with *A. brassicae* in *Alternaria* leaf spot disease development and severity. These findings explain how temperature affects *Alternaria* leaf spot severity caused by *A. japonica* as compared with *A. brassicae* on different foliage components of canola and mustard rape.

Keywords: *Alternaria brassicae*, *Alternaria japonica*, *Alternaria* leaf spot, *Brassica juncea*, *Brassica napus*, canola, mustard rape, temperature

Alternaria leaf spot, caused by *Alternaria brassicae*, is a major pathogen of oilseed Brassicas (Saharan et al. 2016) and highlighted as a significant issue in Canada (Degenhardt et al. 1974), France (Marchegay et al. 1990), India (Saharan and Kadian 1983), and the United Kingdom (Evans and Gladders 1981). In Lithuania, 87 to 100% and 100% of winter and spring oilseed rape siliques (*Brassica napus*), respectively, can be damaged (Brazauskienė and Petravičienė 2006). In India, reported yield losses in Indian mustard (mustard rape, *B. juncea*) include 20 to 22% (Kumar 1997; Prasad et al. 2003) and, in Canada, yield losses of 42% in canola (*B. napus*) have been reported (Degenhardt et al. 1974). In Australia, *Alternaria* leaf spot caused by *A. brassicae* has been widely reported on species of Brassicaceae, including in Western Australia on cauliflower (*B. oleracea* var. *botrytis*), canola, wild radish (*Raphanus raphanistrum*) (Shivas 1989), and crambe (*Crambe abyssinica*) (You et al. 2005); in Victoria on both canola and mustard rape (Van de Wouw et al. 2016); and in Tasmania on rutabaga (*B. napus* var. *napobrassica*) (Sampson and Walker 1982). *A. japonica* has been reported in Western Australia on wild radish; in New South Wales on canola,

mustard rape, and hoary stock (*Matthiola incana*) (Al-lami et al. 2019a; APPD 2019); and in Victoria on pak-choi (*B. chinensis*) (APPD 2019). Recent surveys of canola crops across southern Australia highlighted that *Alternaria* leaf spot on canola across southern Australia is not solely caused by *A. brassicae* but that a range of other *Alternaria* spp. are also involved to varying degrees, including *A. japonica* (Al-lami et al. 2019a). Furthermore, the range of *Alternaria* spp. involved also depends upon the year and the geographic locality (Al-lami et al. 2019a).

A. brassicae, along with *A. alternata*, *A. brassicicola*, and *A. raphani*, cause symptoms across different plant organs, including cotyledons (embryonic leaves), true leaves, petioles, stems, florescences, siliques, and seed (Humpherson-Jones and Maude 1982; Saharan et al. 2016). In the field, conidia are distributed through the air and by rain splash and the initial infections, that can begin on the cotyledons, become a source for secondary infections across the crop (Verma and Saharan 1994). Providing there is favorable temperature and moisture, the entire crop can become infected (Humpherson-Jones and Maude 1982; Humpherson-Jones and Phelps 1989; Verma and Saharan 1994).

Both temperature and relative humidity (RH), along with the photoperiod, play significant roles in *Alternaria* leaf spot development caused by *A. brassicae* (Humpherson-Jones and Phelps 1989; Meena et al. 2010). For example, the severity of *Alternaria* leaf spot on mustard rape cultivar Varuna leaves under field conditions is correlated with a maximum temperature range of 18 to 27°C, minimum temperature of 8 to 12°C, and >92 and 40% RH morning and afternoon, respectively (Chattopadhyay et al. 2005). However, this can vary with host or host species. For example, on mustard rape, cauliflower, and cabbage (*B. oleracea* var. *capitata*), severity of *Alternaria* leaf spot (*A. brassicae*) is correlated with average field maximum and minimum temperatures of 10 to 28 and 1.5 to 15.3°C, respectively

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(Selvamani et al. 2014). On yellow sarson cultivar T-151 (*B. campestris*) and mustard rape cultivar Varuna, severity of *Alternaria* leaf spot caused by *A. brassicae* was correlated with maximum and minimum temperatures of 20 to 23°C and 7 to 10°C, respectively, with >67% RH, more than six total rainy days, and rainfall > 70 mm ($r=0.51$ to 0.81) (Awasthi and Kolte 1990). In another study on mustard rape cultivar RLM 619, disease development from *A. brassicae* was most severe across a temperature range of 13.5 to 19.3°C with >70% RH (Bal and Kumar 2014). Temperature effects have also been reported for other *Alternaria* spp. such as *A. brassicicola*, with disease most severe in the spring season (17 to 32°C, average daily mean) as compared with the winter season (7 to 23°C, average daily mean) of three cultivars of cabbage (viz., Abbott & Cobb No. 5, Gourmet, and Market Prize) in the field (Fontem et al. 1991). For *A. japonica*, *Alternaria* leaf spot disease development at the particularly favored temperature of 23°C was correlated with disease development, while 13 to 31°C was correlated with spore germination and secondary conidial production for *A. japonica* (Rimmer et al. 2007). Disease from *A. japonica* was first reported on turnip cultivar Virtudes-Martillo (*B. rapa* subsp. *rapa*) in fields of Alicante Province in Spain when the temperature ranged from 20 to 30°C (Bassimba et al. 2013), and also on wild and cultivated rocket (*Diplotaxis tenuifolia* and *Eruca vesicaria*, respectively) under greenhouse conditions during autumn and winter in northern Italy (Garibaldi et al. 2011).

In Australia, canola production increased from 3,900,000 metric tons (t) in 2013–14 to 4,200,000 t in 2016–17 (AOF 2018). Quality mustard rape is also considered a promising oleaceous *Brassica* crop for low-rainfall Australian regions, where it was first grown commercially in 2007 as a more thermal and drought tolerant “juncea canola” (Elliott et al. 2015; Oram et al. 2005) along with smaller areas of conidiment mustard rape (Oram et al. 2005). However, although Van de Wouw et al. (2016) and Al-lami et al. (2019a) both highlighted that *Alternaria* leaf spot occurs widely on canola in Australia, no information was available in relation to mustard rape. Furthermore, Al-lami et al. (2019a, 2020) showed that *Alternaria* leaf spot in Australia is not solely caused by *A. brassicae* but also by a range of other *Alternaria* spp., including *A. japonica*, which displayed variation in relative levels of virulence. *A. japonica* was never previously studied in terms of disease development on canola. More importantly, both *A. brassicae* and *A. japonica* caused adverse field yield losses of >58% on canola (Al-lami et al. 2019b). Hence, studies were undertaken into the effects of temperature (14 and 10°C day and night, respectively, or 22 and 17°C day and night, respectively) on development of *Alternaria* leaf spot as relevant to *A. japonica* versus *A. brassicae*. We believe that these are the first studies to highlight the critical roles played by temperature for *A. japonica* as compared with *A. brassicae* in *Alternaria* leaf spot disease development and severity.

Materials and Methods

Alternaria isolates. A mixture of five different hyphal-tipped isolates of *A. brassicae* and six different hyphal-tipped isolates of *A. japonica* were used. All isolates were obtained during a southern Australian 2016 canola disease survey, and were chosen to represent a wide geographical area affected by *Alternaria* leaf spot. For *A. brassicae*, isolates utilized included three isolates (viz., WA-21.8, WA-47.6, and WA-I) isolated from different locations in Western Australia (WA), a single isolate (SA-84.4) from South Australia (SA), and a single isolate (NSW-PSW.20) from New South Wales (NSW). For *A. japonica*, isolates utilized included three isolates (viz., WA-45.2.1, WA-46.8.A, and WA-47.1) from Western Australia derived from diseased canola leaves and three isolates (NSW-PSW.47, NSW-PSW.60, and NSW-PSW.77) from New South Wales collected from infected wild radish leaves. Isolates were chosen based on their high virulence either in tests conducted earlier on both canola cultivar Thunder TT and mustard rape cultivar Dune (Al-lami et al. 2019a) or in tests conducted for *A. japonica* isolates NSW-PSW.60 and NSW-PSW.77 in another study (Al-lami et al. 2020). For each *Alternaria* sp., isolates were mixed in equal proportions for use as inoculum to

eliminate any potential pathotype-specific responses on the two inoculated test *Brassica* cultivars, as has been done with other *Brassica* pathogen studies such as *Pseudocercospora capsellae* (Gunasinghe et al. 2017a,b) and *Hyaloperonospora brassicae* (Mohammed et al. 2017, 2018a), including temperature studies for the latter pathogen (Mohammed et al. 2018b).

Inoculum preparation and inoculation method. For production of hyphal inoculum, isolates were subcultured onto potato dextrose agar (PDA; Merck) for 7 days at $23 \pm 2^\circ\text{C}$ under cool white fluorescent light. Five agar discs of 4 mm² containing growing mycelia from PDA were transferred into 150 ml of liquid growth medium (24 g of potato dextrose broth and 10 g of peptone per liter of water) (Uloth et al. 2015a). Cultures were placed on a rotary shaker at 150 rpm at 25°C. After 5 days, actively growing mycelium was harvested by straining the broth through four layers of cheesecloth and washing well with sterile deionized water (SDW). The mycelium was macerated in SDW using a hand-held blender for 5 to 10 min; the initial inoculum concentration was estimated using a hemocytometer counting chamber and then adjusted to obtain mycelial fragments of 90 ± 10 µm in length at a concentration of 4×10^6 fragments/ml (Gunasinghe et al. 2017b). This hyphal inoculum was then used to initiate production of conidial inoculum as described below.

Production of conidial inoculum was carried out as described by Gunasinghe et al. (2017b) for the white leaf pathogen, *P. capsellae*. Seed of susceptible canola cultivar Thunder TT (Al-lami et al. 2019a) were sown in 7-by-7-by-18-cm pots (six seeds per pot), seedlings thinned to 1 plant per pot, and pots maintained at 22°C (day) and 18°C (night) in a controlled-environment room with a 12-h photoperiod. Plants were fertilized weekly with Thrive (Yates) at the manufacturer’s recommended rate, a complete nutrient solution dissolved in water. At 6 weeks of age, plants were separated into two groups and spray inoculated with either a mixture of *A. brassicae* isolate mycelial suspension for one group or a mixture of *A. japonica* isolate mycelial suspension for the second group, using a hand-held and operated aerosol sprayer. Inoculated plants were covered with clear plastic bags for 96 h postinoculation (hpi) to maintain high humidity and misted with a SDW spray applied once per 24 h for 4 days. When disease symptoms became apparent, by approximately 7 to 14 days postinoculation (dpi), leaves with typical *Alternaria* leaf spot symptoms were collected and washed gently under SDW to remove any surface contamination. Lesions were then cut and placed into glass vials containing 20 to 30 ml of SDW, and shaken vigorously by hand for 4 min to release conidia. The conidial mixture was then centrifuged for 5 min at $400 \times g$ and the supernatant discharged to concentrate conidia. Conidia were washed twice in SDW and resuspended in 2 to 3 ml of SDW. Conidial concentration was adjusted to 5×10^5 ml⁻¹ using a hemocytometer counting chamber.

Effect of two different temperature regimes on Alternaria leaf spot disease. Two susceptible *Brassica* sp. genotypes, canola cultivar Thunder TT and mustard rape cultivar Dune (Al-lami et al. 2019a), were used for each pathogen (*A. japonica* and *A. brassicae*). Four seeds each per pot were sown in 6-by-6-by-9-cm pots; seedlings were thinned to one plant per pot after germination. Plants were maintained at 22°C (day) and 18°C (night) in a controlled-environment room with a 12-h day length and fertilized weekly with Thrive, as described above. Seven days after seedling emergence, half of the pots were shifted into a growth chamber maintained at 14 and 10°C day and night, respectively, and the other half into a growth chamber at 22 and 17°C day and night, respectively, with average RH of 70% in both chambers. These temperatures were similar to those used by Mohammed et al. (2018b) for temperature studies with the downy mildew pathogen *H. brassicae*. Plants in both chambers were placed into clear plastic boxes (770 by 570 by 475 mm, length by width by height) with lids; 2 days later, cotyledons (embryonic leaves) were drop inoculated (10 µl), two drops per leaf, with the conidial inoculum (5×10^5 ml⁻¹) of either pathogen (or SDW as a negative control check). At 21 days after sowing, the true leaves (first leaves) were also inoculated with either pathogen (or SDW). Plastic boxes were internally misted with deionized water and lids closed for 96 hpi to maintain high humidity (wet period duration). Disease

reactions on cotyledons and true leaves were recorded at 14 dpi to accommodate the slower disease development at 14 and 10°C.

Disease assessment. A 0-to-5 scale developed by Kolte (1999) was used to assess the disease, where 0 = no visible symptoms, 1 = 1 to 10% leaf or pod area covered with small spots, 2 = 11 to 25% leaf or pod area covered with spots >3 mm, 3 = 26 to 50% leaf or pod area covered with spots >3 mm but with initiation of coalescing of lesions on leaves and deep lesions on pods, 4 = 51 to 75% leaf or pod area covered with commonly coalescing lesions, and 5 = 76 to 100% leaf or pod area covered with very large spots producing a typical blighted appearance. Each 0-to-5 diseased leaf score was converted into a percent cotyledon disease index (%CDI) or percent true leaf disease index (%TDI), with each disease index (DI) calculated based on the method described by McKinney (1923), where $\%DI = \{[(a \times 0) + (b \times 1) + (c \times 2) + \dots + (f \times 5)] \times 100\} / [(a + b + c + \dots + f) \times 5]$ and where *a*, *b*, *c*, ..., *f* are the number of plants with severity scores of 0, 1, 2, ..., 0.5, respectively.

Experiment design and statistical analyses. The experiment was fully repeated once. Each experiment had 10 replicates arranged in a randomized complete block design by using the 'Generate a Standard Design' function of GenStat Release 18.1 (VSNi 2019). A Shapiro-Wilk test was conducted to test the normality of the data and showed nonnormal distribution. Although several logarithmic transformations were conducted to normalize the data, they failed to apply normality to the data. Therefore, nonparametric analyses were performed using XLSTAT. The homogeneity of initial and repeat experiments was assessed using a Mann-Whitney *U* test. These tests showed no significant difference ($P > 0.05$) between initial and repeated experiment data; hence, data sets from initial and repeat experiments were combined and reanalyzed as a single data set. Friedman's test was performed to calculate means and *P* value of variables. Multiple pairwise comparisons were made using the Nemenyi procedure and two-tailed test (Hollander and Wolfe 1999; Nemenyi 1963).

Results

Symptoms. At 14 and 10°C, the first appearance of *Alternaria* leaf spot symptoms from *A. japonica* on canola was 3 dpi, with 40% of

true leaves showing symptoms after 8 dpi; however, 25 and 5% of cotyledons and true leaves, respectively, never showed any disease symptoms. On mustard rape, the first appearance of the symptoms was 8 dpi across different foliage components but only 20% of cotyledons showed symptoms after 3 dpi and 15% of true leaves showed no symptoms. For *A. brassicae*, the first appearance on both host species was at 3 dpi across different foliage components, with 5% of cotyledons of canola showing symptoms after 8 dpi but with 45% of cotyledons showing a hypersensitive reaction at that same time period. At 22 and 17°C, the first appearance of *Alternaria* leaf spot of either pathogen (*A. brassicae* or *A. japonica*) on both host species and across different foliage components was 3 dpi. With both *A. japonica* and *A. brassicae*, the lesions appeared as gray to dark-gray in color at different temperatures but with an associated chlorotic effect at 22 and 17°C (Fig. 1).

Cotyledons versus true leaves and their response in relation to canola and mustard rape. Data showed no significant difference at $P \leq 0.05$ between canola (mean %DI = 57.6) and mustard rape (mean %DI = 57.4) (Fig. 2A). There was a statistically significant difference at $P = 0.015$ between foliage components. Cotyledons showed overall more disease (overall mean of %CDI = 61.5) compared with the true leaves (%TDI = 53.5) (Fig. 2B). Subsequently, a significant difference at $P < 0.001$ was noted in cotyledons and true leaves across host species. On canola, the true leaves exhibited the greatest overall mean %DI (61.6) compared with %DI = 53.7 for cotyledons. In contrast, cotyledons of mustard rape showed the greatest %DI (69.4) compared with the true leaves (%DI = 45.3) (Fig. 2E).

Virulence of pathogens. There was a statistically significant difference at $P < 0.001$ across pathogens. *A. japonica* caused less disease (overall mean %DI = 49.9) compared with *A. brassicae* (%DI = 65.0) (Fig. 2C), and there was a statistically significant difference at $P < 0.001$ between pathogens in terms of their virulence on canola and mustard rape, where *A. japonica* caused the most disease on canola (%DI = 58.3) as compared with mustard rape (%DI = 41.6), whereas *A. brassicae* caused more disease on mustard rape (%DI = 73.1) than on canola (%DI = 57.0) (Fig. 2F). In

Fig. 1. *Alternaria* leaf spot disease symptoms on canola (*Brassica napus* 'Thunder TT') at different temperatures under controlled environmental conditions. **A** and **B**, Negative control of cotyledons and true leaves, respectively (inoculated with sterile deionized water). **C** and **D**, *Alternaria brassicae* on cotyledons and true leaves at 14 and 10°C. **E** and **F**, *A. brassicae* on the same foliage components, respectively, at 22 and 17°C. **G** and **H**, *A. japonica* on the same foliage components, respectively, at 14 and 10°C. **I** and **J**, *A. japonica* on the same foliage components, respectively, at 22 and 17°C.

terms of the foliage component at the $P < 0.001$ significance level, *A. japonica* caused more disease on cotyledons (%CDI = 59.0) than on true leaves (%TDI = 40.9). In contrast, *A. brassicae* caused more disease on true leaves (%TDI = 66.1) compared with cotyledons (%CDI = 64.0) (Fig. 2G). In relation to host species across different foliage components at the $P < 0.001$ significance level, *A. japonica* caused severe disease on cotyledons of canola (%CDI = 63.1) and mustard rape (%CDI = 54.9) and on the true leaves of canola (%TDI = 53.4), whereas the least disease was on the true leaves of mustard rape (%TDI = 28.31). In contrast, for *A. brassicae*, disease was most severe on cotyledons of mustard rape (%CDI = 83.8) and on true leaves of both canola (%TDI = 69.7) and mustard rape (%TDI = 62.4), whereas canola cotyledons showed the least disease (%CDI = 44.2) (Fig. 2N).

Day and night temperatures of 14 and 10°C, respectively, versus 22 and 17°C, respectively, and their influence on pathogen. Across the two temperatures at the $P < 0.001$ significance level, lower temperatures (14 and 10°C day and night, respectively) resulted in the least disease (mean %DI = 30.4) compared with %DI = 84.5 at the higher temperatures (22 and 17°C day and night, respectively) (Fig. 2D). The disease severity across low and high temperatures showed similarity between *A. japonica* and *A. brassicae*; both caused significantly less disease at 14 and 10°C (%DI values of 19.1 and 41.8, respectively) as compared with more severe disease at 22 and 17°C (%DI values 80.8 and 88.2, respectively) (Fig. 2I).

Influence of temperature on foliage components of canola and mustard rape. At the $P < 0.001$ significance level, cotyledons and true leaves at the higher temperature exhibited more disease

(%DI = 88.6 and 80.5, respectively) compared with the lower temperature (%DI = 34.5 and 26.4, respectively) (Fig. 2J). At the lower temperature, both host species showed the least disease with mean %DI = of 27.3 and 33.5 for canola and mustard rape, respectively, as compared with the higher temperatures, where there was a greater disease incidence with %DI = values of 87.9 and 81.2 for these same host species, respectively, (Fig. 2H). In relation to different foliage components across cultivars at the $P < 0.001$ significance level, cotyledons and true leaves expressed more disease across cultivars at 22 and 17°C day and night, respectively, compared with the low disease incidence of the same foliage components at 14 and 10°C. On canola, the disease severity on cotyledons and true leaves (%DI = 84.9 and 90.9, respectively) was greater at the higher temperatures compared with the lowest %DI values of 22.44 and 32.3, respectively, at the lower temperatures (Fig. 2M). Similarly, on mustard rape, the highest %DI values on cotyledons and true leaves (%DI = 92.2 and 70.2, respectively) were at the higher temperatures compared with %DI values of 46.6 and 20.5, respectively, at the lower temperatures.

Influence of temperature on disease severity across different foliage components of canola and mustard rape. At the $P < 0.001$ significance level, the disease severity of either *A. japonica* or *A. brassicae* was much less at 14 and 10°C compared with 22 and 17°C across different foliage components. At 14 and 10°C, *A. japonica* displayed very low %DI values of 22.6 and 15.5 on cotyledons and true leaves, respectively, as compared with at 22 and 17°C, where there were much greater %DI values of 95.4 and 66.2 on these same foliage components, respectively (Fig. 2K). Similarly,

Fig. 2. Impact of two different temperatures (14 and 10°C day and night, respectively, versus 22 and 17°C day and night, respectively) on *Alternaria* leaf spot development caused by *Alternaria japonica* versus *A. brassicae* across different foliage components (cotyledons and true leaves) of canola (*Brassica napus* 'Thunder TT') and mustard rape (*B. juncea* 'Dune') at 14 days postinoculation. Data represent means $\pm$ standard deviation of disease incidence (%) in relation to **A**, host species or cultivar; **B**, foliage component; **C**, pathogen; **D**, temperature; **E**, cotyledons and true leaves across cultivars; **F**, *A. brassicae* and *A. japonica* across cultivars; **G**, *A. brassicae* and *A. japonica* across different foliage components; **H**, 14 and 10°C day and night, respectively, versus 22 and 17°C day and night, respectively, across cultivars; **I**, *A. brassicae* and *A. japonica* at low and high temperatures; **J**, 14 and 10°C, respectively, versus 22 and 17°C day and night, respectively, across foliage components; **K**, *A. brassicae* and *A. japonica* at low and high temperatures on cotyledons and true leaves; **L**, *A. brassicae* and *A. japonica* at low and high temperatures across cultivars; **M**, influence of low and high temperatures on cotyledons and true leaves of canola and mustard rape; **N**, pathogenic capability of both pathogens on cotyledons and true leaves of canola and mustard rape; and **O**, disease development at low and high temperature in relation to foliage components of canola and mustard rape. Bars labeled with different letters across factors show the significant difference with multiple pairwise comparisons using Nemenyi's procedure and two-tailed test at $P < 0.05$.

A. brassicae showed relatively low %DI values of 46.3 and 37.3 on cotyledons and true leaves, respectively, at 14 and 10°C as compared with 22 and 17°C, where the %DI values were 81.7 and 94.8 for the same foliage components, respectively. Subsequently, in relation to the virulence of two pathogens across host species at the $P < 0.001$ significance level, *A. japonica* caused more disease on canola (%DI = 91.6) compared with mustard rape (%DI = 70.1) at 22 and 17°C, whereas *A. brassicae* caused more disease on mustard rape (%DI = 92.3) compared with canola (%DI = 84.2) at the same high temperatures. At 14 and 10°C, despite both *A. japonica* and *A. brassicae* causing significantly less disease on mustard rape (%DI = 13.2 and 53.9, respectively) and canola (%DI = 24.9 and 29.7, respectively), *A. brassicae* showed more disease on mustard rape and canola compared with *A. japonica* (Fig. 2L).

Disease development at low and high temperatures in relation to foliage components of canola and mustard rape. There was significant difference at $P < 0.001$ in terms of the effect of the two pathogens at low and high temperatures on different foliage components across host species. At the lower temperature, cotyledons (%CDI = 27.1 and 17.6) and true leaves (%TDI = 22.7 and 41.9) of canola showed less disease from *A. japonica* and *A. brassicae* (Fig. 2O). On mustard rape, low disease incidence was also observed on cotyledons (%CDI = 18.1 from *A. japonica*) and the true leaves (%TDI = 8.2 and 32.7 from *A. japonica* and *A. brassicae*, respectively). However, at higher temperatures, the disease severity was significantly greater. On canola and mustard rape, high disease severity was observed across different foliage components; namely, cotyledons (%CDI = 99.1 and 70.7 for canola and %CDI = 91.7 and 92.6 for mustard rape) and true leaves (%TDI = 84.1 and 97.6 for canola and %TDI = 48.4 and 92.0 for mustard rape) from *A. japonica* and *A. brassicae*, respectively. Cotyledons of mustard rape showed more disease (%CDI = 75.0) from *A. brassicae* at the lower temperatures but expressed greater disease at the higher temperatures (%CDI = 92.6) (Fig. 2O). Subsequently, there was significant difference at $P < 0.0001$ in terms of the effect of the two pathogens on cotyledons of mustard rape at the low temperatures; *A. japonica* produced less disease (%CDI = 18.1) as compared with more disease (%CDI = 75.0) from *A. brassicae* (Fig. 2O).

Discussion

We believe that these are the first studies to highlight the critical roles played by temperature for *A. japonica* as compared with *A. brassicae* in *Alternaria* leaf spot disease development and severity on canola and mustard rape. *A. brassicae* is globally considered the most significant *Alternaria* sp. on canola and has been widely studied for infection requirements as related to different temperatures, RH, and host age (Humpherson-Jones and Phelps 1989; Saharan et al. 2016). In contrast, *A. japonica* has not been previously studied in terms of disease development on canola or mustard rape. Because both diseases co-occur on canola in Australia (Al-lami et al. 2019a), it was important that comparison be made between *A. japonica* and *A. brassicae*. Furthermore, for Australia, prior to this study, there was little understanding as relates to temperature for *A. brassicae* in terms of disease severity on canola or mustard rape. *A. brassicae* has been widely highlighted as a major and common pathogen of oilseed Brassicas, while *A. alternata*, *A. brassicicola*, and *A. raphani* are of more concern in relation to vegetable crops (Saharan et al. 2016). However, *A. japonica* was not only isolated from surveys of canola crops across southern Australia in 2016 (M. P. You and M. J. Barbeti, unpublished data) but was pathogenic on both canola and mustard rape (Al-lami et al. 2019a). The current study emphasizes the potential for *A. japonica* to become a significant pathogen on members of the Brassicaceae family, along with the previously described *Alternaria* spp. pathogens, especially because *A. japonica* is pathogenic on both Brassicaceae and non-Brassicaceae hosts, both in Australia and elsewhere (Farr and Rossman 2019).

The current study revealed how temperature affects the development and severity of *Alternaria* leaf spot caused by *A. japonica* on both canola and mustard rape, as compared with *A. brassicae*. Although the effect of temperature showed significant differences across tested variables, including host species, foliage component,

and pathogen, temperature was a dominant determinant of disease development by *A. japonica*, as it is with *A. brassicae*. At the lower temperatures, despite *A. brassicae* being highly pathogenic on cotyledons of mustard rape, neither pathogen caused severe disease until the temperature increased; however, this effect was not independent of foliage component or the causal *Alternaria* sp. Degenhardt et al. (1982), working with different pathotypes of three *Alternaria* spp. also noticed a strong effect of different temperatures (11, 15, 19, 23, and 27°C) on the third leaf of 4-week-old canola cultivar Zephyr and that the effect was dependent on the *Alternaria* sp. They found that *A. brassicae* was pathogenic across these different temperatures, that *A. raphani* was pathogenic at 17°C and above, that *A. brassicicola* was pathogenic at 19°C and above; and that most severe disease was at 19 to 23, 23, and 23 to 25°C for *A. brassicae*, *A. raphani*, and *A. brassicicola*, respectively. Likewise, Fontem et al. (1991) observed similar effects of different temperatures (spring and winter seasons) on three cultivars of cabbage (Abbott & Cobb No. 5, Gourmet, and Market Prize) in the field from *A. brassicicola*. They distinguished the disease progression and epidemic development between spring (17 to 32°C, average daily mean) and winter (7 to 23°C, average daily mean) seasons, with area under disease progress curve values of 13.0, 24.6, and 25.7 in spring, and 7.7, 8.5, and 12.3 in winter for the same cultivars, respectively. They showed that the disease epidemic was slower in winter than in warmer springtime. Furthermore, Mridha and Wheeler (1993) noted that, although optimal infection temperature of *A. brassicae* on *B. napus* ‘Jet Neuf’ third, fourth, and fifth leaves was 25°C, infection also was observed at 15, 20, and 29°C, with very slight infections at 10°C. Bassimba et al. (2013) first reported *A. japonica* leaf spot on turnip in Alicante Province in Spain when the temperature ranged from 20 to 30°C, with approximately 20% disease infestation. Furthermore, Garibaldi et al. (2011) first reported *A. japonica* leaf spot on wild and cultivated rocket under greenhouse conditions during autumn and winter in northern Italy, with 30 to 40% disease infestation. Moreover, Rimmer et al. (2007) demonstrated that a temperature of 23°C was correlated with disease development and 13 to 31°C was correlated with spore germination and secondary conidial production for *A. japonica*. In contrast, Humpherson-Jones and Phelps (1989) illustrated that temperature ranging from 5 to 30°C resulted in no significant differences in the average sporulation duration of *A. brassicae* in lesions generated on canola cultivar Bienvenue or sweede cultivar Best of All. Despite this, in general, the abundance of spores likely increased the disease epidemic in field crops once the favorable conditions for infection were available. *Alternaria* leaf spot development and severity caused by *A. brassicae* disease on species of Brassicaceae is strongly affected by environmental conditions (Awasthi and Kolte 1990; Bal and Kumar 2014; Chattopadhyay et al. 2005; Selvamani et al. 2014) and this likely explains the highly variable nature of *Alternaria* leaf spot epidemics, including in Australia, regardless of the *Alternaria* spp. involved.

The current study not only highlights how temperature, host species, and foliage component influence *Alternaria* leaf spot development on oilseed Brassicas but also shows that this occurs across different species of *Alternaria* (namely, *A. japonica* as compared with *A. brassicae*). Despite *A. japonica* not being as pathogenic at lower temperatures on either cotyledons or true leaves across tested cultivars, the greater disease incidence in the current study of mustard rape from *A. brassicae* at an earlier stage (cotyledons) and at lower temperatures implies both a greater overall crop risk of severe infection by the latter species and its ability to be a significant inoculum source for infecting other oilseed and horticultural crops growing in the same vicinity. In Australia, the Mediterranean regions of southwestern and southern Australia are highly variable in terms of environmental conditions, and this variability greatly and differentially influences the development of a range of Brassica diseases (Barbeti et al. 2012). That *Alternaria* spp. are not generally regarded as particularly major pathogens of oilseed Brassicas in Australia is likely due to these variable environmental conditions, which are less conducive to epidemics there than in some other countries

such as India, where *Alternaria* leaf spot is considered a very serious and, at times, almost “unmanageable disease” (Chattopadhyay et al. 2005; Saharan et al. 2016). However, the finding that *A. japonica* and *A. brassicae* infections in both canola and mustard rape were favored by warmer temperatures in the current study is important for both crops, because temperatures readily reach or exceed 18 to 24°C during the latter part of the growing season in many regions in Australia and worldwide. Furthermore, temperature affects the disease severity of a range of other Brassicaceae diseases. Among other examples, Li et al. (2006) highlighted how Phoma stem canker (blackleg; *Leptosphaeria maculans*) is strongly influenced by temperature, with more severe crown canker at higher temperatures (18 to 24°C) compared with lower temperatures (11 to 18°C); Mohammed et al. (2018b) studied downy mildew at 22 and 17°C versus 14 and 10°C and showed that higher temperatures accelerated the disease severity, well illustrated with *B. napus* ‘Atomic’ that showed less disease at 14 and 10°C but greater disease at 22 and 17°C; and Uloth et al. (2018) studied powdery mildew development and severity on different *B. napus* cultivars and found a similar pattern of high disease severity at 22 and 17°C compared with low disease severity at 14 and 10°C. These latter studies on other Brassicaceae pathogens highlight the concern that temperature increases from climate change will increase severity of some Brassicaceae diseases, as highlighted earlier by Barbetti et al. (2012). It is likely that the same applies for *Alternaria* leaf spot under future warming climate predictions for Australia and elsewhere. However, it remains to be determined if there are temperature phenotypes for *A. japonica* or *A. brassicae* as exist for *Sclerotinia sclerotiorum* where, for example, isolates collected from warmer or cooler areas show adaptation to higher or lower temperatures, respectively, when infecting *B. carinata* (Uloth et al. 2015b).

In conclusion, this study demonstrates the important influence of temperature across different *Brassica* host species and foliage components on the development and severity of *Alternaria* leaf spot caused by *A. japonica* versus *A. brassicae*. The findings show that disease severity from *A. japonica* increases with warmer temperatures and decreases with colder temperatures, as in *A. brassicae*, but the cold temperature effect was not independent of host species, foliage component, or causal *Alternaria* sp. Furthermore, the current study suggests likely greater *Alternaria* leaf spot development and severity in future decades during the spring season across southern Australia, where temperatures are rising due to climate change (Barbetti et al. 2012). In addition, precautions are needed for canola and mustard rape breeders to avoid inadvertently selecting for genotypes more susceptible to one or more *Alternaria* spp., as can easily occur in screening programs intently focused on maximizing resistance to blackleg and where other diseases are “masked” by extremely severe blackleg. The increasing temperatures along with any inadvertent selection for heightened susceptibility to *A. japonica* or *A. brassicae* would significantly enhance their opportunity to become much more important and damaging pathogens to both canola and mustard rape.

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