Effect of wounding and wound age on infection of canola cotyledons by *Leptosphaeria maculans*, interacting with leaf wetness

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**Abstract:** Blackleg, caused by the fungus *Leptosphaeria maculans*, is an important disease of canola. Both ascospores and pycnidiospores of the fungus can infect intact leaves under conditions of extended leaf wetness, with infection then progressing further into the stem. In western Canada, spring is typically cool and dry and additional factors may be involved for successful infection. This study was designed to assess the effect of wounding and wound age on cotyledon infection, to evaluate the likelihood that flea beetle-feeding injuries could contribute to increased disease. Infection of canola cotyledons occurred readily via mechanical wounds in the absence of leaf wetness when seedlings were spray inoculated with pycnidiospores of *L. maculans*, whereas no infection occurred on intact cotyledons even under 6-h leaf wetness. Wound age also played a role in susceptibility; wounded tissues were less susceptible when plants were kept in a greenhouse for 8 h or longer before inoculation, with substantially reduced infection success relative to fresh wounds. This wound-age effect was similar on susceptible and resistant canola varieties. A high temperature (25°C) seemed to favour the healing of wounds, reducing susceptibility when compared with control (21/16°C) and low (10°C) temperatures. Leaf wetness post-wounding may hinder healing, and high post-inoculation humidity (80–90% relative humidity) increased infection via older wounds slightly, relative to lower (50–60%) relative humidity. These data provide a rationale for studying the effect of improved flea beetle control on early *L. maculans* infection of canola.

**Keywords:** blackleg, *Brassica napus*, canola, flea beetle, *Leptosphaeria maculans*, wounding

**Résumé:** La nécrose du collet, causée par le champignon *Leptosphaeria maculans*, est une importante maladie du canola. Autant les ascospores que les pycnidiospores du champignon peuvent infecter les feuilles intactes dans des conditions prolongées d’humidité, puis, éventuellement, la tige. Dans l’Ouest canadien, le printemps est généralement frais et sec et des facteurs additionnels peuvent contribuer à l’infection des plants. Cette étude a été conçue pour évaluer l’effet des blessures et de l’âge des blessures sur l’infection des cotylédons de même que la probabilité que les blessures causées par l’alimentation des altises pourraient accroître l’incidence de la maladie. L’infection des cotylédons du canola s’est produite couramment à la suite de blessures d’origine mécanique en l’absence d’humidité sur les feuilles quand les plantules étaient inoculées par aspersion avec des pycnidiospores de *L. maculans*, tandis qu’aucune infection n’est apparue sur les cotylédons intacts, même si les feuilles avaient été humectées pendant six heures. L’âge des blessures a également joué un rôle dans la réceptivité de l’hôtes; les tissus blessés étaient moins réceptifs lorsqu’ils étaient gardés dans une serre pendant huit heures ou plus avant l’inoculation, affichant un taux d’infection substantiellement réduit comparativement aux blessures fraîches. Une température élevée (25°C) a semblé favoriser la guérison des blessures, réduisant la réceptivité comparativement aux températures témoins (21/16°C) et basses (10°C). Le taux d’humidité des feuilles après la blessure peut entraîner la guérison, et des taux élevés d’humidité après l’inoculation (80-90% d’humidité relative) ont légèrement accru l’infection résultant des plus vieilles blessures, et ce, comparativement à un taux d’humidité.
relative plus faible (50-60%). Ces données justifient l’étude de l’effet d’une meilleure gestion de l’altise quant à l’infection précoce du canola causée par *L. maculans*.

**Mots clés:** Brassica napus, nécrose du collet, canola, altise, Leptosphaeria maculans, blessure

**Introduction**

Blackleg, caused by the hemibiotrophic fungal pathogen *Leptosphaeria maculans* Ces. & de Not., is a serious disease of canola/oilseed rape (*Brassica napus* L.) in Australia, Europe, and Canada (Fitt et al. 2006; Howlett et al. 2001; Hwang et al. 2016; Wang et al. 2020). The pathogen survives saprophytically in canola residue and produces both ascospores and pycnidiospores that germinate on cotyledons or lower leaves and penetrate the tissue via stomata or wounds (West et al. 2001; Ghanbarnia et al. 2009). After a brief biotrophic phase following penetration, the fungus colonizes intercellular space rapidly, causes necrotic leaf lesions (Hammond et al. 1985; Hammond and Lewis 1987; Rouxel and Balesdent 2005), and spreads further along the petiole asymptomatically through the vascular tissue into the stem and crown where blackleg or stem canker occurs (Hammond et al. 1985; Hammond and Lewis 1987; Howlett et al. 2001; Rouxel and Balesdent 2005). The release of initial inoculum often coincides with the emergence and establishment of young canola plants (Travadon et al. 2007; Savage et al. 2013). As early infection is critical to severe epidemics of blackleg, the infection is also considered monocyclic (Huang et al. 1999), and early infection is possibly necessary for blackleg to impact the crop health and economy.

Wounds may allow *L. maculans* to infect canola leaves readily in the absence of wetness, as shown in the cases of root (Sprague et al. 2007), leaf, petiole, and stem infection (West et al. 2001; Huang et al. 2006). Inoculation via mechanical wounds has also been used routinely to establish infection on canola cotyledons under controlled-environment conditions without leaf wetness (Liban et al. 2016; Liu et al. 2021; Soomro et al. 2020; Zhang et al. 2016). It has been long suspected that mechanical wounds caused by windstorms or flea-beetle feeding contribute to early blackleg infection of canola in western Canada, as conditions conducive to infection of intact leaves do not occur frequently. However, there has been no specific investigation reported on wound infection and the conditions that may affect it. *Phyllotreta cruciferae* Goeze and *P. striolata* Fabricius are the most common flea beetle species found in western Canada (Soroka et al. 2018), and their first-generation typically feed on canola seedlings right from the cotyledon stage (Fig. 1). Flea-beetle control relies primarily on seed-applied insecticides; in-crop insecticide is generally not recommended due to little yield benefit

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**Fig. 1** Feeding damage to canola (*Brassica napus*) cotyledons caused by flea beetles on the Canadian Prairies in early spring.
(Sekulic and Rempel 2016). However, if the feeding damage substantially facilitates blackleg infection, flea-beetle control may need to be viewed in a different light.

Another consideration is the environmental conditions during infection that may influence the outcome of *B. napus*- *L. maculans* interactions; blackleg infection may develop more rapidly at higher temperatures on susceptible canola varieties but not so on varieties with quantitative resistance (QR) (Hubbard and Peng 2018). QR may not stop blackleg infection completely but often decreases the growth and spread of pathogen hyphae, resulting in less stem infections and lower blackleg severity (Travadon et al. 2009; Huang et al. 2019). Despite its versatility and potential durability, QR can sometimes be affected by environmental conditions (Fitt et al. 2006; Kumar et al. 2018; Yang et al. 2021).

Specific resistance emanated from the *Rlm1-AvrLm1* interaction at 18°C was lost at 27°C (Ansar-Melayah et al. 1995; Badawy et al. 1992; Balesdent et al. 2002). Similarly, the *Rlm6* resistance was also compromised by the high temperature of 25°C and more than 48 h leaf wetness relative to that at 20°C and 15°C (Huang et al. 2006). High-relative humidity also enhanced infection by *L. biglobosa* on cotyledons under controlled environments (Hadrami et al. 2010). These results showed the influence of the environment on blackleg infection.

The objectives of this study were to (1) investigate infection of intact and wounded canola cotyledons by *L. maculans* under controlled-environment conditions to better understand the relative importance of mechanical wounding in the context of dry spring weather in western Canada; (2) assess the infection efficiency through wounds on susceptible and resistant canola varieties; and (3) determine the impact of post-wounding temperature and wetness conditions on infection. The information will be used to help assess the merit of further investigating flea-beetle feeding and its control on early blackleg infection in western Canada.

**Materials and methods**

**Fungal culture and inoculum preparation**

The isolates of *L. maculans* used for the study were maintained following the protocol described by Chen and Fernando (2006). Briefly, for each isolate, a single pycnidium was transferred to V8-agar medium made with 200 mL V8 juice, 800 mL distilled water, 15 g agar, 0.75 g calcium carbonate, and 0.035 g streptomycin sulphate salt. Culture plates were placed under cool-white fluorescent light at 22–24°C for 10–14 days for inoculum production. Sporulating cultures were flooded with sterilized water to release pycnidiospores and suspensions stored at −20°C until use.

The suspension for inoculation was diluted to the final concentration of 2 × 10^7 spores mL^−1^ estimated using a haemocytometer (Zhang et al. 2016). The isolate ‘12CC09’ carrying *AvrLm6, AvrLm7*, and *AvrLmS* (Supplemental Table S1) was used throughout the study. These avirulence genes were confirmed based on the cotyledon inoculation of 13 differential hosts of *Brassica* varieties/lines (Supplemental Table S1). The isolate ‘12CC09-GFP’ is transformed from 12CC09 with a binary vector containing a GFP fragment via *Agrobacterium*-mediated transformation (Hubbard and Peng 2018).

**Plant materials and growth conditions**

‘Westar’ and ‘74–44 BL’ (Bayer Crop Science) were used to represent susceptible and resistant canola cultivars, respectively. 74–44 BL carries the race-specific *R* genes *Rlm1/LepR3, Rlm3*, and possibly *RlmS*, as well as a level of race-non-specific resistance against blackleg (Hubbard et al. 2020). The resistance conferred by *RlmS* against the corresponding *AvrLmS*, however, was only moderate with average infection severity scores ranging from 4.6 to 6.0 when cotyledons were inoculated with isolate ‘12CC09’ (Supplemental Fig. S1) following the commonly used rating scale in which severity < 5 would be considered a resistant reaction (Zhang et al. 2017). Unless otherwise specified, canola seedlings were grown in a growth chamber set at 21°C and 16°C with a 16 h photoperiod during the higher temperature. Relative humidity was about 50–60% in the growth chamber. Plants were watered regularly and fertilized lightly with the 20–20–20 (N-P-K) water-soluble fertilizer weekly after plant emergence.

**Inoculation and disease assessment**

Two inoculation methods were used to initiate infection. Cotyledons of 7-day-old canola seedlings were pricked using a mechanical pencil or a pair of tweezers with bent pointed tips. In an earlier experiment, wounded cotyledons were spray inoculated with a pycnidiospore suspension (1 × 10^6 or 1 × 10^5 spores mL^−1^) using a misting bottle. About 0.4 mL was spayed on each plant. This appeared to exceed the amount of suspension required to cover the cotyledons as visible runoff occurred. In later experiments, a 10 μL droplet of spore suspension (2 × 10^7 spores mL^−1^) was applied to each pricked
wound. Non-wounded plants or wounded plants inoculated with sterilized water were used as negative controls, depending on the experiment.

Unless otherwise specified, inoculated seedlings were returned to the growth chamber mentioned above for disease development. True leaves were removed at 7 days post-inoculation (dpi) and weekly after that to delay the senescence of cotyledons. The infection symptoms on cotyledons were assessed using a 0–9 scale at 14 dpi (Zhang et al. 2016), assisted with the use of an HP ScanJet for image collection. The fluorescent signals of 12CC09-GFP hyphae in cotyledon tissues were acquired with an epifluorescence microscope or the EVOS M5000 Imaging System (Thermo-Fisher Scientific, Mississauga, ON) with a GFP light cube and excitation/emission maxima near 470/510 nm. The intensity of GFP fluorescence was measured using the image-processing software ImageJ (https://imagej.net/). Disease lesion sizes were also determined using ImageJ and disease incidences were expressed as the percentage of diseased lobes over total inoculated cotyledon lobes.

Infection on intact and wounded canola cotyledons

The cultivars ‘Westar’ (susceptible) and ‘74–44BL’ (resistant) were used for this experiment. About 7 days after planting, each lobe of a cotyledon was pricked with a mechanical pencil resulting in 4 or 16 wounds per cotyledon. This was to simulate light and heavy flea-beetle feeding damage. Cotyledons without wounding were used as controls. Conidiospore suspensions of L. maculans isolate 12CC09-GFP, at $1 \times 10^4$ and $2 \times 10^5$ spores mL$^{-1}$, were sprayed on cotyledons as a fine mist until runoff. Some of the inoculated plants were placed in a dew chamber (Percival Scientific Inc., Boone, IA) set at 21°C for 6 h for a period of leaf wetness, and others in a growth chamber (21°C) without dew. The plants were then moved to another growth chamber set at 21°C (day)/18°C (night) with a 16-h photoperiod provided with cool fluorescent tubes (280–575 μmol m$^{-2}$s$^{-1}$). Each treatment consisted of 8 plants (experimental units) with six of them being assessed for disease severity at 14 dpi and two examined for GFP expression at 9 dpi with a Zeiss Stereo-Lumar epifluorescence microscope.

Effect of wound age

Wounded cotyledons of ‘Westar’ and ‘74–44 BL’ were inoculated with the isolate 12CC09 or 12CC09-GFP at 0, 2, 4, 8, 12, and 24 hours post wounding (hpw). It was hypothesized that older wounds would be less susceptible than fresh wounds. A 10 μL drop of inoculum was applied to each wound, and inoculated seedlings were kept in the growth chamber for 14 days before being examined for disease incidence, lesion size, and fluorescence intensity in cotyledon tissues inoculated with the isolate 12CC09-GFP.

Effect of post-inoculation temperature and humidity on wound infection

‘Westar’ and ‘74–44 BL’ seedlings were inoculated with the inoculum droplets of 12CC09 or 12CC09-GFP via wounds. Three separate sets of seedlings were inoculated at 0, 2, 4, 8, 12 and 24 hpw and transferred to growth chambers with control (21°/18°C), high (25°C continuously) and low (10°C) temperatures, respectively, for disease development. The photoperiod (16 h) and RH (50–60%) were similar in these growth chambers. Additional seedlings, also inoculated at these wound ages (0–24 hpw) were transferred to growth chambers with similar temperature (21°/18°C) and photoperiod (16 h), but high RH (80–90%) created by covering seedling flats with transparent lids for 12 hours post-inoculation. Disease incidence and severity (lesion size) were assessed at 14 dpi.

Effect of post-wounding temperature and leaf wetness on the susceptibility of wounds

Cotyledons of ‘Westar’ and ‘74–44 BL’ seedlings were wounded and maintained initially under the control condition (21°/18°C, 16-h photoperiod and 50–60% RH) for 0, 4 or 8 hours. Some of the plants were then subjected to different conditions for 4 or 8 hours, either at a high (25°C) or low (10°C) temperature or with a period of leaf wetness at 21°C. These wounds would be 4 or 8 hours old at inoculation and this was to assess the potential effect of post-wounding conditions that might affect healing and susceptibility of wounds, as earlier results showed a substantial change in susceptibility between 4 and 8 hpw wounds. The leaf wetness was created by hourly misting with sterilized water until runoff and covering each flat with a lid. Inoculated plants were maintained under the control condition for 14 days before the assessment of disease incidence and severity.

Effect of re-wounding

‘Westar’ and ‘74–44 BL’ seedlings with pricked wounds on cotyledons were maintained under the control condition (21°/18°C, 16-h photoperiod and 50–60% RH) for
0, 2, 4, 8, 12 and 24 hours, and these wounds of varying ages were then re-wounded at the original punctured locations. Re-wounded seedlings were inoculated immediately with the isolate 12CC09, and inoculated plants were kept under the control condition for 14 days before being assessed for disease incidence and severity.

**ROS histochemical detection and caspase 3-like activity**

To examine ROS associated with wounding, histochemical staining of hydrogen peroxide (H$_2$O$_2$) production was carried out on cotyledon wounds of 12 and 24 hpw using 3,3’-diaminobenzidine (DAB) following the protocol described by Thordal-Christensen et al. (1997). These two wound ages appeared to reduce *L. maculans* infection substantially and consistently. H$_2$O$_2$ acts as a cell-death inducer. The Caspase 3-like activity was also measured for these wounds following Huang et al. (2014) using the substrate Ac-DEVD-AFC (N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-Trifluoromethylcoumarin). The activity acts like a cell-death executioner. Wounds of 0 hpw were tested similarly for controls.

**Statistical analysis**

All the experiments were conducted using a completely randomized design, with each treatment comprising three replicates (32 inoculated lobes of 8 plants per replicate). Each experiment was repeated twice. The ANOVA analysis ($P \leq 0.05$) was performed on all data sets in SAS (Version 9.4), and means of disease incidence, lesion size/disease severity, and GFP fluorescence intensity were analysed with Tukey’s HSD test ($P \leq 0.05$) when ANOVA was significant.

**Results**

**Infection of wounded and non-wounded cotyledons by *L. maculans***

In general, spray inoculation of non-wounded cotyledons of ‘Westar’ or ‘74–44BL’ failed to cause infection regardless of the inoculum concentrations used ($10^4$ or $10^5$ spores mL$^{-1}$) or the presence of a 6-h dew period post-inoculation (Fig. 2). In contrast, all cotyledons with pricked wounds were infected, evidenced by spreading fungal hyphae on ‘Westar’ at 9 dpi and leaf lesions on both cultivars at 14 dpi (Figs 2, 3). The higher number of wounds (16 per cotyledon) caused slightly greater average infection severity on ‘74–44BL’, especially at the higher inoculum level.

**Effect of the wound age on infection**

Wound age showed a clear effect on infection of both ‘Westar’ and ‘74–44BL’; older wounds, especially those inoculated 8 hpw or later, were substantially less susceptible, as evidenced by reduced infection success at 14 dpi (Fig. 4). Symptoms from inoculation of wounds 2 hpw were almost the same as those of fresh wounds (0 hpw,

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![Fig. 2](image-url) Intact (upper) and wounded (lower) canola (*Brassica napus*) cotyledons spray inoculated with pycnidiospore suspension of *Leptosphaeria maculans* at 9 days post-inoculation. Photos on the left show early infection development from the pricking wounds with visible green-fluorescent fungal hyphal spreading (lower right). No fungal fluorescence was detected in the non-wounded cotyledon tissue (upper right).
control); large lesions with diffuse margins and numerous pycnidia were observed on ‘Westar’. Smaller lesions delimited by dark necrotic margins and fewer pycnidia occurred on ‘74–44 BL’, a typical reaction of intermediate resistance. Average disease incidence and lesion size (severity) showed a similar pattern of response to wounding age (Fig. 5); both were lower for wounds older than 4 h relative to fresh wounds or those inoculated 2 hpw. Wounds inoculated 8 hpw or older had substantially reduced disease incidence and severity; the reduction was > 90% for wounds inoculated 12 or 24 hpw, on both cultivars.

Effect of temperature on colonization by L. maculans resulting from wound infection

Based on the extensiveness of the GFP-tagged L. maculans ‘12CC09-GFP’ hyphal growth and relative fluorescence intensity in cotyledon tissues, the higher post-inoculation temperature at 25°C did not change the effect of wound age on infection, with a similar pattern to that under the control temperature (21/18°C); infection success was significantly lower through the wounds of 8 hpw and older although the hyphal growth was more extensive with greater fluorescence intensity at the higher temperature once the infection took
Effect of wounding and wound age on infection by L. maculans

Fig. 4 Effect of wound age on cotyledon (Brassica napus canola) infection by Leptosphaeria maculans. Representative symptoms resulting from the inoculation of wounds of 0 to 24 hours post wounding (hwp) at 14 days post-inoculation.

place, especially through the wounds of 0–4 hwp (Figs 6, 7). At the lower post-inoculation temperature (10°C), the same pattern of wound-age effect was also observed although the hyphal growth was substantially limited despite successful infection. The strength of GFP signals appeared to correspond to the severity of symptoms on cotyledons inflicted by the inoculation with the wild type ‘12CC09’ (Fig. 4) under the control condition (21/18°C) on both cultivars. The relative GFP fluorescence intensity accounted for the background signal using cotyledons treated with ddH₂O as a negative control (Supplemental Fig. S2).

Effect of high humidity on infection of wounds of different ages

The 12-hour high RH (80–90%) provided immediately post inoculation did not substantially change the effect of wound age on infection; wounds of 8 hwp or older showed much-reduced disease incidence and severity (lesion size) on both cultivars, relative to younger wounds (Fig. 8). The pattern of response for the wound-age effect against infection was similar to that in the earlier experiment with lower post-inoculation RH, although the disease levels appeared visually higher on older wounds than those observed before.

Effect of post-wounding conditions on the susceptibility of wounds

Post-wounding temperature or moisture conditions showed an only slight effect on the susceptibility of wounds and the key factor appeared to be the wound age (hwp); wounds inoculated 8 hwp consistently showed lower infection incidence or severity than those inoculated 4 hwp, regardless of the post-wounding condition applied (Fig. 9). The pattern was also similar between the two canola cultivars, although ‘74–44 BL’ often showed noticeably smaller lesions. Wounds of 8 hwp exposed to the higher post-wounding temperature (25°C) appeared slightly less susceptible than those of the same age not exposed to the higher temperature; this was on ‘Westar’ and the exposure to the low (10°C) post-wounding temperature or leaf wetness may increase wound susceptibility slightly relative to that under control conditions (21/18°C). These results suggested environmental conditions during the wounding process also affected blackleg infections, and the observed effects were similar between susceptible and intermediate reactions.

Effect of re-wounding at wounds of different ages on infection

Localized re-wounding of wounds of 0 to 24 hwp followed with immediate inoculation generally erased the wound age effect on infection, causing typical susceptible and intermediate resistant reactions, respectively, on ‘Westar’ and ‘74–44 BL’ at 14 dpi (Fig. 10). The lesion sizes were much smaller on ‘74–44 BL’, although the disease incidence reached 100% on both cultivars.
Compared to the wounds of 0 h pw, those of 12 h pw and 24 h pw showed higher \( \text{H}_2\text{O}_2 \) levels in both ‘Westar’ and ‘74–44 BL’, with a slightly higher accumulation found at 24 h pw relative to that of 12 h pw (Supplemental Fig. S4A). However, little difference was found in the caspase 3-like activity associated with the wound age in either ‘Westar’ or ‘74–44 BL’ (Supplemental Fig. S4B).

**Discussion**

This study was designed to assess the infection of canola by *L. maculans* in the context of western Canadian environmental conditions where the weather is typically cool and dry in early spring. Infection of intact canola leaves, by both ascospores and pycnidiospores, may be hindered by a lack of leaf wetness and/or warm temperatures required for germination/penetration (Huang et al. 2006; Sosnowski et al. 2005; Toscano-Underwood et al. 2001). With a relatively short growing season in the region (Walton et al. 1999), early infection is likely important for blackleg to cause a serious impact on the crop. It has been suspected that flea-beetle feeding (Fig. 1), which happens commonly on the Canadian Prairies in early spring (Soroka et al. 2018), may contribute to the infection of canola seedlings as feeding...
Fig. 6 Effect of post-inoculation temperature on infection development resulting from wounds of different ages (hour post wounding – hpw). A GFP-tagged *Leptosphaeria maculans* isolate (12CC09-GFP) was used for inoculation and the colonization was visualized based on the extensiveness of fluorescent fungal hyphae in the leaf tissue of susceptible (‘Westar’) and resistant (‘74–44 BL’) canola (*Brassica napus*) cultivars under control, high and low-temperature conditions at 14 days post-inoculation. The arrows mark the wounding sites.

Fig. 7 Quantification of relative fluorescence intensity of *Leptosphaeria maculans* 12CC09-GFP hyphae in cotyledon tissues resulting from the infection via wounds of different ages under control, high and low-temperature conditions. Values are fluorescent signal intensity ±SE averaged over three replicates, each consisting of at least 8 plants, and the same letters indicate no difference (P < 0.05) within the temperature and genotype group.
Fig. 8 Lesion size (A) and disease incidence (B) caused by the inoculation of wounds with *Leptosphaeria maculans* 0 to 24 hours post wounding (hpw) on cotyledons of susceptible (‘Westar’) and resistant (‘74–44 BL’) canola (*Brassica napus*) cultivars at 14 days post inoculation. High Values are means ±SE, of 3 replicates each consisting of at least 8 plants, and the same letters indicate no differences (P < 0.05) within the cultivar.

Fig. 9 Effect of post-wounding high and low temperatures (designated as high and low, respectively), or leaf wetness (wet) on the susceptibility of wounds. ‘Westar’ and ‘74–44 BL’ canola (*Brassica napus*) seedlings with wounds of 0, 4, and 8 hpw were subjected to 0, 4, or 8 hours of treatment, resulting in wounds of either 4 hpw or 8 hpw for inoculation with *Leptosphaeria maculans*. Lesion sizes (A, C, E) and disease incidences (B, D, F) were assessed at 14 dpi. Means ±SE were from three replicates, each consisting of at least 8 plants. The same letters indicate no difference within the genotype and post-wound condition (P < 0.05).
wounds might allow *L. maculans* to enter leaf tissues without the presence of surface wetness (Huang et al. 2006; West et al. 2001).

The results clearly showed that mechanical wounds facilitated *L. maculans* pycnidiospore infection on both susceptible and resistant canola cultivars without the presence of leaf wetness, while intact cotyledons could not be infected even when a 6-h dew period was provided post-inoculation. This indicates the potential that flea-beetle feeding wounds may be a factor for the infection in early spring, and may explain why an early fungicide application between 2- and 4-leaf stages is more effective than a later treatment at early bolting (Peng et al. 2020, 2021). Further results suggested that wound age also plays a role; wounds > 4 hwp may become less susceptible than fresh wounds and those of 8 hwp and older reduce the infection success substantially. This adds variability under field conditions concerning the timing of flea-beetle feeding activities. In other pathosystems, wounds facilitated the invasion of tomato roots by the bacterial pathogen *Pseudomonas solanacearum* (Vasse et al. 1995), but induced localized defence responses on Arabidopsis to *Botrytis* (Chassot et al. 2008). The wound age has also been shown to affect infection of tomato roots by several pathogens (Francia et al. 2008), as well as that of apple shoots by the fungal pathogen *Valsa Mali* (Chen et al. 2016). Our results demonstrated the effect of wounding and wound-age on blackleg infection, which may aid the blackleg pathogen *L. maculans* to establish in canola seedlings under dry spring conditions. This is potentially important information for western Canada where early-spring conditions often are not conducive for infection of intact canola leaves.

The results also showed that post-wounding conditions could affect wound susceptibility, interacting with wound age; a healing process ≥ 8 hwp appears to be the key for substantial reduction of wound susceptibility, regardless of post-wounding conditions. The high-temperature did not appear to affect the colonization of ‘74–44BL’ (*Rlm1-AvrLm1 interaction*) by *L. maculans*, which is similar to the effect on hypersensitive responses mediated by *Rlm6* against *AvrLm6* in *L. maculans* (Huang et al. 2006), as well as to those observed in other pathosystems (Wang et al. 2009) where the host resistance is largely sustained under high temperatures. This, however, is contrasted to the case of *Rlm1-AvlLm1* where high temperatures seemed to undermine the resistance (Ansari-Mehaye et al. 1995; Badawy et al. 1992; Balesdent et al. 2002). The effect of high temperature on the manifestation of resistance can vary from seedlings to adult plants of canola; in this study, the escalation of infection trended similarly on seedlings of both ‘Westar’ and ‘74–44 BL’ under high-temperature conditions (Figs 6, 7, 8), while high-temperature stresses increased the infection on adult plants of ‘Westar’ only but not on those of ‘74–44 BL’ (Hubbard and Peng 2018). The reduced infection development at the lower temperature is consistent with earlier results (Toscano-Underwood et al. 2001).
The lower susceptibility of older wounds is likely related to the physiological status of the tissue at the time of inoculation (Sharabani et al. 2013). It is not a surprise that the wound-age effect can be removed by localized re-wounding on both cultivars before inoculation, implying the obstruction of infection likely stems from the healing of wounds. Older wounds (12 hwp and 24 hwp) showed slightly higher H$_2$O$_2$ levels in both cultivars (Supplemental Fig. S4A). The accumulation of H$_2$O$_2$ is the primary trigger for ROS to mediate wound signalling (Leon et al. 2001; Lew et al. 2020) which often overlaps among abiotic and biotic stress responses (Perez and Brown 2014). However, little difference was found in association with wound age and the caspase 3-like activity, the most prevalent cell-death executioner (Supplemental Fig. S4B). The caspase 3-like activity has been observed on large (Iakimova and Woltering 2018) and old (Cui et al. 2019) wounds. The role of ROS in reducing the susceptibility of older wounds to *L. maculans* infection is still inconclusive.

Similar to some of the earlier results (Nnodu et al. 1982; Fugate et al. 2016), higher post-wounding temperatures may aid in wound healing on canola cotyledons, evidenced by slightly reduced disease incidence for the 8 h treatment at 25°C on ‘Westar’ relative to shorter exposure to the high temperature in this study (Fig. 9). The lower (10°C) temperature or the leaf wetness appeared to delay the healing, resulting in slightly higher disease levels than a shorter exposure to these conditions. Overall, older wounds tend to be less susceptible to infection, even between 4 hwp and 8 hwp.

This study was conducted to help determine the merit of investigating flea-beetle feeding on canola seedlings concerning early blackleg infection under field conditions in western Canada where it is relatively dry and cool with only occasional rain in early spring. Flea beetle feeding is common in many areas each year. The data showed that mechanical wounds would facilitate the infection of canola seedlings by *L. maculans* when there is a lack of moisture, especially for the fresh wounds of $< 4$ hwp. Low-temperature/leaf wetness conditions may prolong the healing of wounds, making them slightly more vulnerable to infection than under high-temperature conditions. The data provide a strong rationale for further studies to assess the influence of flea-beetle feeding on blackleg infection, as well as potential benefits of improving flea-beetle control to blackleg management.

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**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Supplementary material**

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