



Virulence/avirulence patterns among *Leptosphaeria maculans* isolates determines expression of resistance, senescence and yellowing in cotyledons of *Brassica napus*

A. Dolatabadian · J. Batley · D. Edwards · M. J. Barbetti

Accepted: 26 February 2020 / Published online: 13 March 2020
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Abstract Blackleg, (stem canker, *Leptosphaeria maculans*), is the most economically important disease on oilseed rape (*Brassica napus*). Studies were undertaken to determine the effect of three different *L. maculans* isolates with different virulence/avirulence patterns on the expression of qualitative resistance and senescence on cotyledons of 11 *B. napus* genotypes. There were significant differences between genotypes ($P < 0.01$), isolates ($P < 0.01$) and a significant genotype by isolate interaction ($P < 0.01$) in terms of disease index and lesion diameter. Overall, *B. napus* LSF0530 was more resistant than Barrel > Scotia > Amherst x Commande DH Line. Westar, Drummonds Purple Top, and Parkside were the most susceptible. *L. maculans* isolates D5 and D13 were the most pathogenic, showing the greatest disease index and lesion diameters. Genotypes such as LSF0530, Scotia, Duell and Barrel responded with a distinct hypersensitive response, whereas Parkside, Drummonds Purple Top, Amherst x Commande DH Line and Westar all showed a distinct yellow halo around the lesions, indicative of cotyledon senescence likely involving programmed cell

death (PCD). However, expression of the distinct yellow halo symptom was not observed in all *B. napus* genotypes infected by one specific *L. maculans* isolate nor in one specific *B. napus* genotype challenged with all the isolates. We believe that this is the first report, i), showing the involvement of a distinct yellow halo (senescence/PCD) associated with both ‘typical’ lesions and with an expression of HR in cotyledons of winter-type *B. napus* genotypes challenged with *L. maculans* isolates and, ii), that expression of the distinct yellow halo is clearly dependent on the interaction between particular isolate virulence(s) x particular winter-type *B. napus* genotype combinations.

Keywords Blackleg · *Brassica napus* · Cotyledon senescence · Disease development · *Leptosphaeria maculans* · Pathogenicity

Introduction

Blackleg or stem canker, caused by a complex of phylogenetically-related ascomycete species, particularly *Leptosphaeria maculans*, and to a lesser extent *L. biglobosa*, is the most important disease on canola (*Brassica napus*) in many regions of the world. In Australia, *L. maculans* predominates, and it causes cotyledon and leaf lesions during seedling growth, followed by a particularly damaging stem canker phase, significantly reducing yield (Li et al. 2006). In Australia, early seedling stage infection, especially on cotyledons, maximises yield loss (Barbetti and Khangura 1999),

A. Dolatabadian · J. Batley () · D. Edwards
UWA School of Biological Sciences and the UWA Institute of Agriculture, Faculty of Science, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia
e-mail: jacqueline.batley@uwa.edu.au

M. J. Barbetti
UWA School of Agriculture and Environment and the UWA Institute of Agriculture, Faculty of Science, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia

there being a strong positive correlation between incidences of cotyledon infection with the extent of subsequent crown canker development (Cargeeg and Thurling 1980).

Generally, in *Brassica* species, resistance to *L. maculans* is divided into two types of resistance: seedling resistance (qualitative) and adult resistance (quantitative); the former being single-gene (monogenic), race-specific and expressed in the cotyledons (Delourme et al. 2006). By contrast, adult resistance is race non-specific and controlled by multiple genes (polygenic). Qualitative resistance against *L. maculans* can be selected in young plants by assessing lesion phenotypes after inoculation on cotyledons or leaves. This type of resistance puts strong selection pressure on the fungal populations to quickly adapt and overcome such resistance(s) (Rouxel et al. 2003).

The interaction between *B. napus* and *L. maculans* on cotyledons is a classic gene-for-gene relationship whereby for each major seedling resistance gene in the plant there is a corresponding avirulence (*Avr*) gene in the fungus. Avirulence genes encode proteins that are specifically recognised by host plants containing the ‘matching’ resistance gene (Rouxel and Balesdent 2010). The products of these genes are known as effector proteins. A wide range of effector proteins contribute to the process of infection and includes the secretion of different compounds into host cells (Schmidt and Panstruga 2011). Effectors also encompass genes that perform a wide variety of functions, such as *Avr* effectors that play a role in toxicity and in triggering host responses or pathogen-associated molecular patterns, and as enzymes responsible for cell wall degradation (Sperschneider et al. 2015).

Changes in the frequency of virulence genes in the *L. maculans* genome can lead to different resistance responses in *B. napus* genotypes (Marcroft et al. 2012), and *L. maculans* populations can change their virulence pattern(s) (Rahman et al. 2016). Previous studies on genetic variation in *L. maculans* populations have found high levels of genetic variation (Hayden et al. 2007; Zander et al. 2013).

To date, 16 *L. maculans* *Avr* genes have been identified: *AvrLm1-Lm11*, *AvrLepR1-LepR3* and *AvrLmS*, and among these *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4–7*, *AvrLm6*, *AvrLm11* and *AvrLm5–9* have been cloned (Gout et al. 2006; Fudal et al. 2007; Parlange et al. 2009; Balesdent et al. 2013; Van De Wouw et al. 2014; Ghanbarnia et al. 2015; Plissonneau et al. 2016; Ghanbarnia et al. 2018). *AvrLm1* confers avirulence towards the *B. napus* resistance

genes *Rlm1* and *LepR3* (two independent *R* loci located on chromosomes A7 and A10, respectively) and *AvrLm2* corresponds with *RLm2*. *LepR3* and *Rlm2* are allelic and encode membrane-bound receptor-like proteins (RLPs) (Ghanbarnia et al. 2018). *AvrLm3* was cloned by Plissonneau et al. (2016) who showed that *AvrLm3* recognition by the *B. napus* *R* protein *Rlm3* is masked in the presence of *AvrLm4–7*. *AvrLm3* and *AvrLm4–7* are physically linked (Plissonneau et al. 2016). *AvrLm4–7* is perceived by both *Rlm4* and *Rlm7* (Parlange et al. 2009), whereas *AvrLm6* confers avirulence towards resistance gene *Rlm6*. Two other *Avr* genes, *AvrLm9* and *AvrLep1*, have been reported to be closely linked to *AvrLm4–7* (Balesdent et al. 2005; Ghanbarnia et al. 2012). Singleton *L. maculans* *Avr* genes have also been reported: *AvrLm11* and *AvrLmJ1* (Balesdent et al. 2013; Van De Wouw et al. 2014). *AvrLm11* confers avirulence towards resistance gene *Rlm11* of *B. rapa* (Balesdent et al. 2013). *AvrLmJ1* was characterized as being avirulent on *B. juncea* carrying an unknown *R* gene. However, Plissonneau et al. (2018) have shown that *AvrLmJ1* is, in fact, *AvrLm5* based on co-localized mapping and the demonstration that *Lm* isolates carrying *AvrLmJ1* are also avirulent on *B. juncea* differential lines carrying *Rlm5*. Recently, the *AvrLm9* phenotype has been mapped to SC7 and shown to be an allele of *AvrLm5* (Ghanbarnia et al. 2018).

Senescence is a regulated terminal stage process of plant development, during which mature cells, tissues, organs and even the whole plant go through a series of programmed cell death (PCD) processes (Liu et al. 2008). Senescence usually corresponds with age, however the process involves complex regulation of pathways that respond to several endogenous and exogenous environmental factors that adjust its onset, progression and completion (Woo et al. 2013). Cotyledon senescence also involves a programmed degradation of cells and tissues (Thomas et al. 2003). Once cotyledons have completed their role in supplying nutrients to the seedlings they generally undergo senescence. Like other abiotic and biotic stresses, fungal infections may accelerate cotyledon or leaf senescence, possibly acting as a resistance mechanism by limiting the infectious period. It has been suggested that resistance may be correlated with the onset of senescence (Develey-Rivière and Galiana 2007).

The first line of defence in plants is provided by pattern recognition receptors (PRRs), which recognize microbe- or danger-associated molecular patterns (MAMPs and DAMPs, respectively), and trigger immune signalling (Coll et al. 2011). The second

intracellular class of innate immune receptors is activated via recognition of pathogen effectors, resulting in effector-triggered immunity (ETI), which is mediated by nucleotide-binding domain, LRR (NB-LRR) disease resistance proteins (Coll et al. 2011). Pathogen recognition via NB-LRRs leads to inhibition of pathogen growth, which is often, but not always, accompanied in plants by the hypersensitive response (HR), a form of PCD. HR is characterized by the rapid death of plants cells at the site of pathogen infection (Lam 2004) and has been well described in *B. napus* (Li et al. 2006, a, b, 2008).

There have been many studies investigating the interaction of *B. napus* genotypes to different *L. maculans* isolates (e.g., Balesdent et al. 2002, 2005; Parlange et al. 2009). Such studies not only improved our understanding of the expression of host resistance in *B. napus*, but also enabled identification of genotypes with resistance to particular individual pathogen isolates, not just for *L. maculans*, but also for other *Brassicaceae* pathogens like *Sclerotinia sclerotiorum* (Ge et al. 2015). Further, Develey-Riviére and Galiana (2007) noted that resistance may be correlated with the onset of senescence, and Ge et al. (2015) had confirmed such for *B. napus* against *S. sclerotiorum*. Hence, we undertook studies to determine the effect of different *L. maculans* isolates with different virulence/avirulence patterns on the expression of qualitative resistance and senescence on cotyledons of 11 *B. napus* genotypes using three *L. maculans* isolates with different virulence/avirulence patterns.

Materials and methods

L. maculans isolates and inoculum preparation

Three previously characterised and prevalent isolates of *L. maculans* (D5, D13 and D16) in Australia (Van De Wouw et al. 2018) were kindly provided by Dr. Angela Van De Wouw, School of BioSciences, The University of Melbourne, Victoria, Australia. Isolate details are given in Table 1. As per the methods of Mengistu et al. (1993), subcultures of these isolates were grown on V8 agar until production of abundant conidia occurred, briefly as follows. 1 ml of sterile deionized water was pipetted into a sterile plastic tube into which a colonised 0.5×1 cm V8 agar strip with pycnidia had been cut out using a sterile scalpel and placed in the tube. The agar strip was left in the tube for 10 mins to

Table 1 Virulence/Avirulence gene pattern of *Leptosphaeria maculans* isolates used

Genes	Isolate		
	D5	D13	D16
<i>AvrLm1</i>	Avr	Vir	Vir
<i>AvrLm2</i>	Avr	Vir	nd
<i>AvrLm3</i>	Vir	Vir	Vir
<i>AvrLm4</i>	Avr	Avr	Vir
<i>AvrLm5</i>	Vir	nd	Avr
<i>AvrLm6</i>	Vir	Avr	Avr
<i>AvrLm7</i>	Avr	Avr	Avr
<i>AvrLm8</i>	Vir	nd	nd
<i>AvrLm9</i>	Vir	Vir	Vir
<i>AvrLmS</i>	Avr	Vir	Avr
<i>AvrLepR1</i>	Avr	Vir	Vir

nd: not defined

allow the conidia to be released. Then 100 μ l of the conidial suspension was transferred onto each of the V8 agar plates and spread over the agar surface using a sterile glass rod. Parafilm™ was used to seal the Petri™ plates which were then incubated at 22 °C under the single cool-white fluorescent light tube and a single black light tube (Phillips TL 40 W/80 RS F40 BLB) until they produced pycnidia and conidia (approximately 1 week). Conidia were then harvested by adding 10 ml of sterile deionized water onto each culture plate and leaving for 10 min to allow conidia to release from pycnidia. Further, additional conidia were harvested by scraping the surface of each culture using a sterile glass rod. The conidial suspension was then filtered into a 15 ml Falcon tube using Mira cloth (Calbiochem, Hoechst. La Jolla, USA). To ensure consistent conidial concentration, 10 μ l of the suspension was placed onto the haemocytometer slide (Calbiochem, San Diego, USA), examined under the microscope, and conidial counts and adjustments made to ensure a final conidia concentration of 1×10^7 ml $^{-1}$. The conidial suspensions were transferred into 1.5-ml tubes and frozen at -20 °C until required.

B. napus genotypes

Ten winter-type genotypes of *B. napus* were chosen from a list of over 200 within the UWA *B. napus* seed collection to represent a range in resistance types. Westar (spring-type) was used as a susceptible control comparison as it is routinely used for this purpose in such experiments. Details of all the genotypes are listed in Table 2.

Table 2 *Brassica napus* genotypes used

Number	Genotype	Type
1	Boston	Winter
2	Scotia	Winter
3	Parkside	Winter
4	Drummonds Purple Top	Winter
5	Chuosenchu	Winter
6	LSF0530	Winter
7	Duell	Winter
8	Barrel	Winter
9	Liglory	Winter
10	Amber x Commande DH Line	Winter
11	Westar	Spring

Growth conditions

Two duplicate experiments were undertaken under controlled environment conditions. *B. napus* genotypes were sown in multi-cell (6×8 cells) trays filled with UWA potting mix consisting of 2.5 m^3 fine composted pine bark, 1 m^3 coco peat, 5 m^3 brown river sand, 10 kg slow release fertilizer Osmoform® NXT 22 N + 2.2 P₂O₅ + 9.1 K₂O + 1.2 Mg + trace elements (Everris International B.V.), 10 kg Dolomite (CalMag®), 5 kg gypsum clay breaker, 5 kg extra fine limestone, 4 kg iron hepta sulphate, and 1 kg iron chelate. Potting mix was pasteurized at 63°C for 30 min. Each tray was placed in a plastic container before placing in a phytotron under $18/13 \pm 2^\circ\text{C}$ day/night and natural light. Seedlings were equally irrigated regularly with tap water to free draining potting mix.

Inoculation procedure

Fourteen days after sowing, inoculation was performed as described by Purwantara et al. (1998), with some modification. Briefly, one lobe of each fully expanded cotyledon was wounded using sterile sharp-pointed forceps and a $10 \mu\text{l}$ droplet of conidial suspension (10^7

conidia ml^{-1}) was deposited on one wounded lobe using a micropipette. For controls, $10 \mu\text{l}$ of sterilized distilled water was similarly deposited on the cotyledon lobe. Inoculated seedlings were covered with a transparent polyethylene cover to maintain high humidity for 72 h post-inoculation. Inoculated seedlings were maintained at constant 22°C in a controlled environment room equipped with cool white fluorescent lamps (Model 840, Philips). Photosynthetically active radiation was $350 \mu\text{mol m}^{-2} \text{ s}^{-1}$, photoperiod of 12 h and relative humidity 70%.

Disease assessment

Days to lesion appearance was recorded when the seedlings commenced showing visible lesions. Two weeks after inoculation, cotyledons were detached and photographed and lesions assessed on the basis of lesion diameter (mm) measured by ImageJ® software, and also colour and presence or absence of pycnidia. Symptoms were scored on inoculated cotyledons 14 days after inoculation using the rating scale described by Williams and Delwiche (1979) with some modifications. There were seven infection classes: 0 (no darkening around wounds, usually indicates missed inoculation), 1 (limited blackening around wounds, lesion diameter 0.5–1 mm), 3 (dark necrotic lesions 1–2 mm in diameter), 5 (lesion diameter 3–5 mm, lesions can vary in colour from dark to pale, however, dark lesions are often an avirulent reaction that will not progress any further in size), 7 (large grey-green lesions, lesion diameter > 5 mm), 8 (large grey-green lesions but with some pycnidia, lesion diameter > 5 mm) and 9 (large grey-green lesions with profuse sporulation, lesion diameter > 5 mm). Note that the scale established by Williams and Delwiche (1979) did not have a category of 8, but this was added for the current study to distinguish between pycnidia development and sporulation. A percent disease index (%DI) was calculated as widely utilized in other studies of rapeseed cotyledon disease (e.g., for white leaf spot by Gunasinghe et al. 2014; and for downy mildew by Mohammed et al. 2019), as follows:

$$\%DI = \{[(a \times 0) + (b \times 1) + (c \times 2) + (d \times 3) + (e \times 4) + \dots + (j \times 9)] \times 100\} / [(a + b + c + d + e + \dots + j) \times 9]$$

And where a, b, c, d, ej are the number of plants with severity scores of 1, 1, 2, 3, 49, respectively.

According to classification by Van De Wouw et al. (2009), lesion score 3 or less was classified as resistant, lesion scores >3 but <5 were classified as moderate

resistance, and scores 5 and > 5 were considered as susceptible.

Statistical analyses

Two identical experiments were undertaken. Each experiment consisted of 11 genotypes and three isolates with three replicates, arranged as factorial and with treatments in a complete randomised design. There were three control plants (inoculated with sterilized distilled water) for each genotype. The relationship between the initial and repeat experiments was assessed using a paired t-test. As there were no significant differences between initial and repeated experiments ($P > 0.05$), the original and repeat experiment data sets were combined and presented following ANOVA conducted using SAS 9.4. Fisher's least significant differences (LSD) were used to separate significant differences between treatments.

Results

There were significant ($P < 0.01$) effects of both *L. maculans* isolate and *B. napus* genotype in relation to the number of days for lesion appearance, for cotyledon lesion diameter and for disease index (Table 3). In addition, there was a significant ($P < 0.01$) isolate \times genotype interaction in terms of disease index and cotyledon lesion diameter (Table 3).

Lesions took significantly longer to appear with the D16 isolate than for D5 and D13 isolates, but there was no significant difference between D5 and D13 isolates (Fig. 1). The greatest number of days to lesions appearance (9.22 days) was for genotype LSF0530 (Fig. 2).

Table 3 Analysis of variance on number of days to lesions appearance, disease index and lesion diameter as affected by three *Leptosphaeria maculans* isolates (D5, D13 and D16) and 11

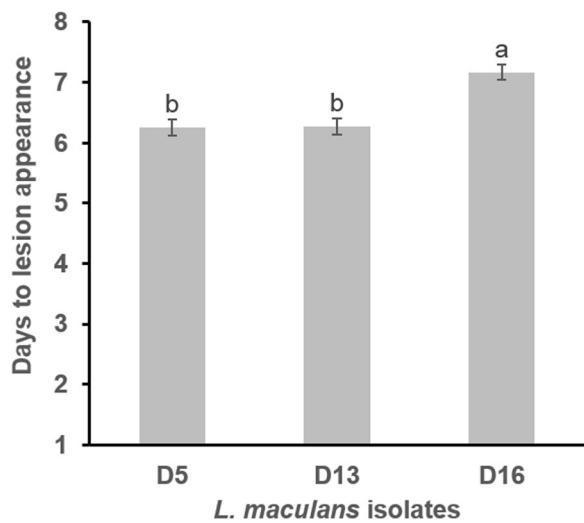


Fig. 1 Effect of *Leptosphaeria maculans* isolates (D5, D13 and D16) on days to lesion appearance. Different letters indicate significant differences ($P \leq 0.05$ (LSD = 0.17)). Bars show standard errors ($n = 66$)

There were significant ($P < 0.01$) differences in virulence among isolates in terms of disease index. Isolates D5 (DI 5.01) and D13 (5.13) were the most virulent, whereas D16 (4.34) was found to be the least virulent isolate (Fig. 3). Isolates D5 and D13 had maximum mean lesion diameters of 4.53 and 5.16 mm, respectively (Fig. 4).

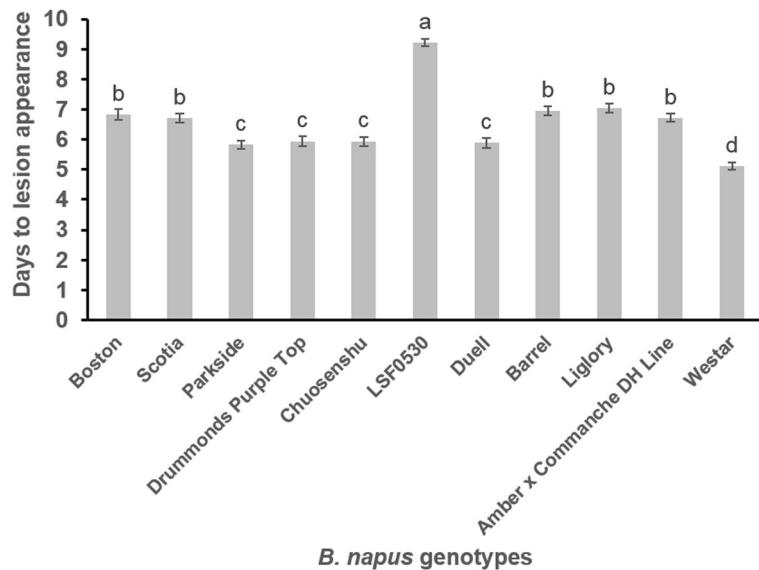
There was a wide range in terms of the level of resistance/susceptibility based on disease index and mean lesion diameter across the test *B. napus* genotypes, ranging from a disease index of 2.88 and lesion diameter of 2.66 mm on the most resistant genotype (LSF0530) to a disease index of 7.50 and mean lesion diameter of 6.44 mm on the most susceptible genotype (Westar) (Table 4). Genotype LSF0530 showed a high level of resistance; in contrast, genotypes Parkside, Drummonds Purple Top, Chuosenshu, Duell and Westar were all

Brassica napus genotypes (Boston, Scotia, Parkside, Drummonds Purple Top, Chuosenshu, LSF0530, Duell, Barrel, Liglory, Amber x Commande DH Line and Westar)

Sources of variation	d.f	Days to lesion appearance	Disease index	Lesions diameter
Isolate	2	17.88**	11.87**	50.36**
Genotype	10	20.59**	33.68**	25.42**
Isolate \times Genotype	20	0.12 ns	11.58**	13.62**
Error	165	0.25	1.21	1.97
C.V (%)		7.71	22.84	32.05

**= significant at $P < 0.01$; ns = not significant

Fig. 2 Effect of *Brassica napus* genotype (Boston, Scotia, Parkside, Drummonds Purple Top, Chuoenshu, LSF0530, Duell, Barrel, Liglory, Amber x Commande DH Line and Westar) on days to lesion appearance. Different letters indicate significant differences ($P \leq 0.05$), (LSD = 0.33). Bars show standard errors ($n = 18$)



consistently highly susceptible. Other genotypes such as Boston, Scotia, Barrel, Liglory and Amber x Commande DH Line, showed a moderate resistance response (Table 4). In terms of disease index, genotypes like Scotia, Drummonds Purple Top, LSF0530 and Westar, showed a consistent and isolate-independent response (Table 5). In contrast, other genotypes (e.g., Boston, Barrel and Amber x Commande DH Line), showed high levels of resistance against one isolate but were quite susceptible to other isolates. Overall, across the three test isolates, the most resistant genotype was LSF0530 with a disease index of ≤ 3 , while Westar, followed by

Drummonds Purple Top, both showed a disease index >5 (Table 5). The greatest disease index was for Westar infected by D5 or D13, followed by Drummonds Purple Top infected with the same isolates (Table 5). In contrast, the least disease index was for Barrel and the Amber x Commande DH Line genotypes infected with isolates D13 or D16, respectively (Table 5).

Lesion diameter varied from 1.33 mm in genotypes Barrel and Amber x Commande DH Line infected by isolates D13 or D16 isolates, respectively; to 7–8.50 mm in Westar and Drummonds Purple Top infected by isolates D5 or D13, respectively (Table 6). In general, the

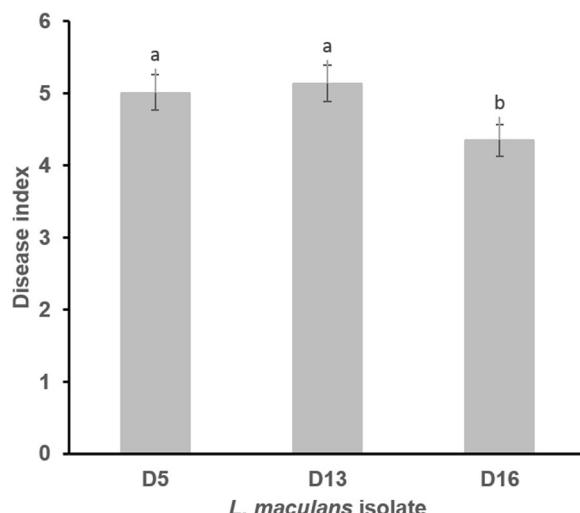


Fig. 3 Effect of *Leptosphaeria maculans* isolates (D5, D13 and D16) on disease index. Different letters indicate significant differences ($P \leq 0.05$), (LSD = 0.37). Bars show standard errors ($n = 66$)

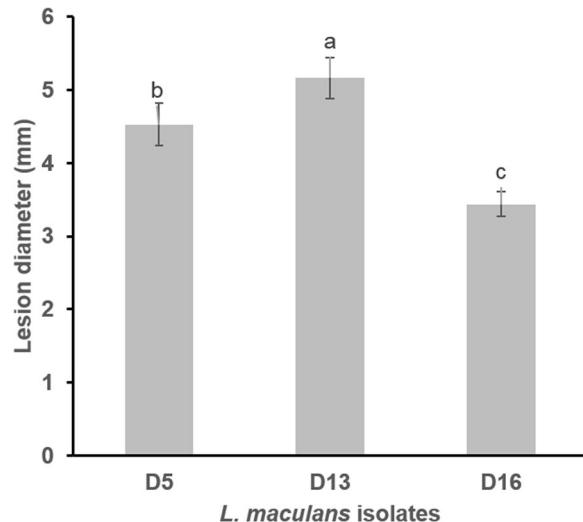


Fig. 4 Effect of *Leptosphaeria maculans* isolates (D5, D13 and D16) on lesion diameter. Different letters indicate significant differences ($P \leq 0.05$), (LSD = 0.17). Bars show standard errors ($n = 66$)

Table 4 Effect of *Brassica napus* genotype on disease index and lesion diameter at 14 days after inoculation of cotyledons across 11 different *B. napus* genotypes

Genotypes	Disease index	Lesion diameter
Boston	4.00c	3.94bcd
Scotia	3.88c	3.11de
Parkside	5.33b	4.66b
Drummonds Purple Top	6.77a	6.38a
Chuosenshu	5.22b	4.38bc
LSF0530	2.88d	2.66e
Duell	5.00b	4.83b
Barrel	3.72c	3.61cd
Liglory	4.88b	4.05bc
Amber x Commanche DH Line	3.94c	4.05bc
Westar	7.50a	6.44a
LSD values	0.72	0.92

Different letters indicate significant differences ($P \leq 0.05$).

■ Resistant ■ Moderate ■ Susceptible

smallest lesions were observed when seedlings were inoculated with D16 (Table 6). Overall, isolate D13 was the most virulent isolate across most test genotypes compared with isolates D5 and D16.

In addition to expected lesion symptoms, atypical symptoms of lesions with a distinct yellow halo were observed across some genotype x isolate combinations (Fig. 5). For example, in Scotia and Barrel genotypes, in contrast to lesions produced by the D13 and D16 isolates where symptoms were characterized by ‘normal’

lesion symptoms, isolate D5 induced lesions displaying a distinct yellow halo around the lesions (Fig. 5). Similarly, when genotype Duell was challenged with different isolates, it only developed a distinct yellow halo around the cotyledon lesions with isolate D13. The distinct yellow halo symptoms were also observed in genotype Boston against isolates D5 and D13; for Chuosenshu and Liglory against D13 and D16; while for Parkside, Drummonds Purple Top, Amber x Commanche DH Line and Westar the distinct yellow

Table 5 Disease index at 14 days after inoculation of the cotyledons of 11 *Brassica napus* genotypes with three different isolates of *Leptosphaeria maculans*

Genotypes	Isolates		
	D5	D13	D16
Boston	2.33lm	6.00cde	3.66hijk
Scotia	4.66fgh	3.66hijk	3.33ijkl
Parkside	6.66bcd	4.66fgh	4.66fgh
Drummonds Purple Top	7.00abc	7.00abc	6.33cde
Chuosenshu	3.66hijk	6.66bcd	5.33efg
LSF0530	2.66kl	3.00jkl	3.00jkl
Duell	4.66fgh	6.33cde	4.00hij
Barrel	6.16cde	1.33m	3.66hijk
Liglory	4.66fgh	4.33ghi	5.66def
Amber x Commanche DH Line	4.66fgh	5.83cde	1.33m
Westar	8.00a	7.66ab	6.83abcd
LSD value 1.25			

Different letters indicate significant differences ($P \leq 0.05$).

■ Resistant ■ Moderate ■ Susceptible

Table 6 Lesion diameter on cotyledons of 11 different *Brassica napus* genotypes at 14 days after inoculation with three different isolates of *Leptosphaeria maculans*

Genotypes	Isolates		
	D5	D13	D16
Boston	2.33kl	6.16bcde	3.33hijk
Scotia	3.33hijk	2.83jkl	3.16ijk
Parkside	6.16bcde	4.66efghi	3.16ijk
Drummonds Purple Top	6.66bc	8.50a	4.00ghij
Chuosenshu	3.00jk	6.00bcde	4.16ghij
LSF0530	2.83jkl	3.16ijk	2.00kl
Duell	5.16cdefg	6.00bcde	3.33hijk
Barrel	6.33bcd	1.33 1	3.16ijk
Liglory	3.00jk	4.83defgh	4.33fghij
Amber x Commanche DH Line	4.00ghij	6.83b	1.33 1
Westar	7.00ab	6.50bc	5.83bcdef
LSD value 1.60			

Different letters indicate significant differences ($P \leq 0.05$)

halo symptoms were observed against all isolates (Fig. 5). LSF0530 was the only genotype that did not show this symptom with any isolate (Fig. 5). The maximum frequency of this yellow halo symptom (8 out of 11 genotypes) was observed against the most virulent isolate (D13), followed by isolates D5 and D16 where such response was observed in 7 or 6 out of 11 genotypes, respectively (Fig. 5).

Discussion

We believe this is the first study to report a distinct yellow halo (senescence/PCD) associated with both ‘typical’ lesions and with an expression of HR around the cotyledon inoculation site in winter-type *B. napus* genotypes challenged with *L. maculans* isolates. Expression of a distinct yellow halo symptom was not observed in all test *B. napus* genotypes infected by one specific *L. maculans* isolate, nor in one specific *B. napus* genotype challenged with all the isolates. It was clear that the expression of a distinct yellow halo was dependent both on virulence differences among *L. maculans* isolates and their interaction with genetically different *B. napus* genotypes.

The distinct yellow halo in winter-type *B. napus* genotypes challenged with *L. maculans* isolates in the current study is likely associated with PCD around the

cotyledon inoculation site. This yellow halo symptom was earlier reported by Ge et al. (2015) in spring-type *B. napus* cotyledons infected by some specific *S. sclerotiorum* pathotypes or isolates. That study highlighted how such a yellow halo reaction of leaf senescence involved PCD (Greenberg 1997; Van Doorn and Woltering 2004; Yen and Yang 1998). Further, Li et al. (2008) also noted the occurrence of PCD (but not a yellow halo symptom) in relation to *L. maculans* infection, but, again, on spring-type *B. napus*. It is noteworthy that this yellow halo symptom expression in the current study depended both on virulence differences among *L. maculans* isolates and their interaction with genetically different *B. napus* genotypes, as yellow halos were not observed in all genotypes infected by one specific isolate nor in one specific genotype challenged with all the isolates. For example, in genotypes, Scotia and Barrel, only isolate D5 induced a distinct yellow halo around the lesions, while for genotype Duell there were distinct yellow halos around cotyledon lesions induced only with isolate D13 but not with isolates D5, D13 or D16. Similar to other senescence processes, cotyledon senescence, as an important constituent of development, is a programmed process (Du et al. 2014) that leads to nutrient recycling and ends in cell death. Senescence is accompanied by colour changes from green to yellow, or from green to red due to anthocyanins, with either red or yellow halo

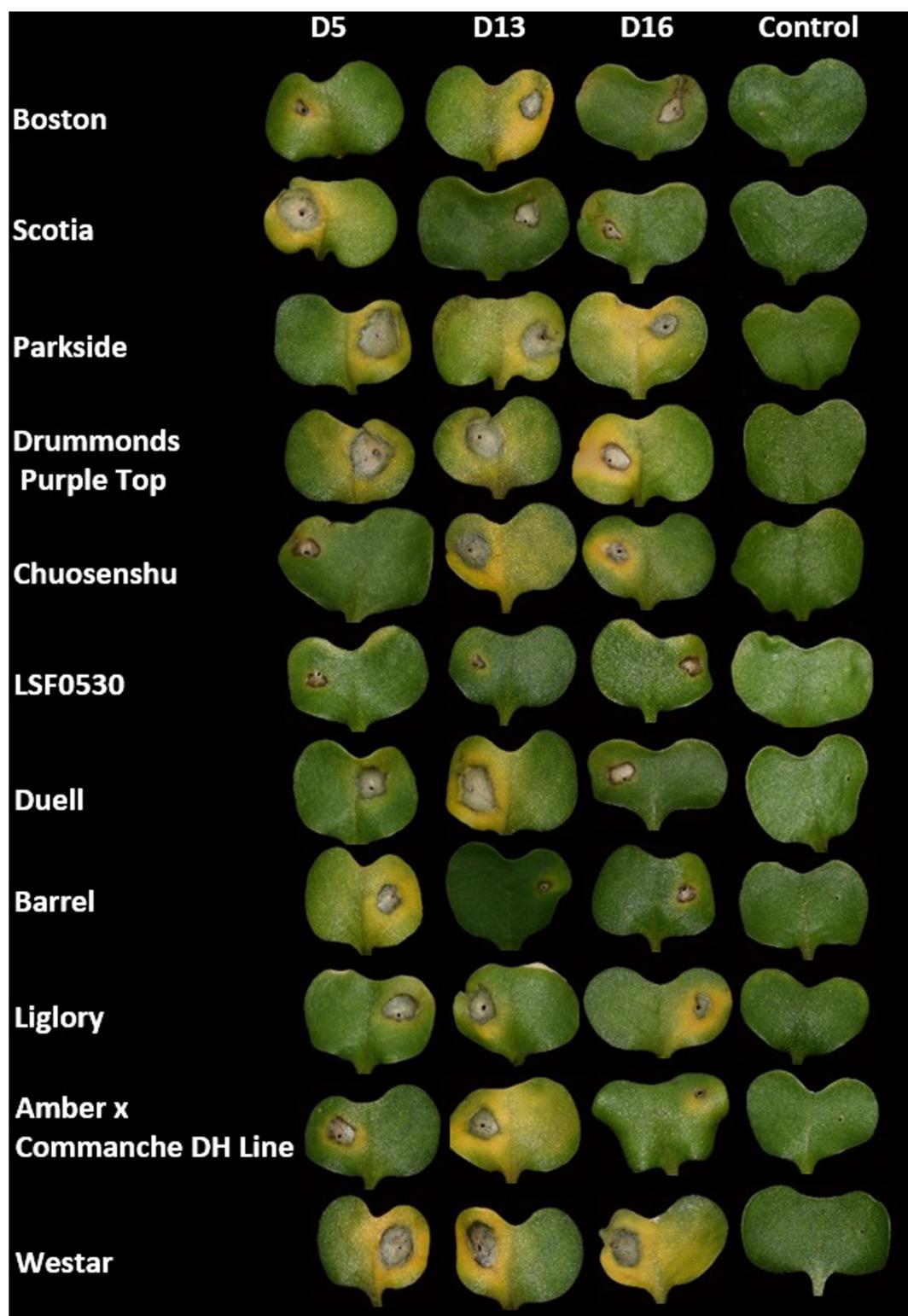


Fig. 5 Examples of inoculation with the D5, D13 and D16 isolates of *L. maculans* and control comparison with distilled water on to *Brassica napus* genotypes at 14 days after inoculation. Yellow halo indicative of cotyledon senescence involving programmed cell death

symptoms observed by Ge et al. (2015) in relation to particular *S. sclerotiorum* pathotype x *Brassicaceae* genotype combinations. Such senescence can involve dismantling of chloroplasts and the degradation of DNA, RNA, and proteins (Rapp et al. 2015). The current results highlight, as also found by Ge et al. (2015), that the link between defence response and senescence likely involves PCD.

In general, but not always, where HR occurred in the current study, the HR showed a distinct dark brown margin around the lesions, as could be expected (Li et al. 2007a, b, 2008; Mur et al. 2008). Initiation of HR is ‘normal’ in highly resistant infected plants, and uninfected and yellowing tissue adjacent to HR lesions may undergo PCD, possibly induced by apoptosis or autophagy, both highly regulated processes by which PCD occurs (Kabbage et al. 2013). HR is highly related to active resistance (Pontier et al. 1998), and initiation of the HR process is mediated as plants perceive pathogen presence through interaction between pathogen avirulence genes and the plant resistance genes (Pontier et al. 1998). HR can include a microscopic area of just a few cells or can spread over a much broader, macroscopic area of necrosis such as an entire leaf (Klingler et al. 2009). Therefore, clearly, there are different forms and levels of cell death and mechanisms leading to HR (Kombrink and Schmelzer 2001). The hypersensitive response related to *L. maculans* has been reported in winter-type *B. napus* (e.g., Roussel et al. 1999) and is also well documented in spring-type *B. napus* (e.g., Li et al. 2007a, b).

We also found that *B. napus* genotypes differ in terms of their level of expression of resistance to the specific *L. maculans* isolates, with the virulence differences observed among *L. maculans* isolates determining the expression of cotyledon resistance responses in different *B. napus* genotypes. In general, and as expected, we found that virulence differences among *L. maculans* isolates determine *B. napus* cotyledon resistance responses across *B. napus* genotypes, as has been shown in many previous studies (e.g., Balesdent et al. 2002, 2005; Parlange et al. 2009). In terms of disease index or lesion diameter, there were significant differences among the *L. maculans* isolates in relation to their pathogenicity. Overall, isolates D5 and D13 were highly pathogenic, whereas D16 was less pathogenic, irrespective of the *B. napus* genotypes. Moreover, some genotypes showed consistent host responses irrespective of *L. maculans* isolate (e.g., LSF0530 showed a resistant

score when challenged with all the isolates), whereas others showed a variable response depending upon the isolate used. Differences observed in terms of number of days to lesion appearance, disease index and lesion diameter are likely due to differences in the *AvrLm* genes harboured by different *L. maculans* isolates and/or differences in *Rlm* genes carried by different *B. napus* genotypes. So far, 18 major resistance genes (*Rlm1–11*, *RlmS*, *LepR1–4*, and *BLMR1–2*) have been identified (Delourme et al. 2006; Van De Wouw et al. 2009; Balesdent et al. 2013). However, while one isolate can carry several *AvrLm* genes, a single *AvrLm* gene can also confer resistance to two independent major resistance genes. For example, *AvrLm1* interacts with both *Rlm1* and *LepR3* genes and *AvrLm4–7* induces resistance after recognition by either *Rlm4* or *Rlm7* (Parlange et al. 2009; Larkan et al. 2013). As qualitative resistance prevents infections through a gene-for-gene interaction between *B. napus Rlm* genes and *L. maculans AvrLm* alleles (Delourme et al. 2006), variation in *AvrLm* and *Rlm* genes between the test genotypes most likely accounts for differences in lesion severity in the current study. Further, it has been reported that *LepR3* perceives *L. maculans AvrLm1* and triggers *Brassica* defence, leading to a HR at the site of infection (Larkan et al. 2013) and, as *LepR3* is a cell surface receptor (Ma and Borhan 2015), this may explain the HR we observed in some instances.

In the current study, there were significant differences observed among different *B. napus* genotypes when challenged with different *L. maculans* isolates and also among different isolates in relation to their pathogenicity as measured by days to lesion appearance, disease index, and cotyledon lesion diameter, on different *B. napus* genotypes. LSF0530 genotype was found to be the most resistant genotype and D5 and D13 isolates were highly pathogenic isolates.

Despite there being considerable isolate dependency in the response of such genotypes, results confirmed that resistance in these winter-type *B. napus* genotypes against *L. maculans* was polygenic, as has already been widely established across other genotypes, e.g., see Delourme et al. (2006) and as noted above.

Winter-type *B. napus* Parkside, Drummonds Purple Top, Amber x Commande DH Line and spring-type Westar all showed yellow halos around the lesions caused by one or more *L. maculans* isolates, indicating a cotyledon senescence response as detailed earlier. We believe is the first report of an association of a yellow

halo response likely involving PCD with cotyledon senescence in winter-type *B. napus* in response to *L. maculans*, suggesting that *B. napus* cotyledon senescence is accelerated due to *L. maculans* invasion as has been widely observed in the field on spring-types (MJ Barbetti, unpubl.). This also perhaps explains the previous report by Travadon et al. (2009) showing that hypocotyl necrosis in seedlings increases with the number of *L. maculans* infection sites on cotyledons. The current study highlights how the yellow halo response and associated cotyledon senescence depend both on virulence differences among *L. maculans* isolates and on genetic variation among *B. napus* genotypes. These yellow halo symptoms should make an interesting focus for future molecular plant-pathogen interactions studies at both physiological and molecular levels.

Acknowledgements The first author gratefully acknowledges a Scholarship from The University of Western Australia (Scholarship for International Research Fees and Ad Hoc Postgraduate Scholarship). We are grateful to the financial assistance of the School of Biological Sciences, University of Western Australia. The authors are also grateful to Robert Creasy and Bill Piasini in the UWA Plant Growth Facilities for their technical assistance in plant growth facilities at UWA. In addition, the authors would like to thank Ting Xiang Neik for preparing the inoculum.

Compliance with ethical standards

Ethical statement This research did not involve any animal and/or human participants.

Conflict of interest All authors declare that they have no conflict of interests.

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