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Variations in relative humidity modulate *Leptosphaeria* spp. pathogenicity and interfere with canola mechanisms of defence

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Abstract Blackleg (phoma stem canker), caused by *Leptosphaeria* spp., is an important disease of canola (oilseed rape, *Brassica napus*). Control strategies rely on the use of resistant cultivars, chemical and disease-reducing cropping practices. In Canada, the pathogen population is represented by *L. maculans* and *L. biglobosa*, which are considered to be highly and weakly aggressive, respectively. It is largely admitted that *L. biglobosa* isolates are not able to cause a significant amount of stem canker and develop on the plant only when it becomes senescent, late in the season. The prevalence of *L. maculans* over *L. biglobosa* has been considered to be linked to the low aggressiveness of the latter. However, in this study, we show that *L. biglobosa* isolates could become highly aggressive in terms of lesion appearance on cotyledons, if the right conditions of temperature and relative humidity (RH) are provided. Percent germination of inoculated pycnidiospores was not affected by the RH regimes tested. This is the first study to show the importance of RH as a factor conditioning the pathogenicity of *L. biglobosa* isolates on canola cotyledons. Concurrent changes in the host defence mechanisms against *L. biglobosa* isolates in response to variations in the RH were also investigated. Under high RH, the increase in disease caused by the weakly aggressive isolates coincided with a reduced accumulation of lignin at the early stages of infection.

Keywords Canola (oilseed rape) · *Leptosphaeria maculans* · *L. biglobosa* · Blackleg · Phoma canker · Relative humidity · Temperature · Phenolics · Lignin · Hypersensitive reaction

Abbreviations

Cf/*Avr*	*Cladosporium fulvum*/*Avirulent*
d.a.i.	Day after inoculation
HR	Hypersensitive reaction
PG	Pathogenicity group
RH	Relative humidity

Introduction

Canola (oilseed rape; *Brassica napus*) is the second largest oilseed crop worldwide (Raymer 2002). It is largely grown in Canada, China, USA, Australia, and several European countries. This crop is known for the quality and health benefits of its oil, but interest has also been growing towards its use as an alternative source for biodiesel and other by-products. The most important fungal disease of canola is blackleg (phoma stem canker; West et al. 2001; Fitt et al. 2006), caused by a complex of

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phylogenetically-related ascomycete species, including *Leptosphaeria maculans* and *L. biglobosa* (anamorph: *Phoma lingam*) (Williams and Fitt 1999; West et al. 2001; Shoemaker and Brun 2001; Mendès-Pereira et al. 2003).

The control of this disease complex relies on the use of resistant cultivars along with chemical and cultural practices (West et al. 2001; Sivasithamparam et al. 2005; Gladders et al. 2006). Previously, the pathogen populations consisted of five pathogenicity groups (PG1, PG2, PG3, PG4 and PGT) as defined by Mengistu et al. (1991). These five PGs were found in western Canada (West et al. 2001) and can survive on naturally-infected rapeseed and canola stubble for a long period (Petrie 1995). PG1 has been reclassified since as *L. biglobosa* (Shoemaker and Brun 2001), and is represented by weakly aggressive isolates developing into a canker only late in the season when plants are senescent. *Leptosphaeria biglobosa* is still prevailing over *L. maculans* in certain canola and rapeseed growing areas i.e., east Europe (Jedryczka et al. 1994, 2002) and has been proven to cause major damage to winter-type cultivars of rapeseed and decrease the yield under field conditions, especially in Poland (Jedryczka 2007). PG2 on the other hand, has been the most prevalent group of *L. maculans*.

Isolates from these two species can co-exist on canola plants (West et al. 2001, 2002; Mahuku et al. 1996a, b). However, the way they interact with each other on plant tissues is poorly understood. Pre-inoculation of cv. Westar seedlings with pycnidiospores of an *L. biglobosa* isolate was shown to induce both local and systemic resistance to an *L. maculans* isolate under controlled conditions (Mahuku et al. 1996a, b). However, conflicting results from co-inoculation of the two types of isolates on cv. Surpass 400, in Australia, have shown that the effect of *L. biglobosa* on *L. maculans* is only local (Li et al. 2006). More recent results have shown both local and systemic decrease of the spot lesion area (Liu et al. 2006). Such an effect was confirmed in the field and was comparable to the effect of certain chemical defence activators (Liu et al. 2006). Based on these and earlier observations, it seems interesting to use isolates of *L. biglobosa* to control subsequent infections by *L. maculans*. However, isolates to be used for such cross-protection should be weakly aggressive under various conditions. Therefore, before selecting an *L. biglobosa* isolate that can be used as a biological control agent (Daayf et al. 2003b), it is important to study its pathogenic variability under different environmental conditions.

The objectives of the present study were to investigate (i) the effects of temperature and relative humidity (RH) on the pathogenic variability of selected isolates; and (ii) how the variation of such conditions impacts on the canola defence in response to these isolates.

Material and methods

Plant materials

Three canola cultivars, Westar, Quinta and Glacier, exhibiting differential responses to *Leptosphaeria* spp. isolates were selected for this study. They were grown in a Metromix soil mixture amended with NPK (20:20:20) under cool fluorescent light with a 16 h photoperiod at 20/16°C day/night and watered regularly.

Leptosphaeria spp. isolates, inoculation and disease assessment

Fourteen *Leptosphaeria* spp. isolates were used in this study. They were all collected from infected canola plants grown in Manitoba, Canada. After single-spore isolation, each isolate was propagated on V8-agar medium containing 200 ml of V8, 15 g of agar and 0.75 g of $CaCO_3$ l^{-1}. Based on an initial screening on differential cultivars, five of these isolates were found to be *L. biglobosa* (former PG1) and nine were *L. maculans* (3 PG2, 2 PG3, 2 PG4, and 2 PGT). The identity and classification of the isolates to either *L. maculans* or *biglobosa* were verified based on the colony morphology as well as using GPI-isozymes along with species-specific primers described by Kuusk et al. (2002) and Liu et al. (2006) (data not shown). Isolates of *L. biglobosa* were found to be *L. biglobosa* 'canadensis' as defined by Mendès-Pereira et al. (2003).

For inoculum production, the isolates were incubated for two weeks under continuous cool-white fluorescent light at 20±2°C. Pycnidiospores were then harvested in water by gently scraping the surface of the cultures. Inoculations were performed either on

cotyledons from 8 day-old plants or on 4–6 leaf-adult plants by placing 10 µl droplets of a spore suspension calibrated at 2×10^7 pycnidiospores ml^{-1} on a freshly made wound. Disease rating was conducted 12 days after inoculation (d.a.i.) following a scale ranging from 0 (no disease) to 9 (large necrotic symptoms producing large number of pycnidia) (Fig. 1).

Relative humidity (RH) and temperature conditions

The pathogenicity of selected isolates was tested, under a range of temperature and RH conditions, on differential cvs Westar, Quinta and Glacier at the cotyledon stage. In a preliminary study, five isolates from *L. biglobosa* (former PG1) were screened against a reference *L. maculans* isolate (former PG2). Conditions tested consisted of three temperature regimes (20/16; 25/23, and 30/27°C day/night) that cover the range of temperatures typically recorded in western Canada under an ambient RH. The same set of six isolates was tested at 20/16°C day/night under two RH conditions: (i) a constant 45–50% RH (low RH), (ii) incubation in saturated atmosphere for three days, then under 70% RH (high RH) until rating at 12 d.a.i.

The 15 isolates were further tested for their pathogenicity in highly-controlled atmosphere cabinets where three different temperature regimes 20/16, 25/23, and 30/25°C day/night were combined with three RH conditions (low RH 45–50%; intermediate RH 70% and high RH≥85%). The growth cabinets were limited to a top RH≈85%, which did not allow for the testing of combinations involving saturated atmosphere (experiment design reported below).

At all times, inoculum from each tested isolate was placed on open water-agar plates and incubated at the same environmental conditions tested, then checked for germination. In all included results, inoculum germination reached 85–92.5%. Additional experiments including reference isolates exhibiting various degrees of pathogenicity were conducted under regular conditions (20/16°C day/night; ambient RH). Both of these measures were carried out to exclude any effect of a poor germination of the inoculum on further development of blackleg symptoms and to assign the observed pathogenicity of the isolates to the environmental conditions tested.

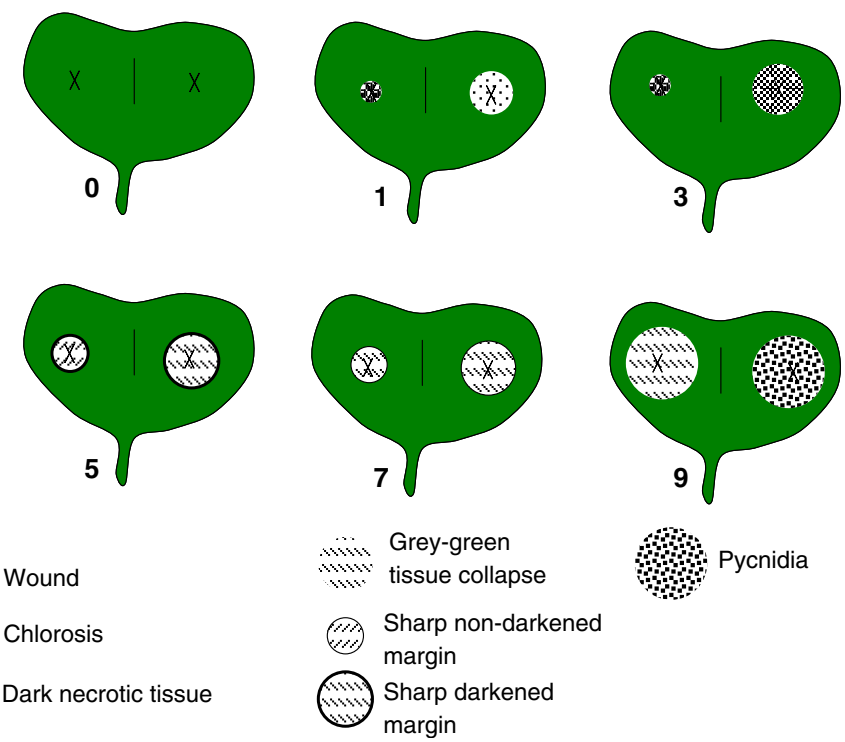


Fig. 1 Rating scale used to assess blackleg disease on canola cotyledons 12 d.a.i.

Histology and lignin staining

Canola cotyledons from all tested cultivars, either healthy or inoculated with various isolates, were freshly harvested and discoloured in Carnoy's solution overnight (ethanol:chloroform:acetic acid, 6:3:1, v/v/v). The following day, cotyledon tissues were rinsed using distilled water, stained in phloroglucinol-HCl and observed under light microscopy (Jensen 1962).

High pressure liquid chromatography (HPLC) analysis of secondary metabolites

Soluble phenolics of canola leaves (500 mg–1 g) were extracted using 80% methanol (Daayf et al. 2003a; El Hassni et al. 2004). After centrifugation, the pellet was re-suspended twice in 1.5 ml of 80% methanol. Pooled methanolic fractions were incubated under a nitrogen stream until the organic phase had evaporated. The remaining aqueous phase was then mixed with petroleum ether to remove chlorophyll, waxes and lipids from the leaf extracts. The cleared aqueous phase was then extracted three times with ethyl acetate. After a final evaporation of the ethyl acetate fraction, the residue was suspended in 200 µl of pure methanol and immediately subjected to analysis or stored at −20°C until used.

For HPLC analysis a Waters 2690 separation module was used. This module is equipped with an autosampler and a Waters 996 photodiode array detector, and fitted with a 5 µm LiChrospher® 100 RP-18 guard column (LiChroCART® 4-4, Germany) and a reverse-phase 5 µm 250-4 LiChrospher® 100 RP-18 column (LiChroCART® 4-4, Germany). The column was eluted at a flow of 1 ml min^{-1} with a gradient using a solvent system composed of A: 0.1% H_3PO_4-acidified water and B: HPLC grade acetonitrile. The gradient used to carry the analysis was as follows: (time [min]/A [%]/B [%]) = 0/100/0, 5/95/5, 10/95/5, 14/90/10, 20/80/20, 23/80/20, 30/65/35, 35/65/35, 43/50/50, 48/25/75, 55/0/100, 60/0/100. Injected volumes were 15 µl per sample and each injection was repeated twice. Data were analysed using the Empower™ 2.0 (Waters, Ville-Saint-Laurent PQ, Canada). Compounds were identified based on their retention time, their typical UV spectra in reference to our database and through co-elution with commercial standards. Quantification of hydroxycinnamates was conducted by reference to a standard curve using a serial dilution of commercial chrologenic acid (Sigma-Aldrich Co.).

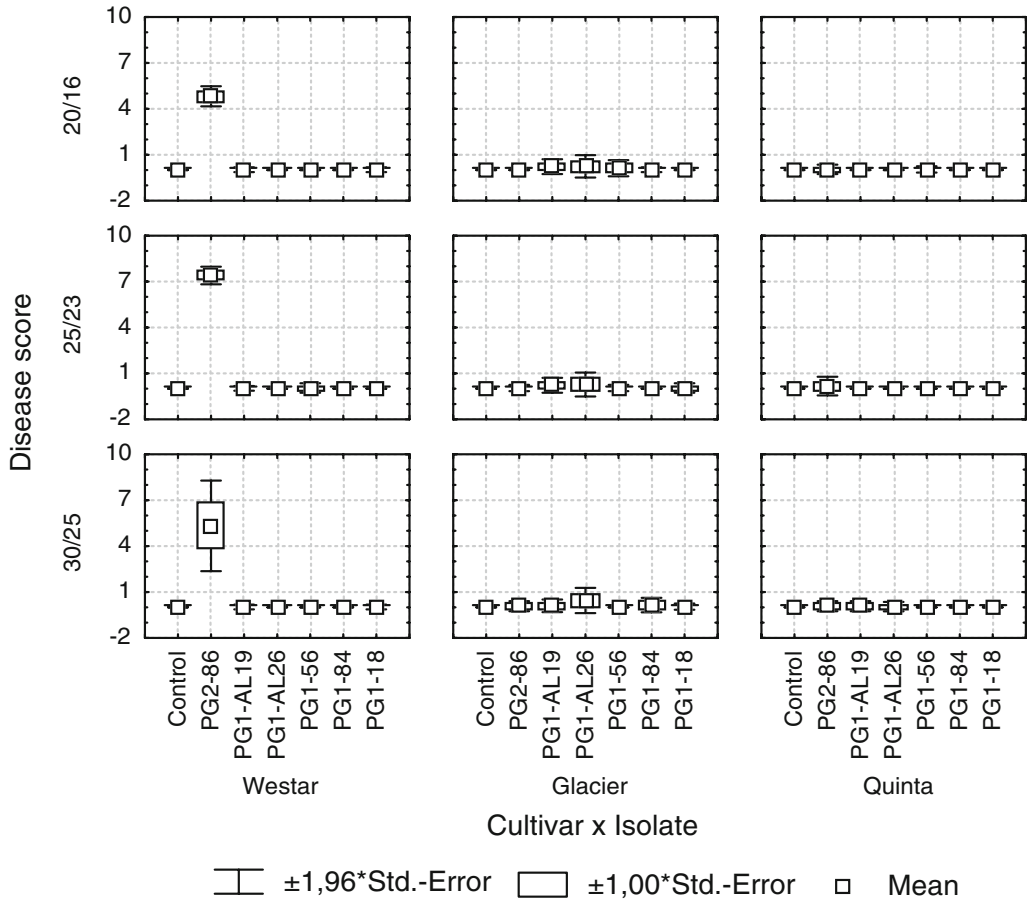
Experiment design and data analysis

All experiments were conducted in a randomised complete block design. For every trial, 20–24 seedlings were used per replicate isolate × cultivar × treatment and each trial was repeated independently three times. Collected data were submitted to variance analysis using the GLM model of the SAS 9.1 programme software (SAS Institute Inc., Cary, N.C., U.S.A. 2004). The homogeneity of the variances and the symmetry of the distribution were checked for each analysed variable included in the ANOVA analysis. When the F-test was significant at $P<0.05$, mean values were compared according to Newman-Keuls test. Non-wounded and wounded and non-inoculated controls were used in different trials and included in the analysis when necessary. Statistica software (StatSoft 1999) was also used to generate graphics from the data. For the soluble phenolics analysis, three bulk sub-samples were formed out of the 20–24 seedlings used per replicate isolate × cultivar × treatment and extracted. Each one of these samples was subjected to HPLC analysis at least twice.

Results

Initial pathogenicity screening

No significant effect of the three tested temperature regimes was detected on the pathogenicity of the selected isolates (Fig. 2). *Leptosphaeria biglobosa* isolates exhibited low pathogenicity on all three tested cultivars regardless of the temperature condition tested. *Leptosphaeria maculans* isolates were highly aggressive on cv. Westar, especially at 20/16 and 25/23°C day/night (Fig. 2). Reactions of the isolates on cvs Quinta and Glacier depended on their pathogenicity grouping (former PG2, 3, 4 or T). However, no significant effect of the temperature was recorded. Isolates formerly known as PG2 produced blackleg symptoms only on cv. Westar, whereas isolates from PG3 and PG4 were highly aggressive on Westar and Glacier and on the three tested cultivars, respectively.

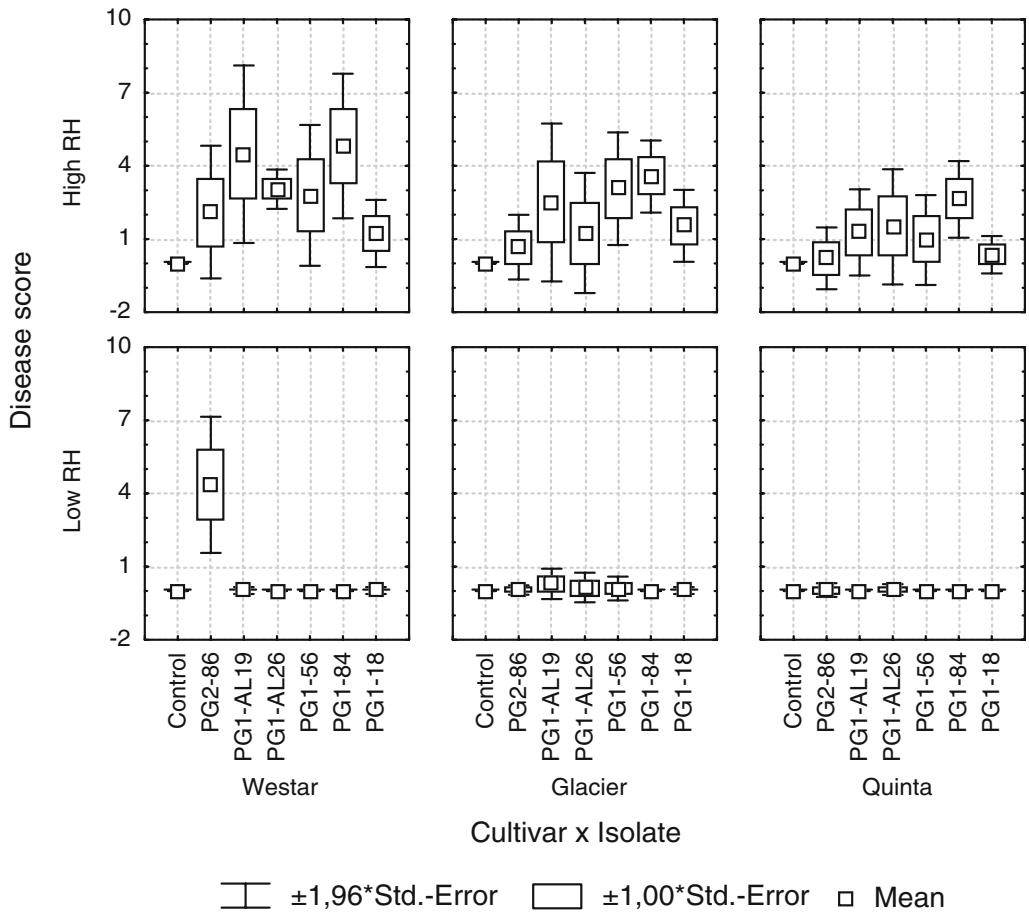


	SS	df	MS	F	P-value
1: Cultivar	86.4	2	43.2	752.5	0.000
2: Isolate	295.4	6	49.2	857.7	0.000
3: Temp.	1.8	2	0.9	15.9	1.82E-07
1*2	584.3	12	48.7	848.1	0.000
1*3	3.6	4	0.9	15.9	2.47E-12
2*3	12.3	12	1.0	17.9	0.000
1*2*3	20.2	24	0.8	14.7	0.000
Error	32.6	567	0.06		

Fig. 2 Disease scores recorded on three canola cultivars (Westar, Glacier and Quinta) after inoculation with either one *L. maculans* (86-14) or one of the five *L. biglobosa* isolates tested (AL19; AL26; 03-56-02; 84-12; 04-18-01) and incubation under three conditions of temperature (20/16°C, 25/23°C and 30/27°C day/night). The ANOVA table summarises the significance of each tested effect and their interactions at $P < 0.05$. SS: sum of squares; df: degree of freedom; MS: mean square

The same set of six isolates has been tested at 20/16°C day/night under high (3 d.a.i. under saturated atmosphere followed by constant 70% RH) and low RH conditions (constant 45–50% RH). Interestingly, under high RH levels, *L. biglobosa* isolates (former PG1) caused severe disease on all three tested cultivars (Fig. 3). Their level of aggressiveness was as high as *L. maculans* isolates (Figs. 3 & 4). In

addition, cotyledons infected with *L. biglobosa* isolates displayed blackleg symptoms as early as 8 d.a.i. and turned senescent at 10 d.a.i. compared to cotyledons kept either as controls or inoculated with *Leptosphaeria maculans* isolate. Pycnidia were present on the cotyledons in both cases. *Leptosphaeria biglobosa* isolate apparent aggressiveness was 2 to 12 times higher under high RH conditions as compared



	SS	df	MS	F	P-value
1: Cultivar	109.2	2	54.6	93.6	0.000
2: Isolate	156.6	6	26.1	44.8	0.000
3: RH	308.4	1	308.4	528.9	0.000
1*2	111.5	12	9.3	15.9	2.32E-28
1*3	23.9	2	11.9	20.5	3E-09
2*3	235.2	6	39.2	67.2	0.000
1*2*3	83.4	12	6.9	11.9	3.96E-21
Error	269.4	462	0.6		

Fig. 3 Disease scores recorded on three canola cultivars (Glacier, Quinta and Westar) after inoculation with either one *L. maculans* (86-14) or one of the five *L. biglobosa* isolates tested (AL19; AL26; 03-56-02; 84-12; 04-18-01) and incubation under two relative humidity (RH) conditions: low RH consisting of continuous 45–50% RH or high RH consisting of 3 d.a.i. at saturated atmosphere followed by an incubation at 70% RH. The ANOVA table summarises the significance of each tested effect and their interactions at $P<0.05$. SS: sum of squares; df: degree of freedom; MS: mean square

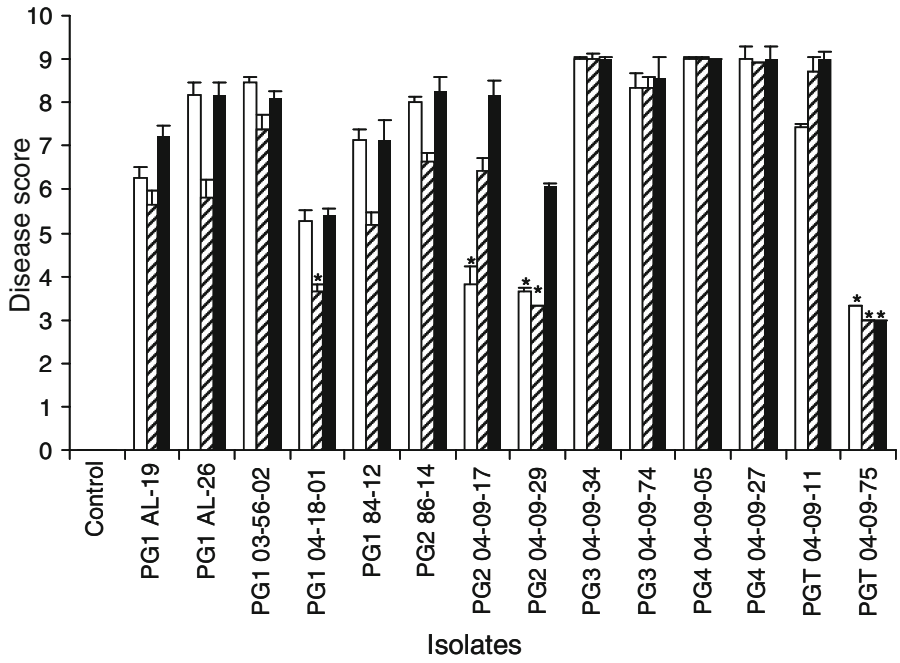


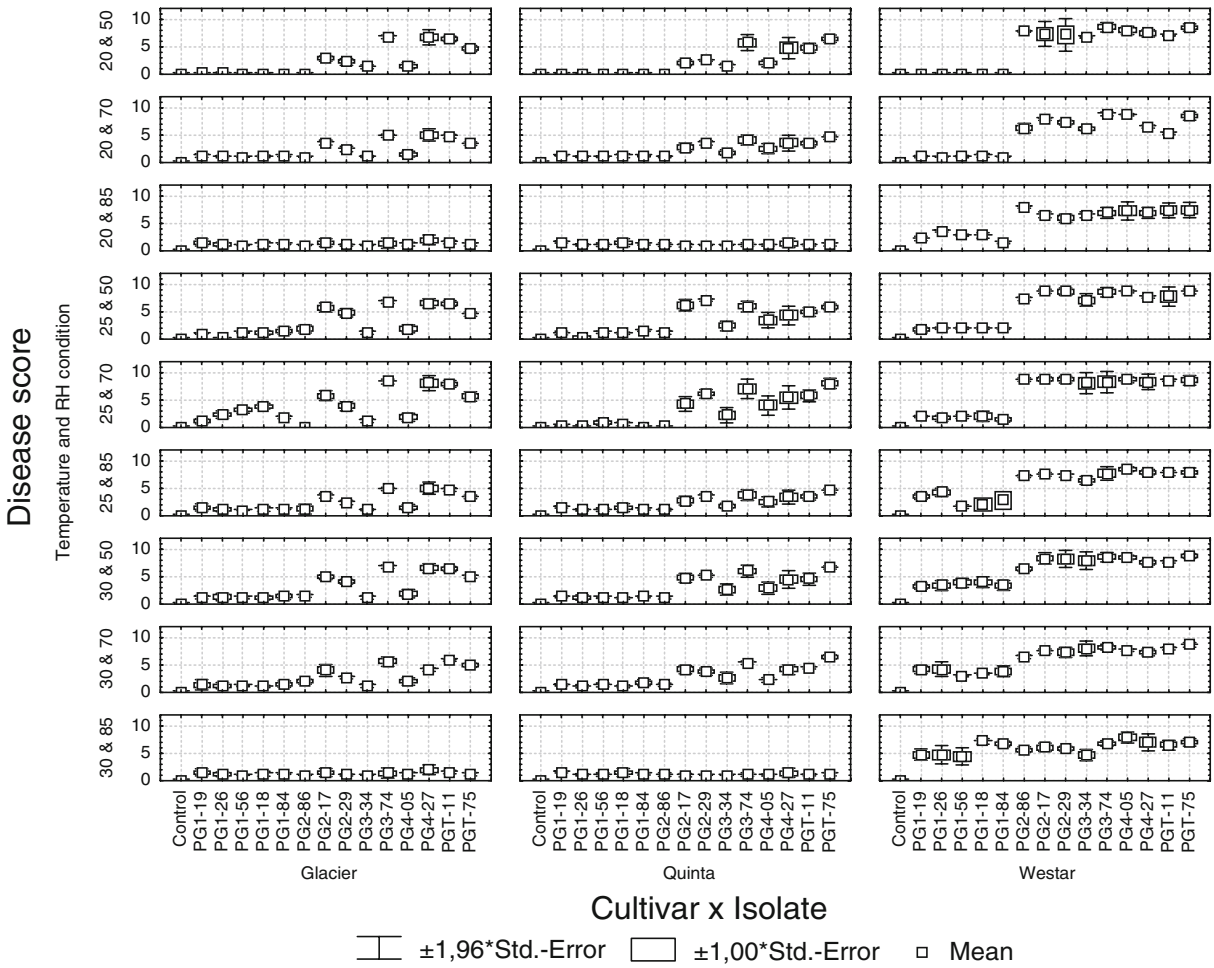
Fig. 4 Disease scores on three canola cultivars (□ Glacier, ▨ Quinta and ■ Westar) after inoculation with *Leptosphaeria* spp. isolates (reported by their former pathogenicity grouping PG1, PG2, PG3, PG4 and PGT) and incubation under high RH conditions. Asterisks show significant decrease in disease score ($P<0.05$) among all of the treatments

to when they were incubated under low RH. The reference *L. maculans* isolate (former PG2), on the other hand, showed a decrease in aggressiveness under high RH conditions even though the percentage of germinating inoculum was high and almost same as the other isolates (89–91%; Fig. 3). Similar observations were also made on other isolates from the same pathogenicity group and in the field during wet summers in western Canada, where former PG2 isolates have been predominant (data not shown). Complementary experiments conducted with the same isolates on detached leaves from adult seedlings (with 4–6 fully-developed leaves) and incubated under saturated atmosphere in the laboratory confirmed the above-mentioned results (data not shown).

For further investigations, the selected set of isolates was extended to 15 and was subjected to pathogenicity screening in highly controlled atmosphere cabinets, where three different temperature regimes 20/16, 25/23, 30/25°C day/night were combined with three RH conditions (low RH 45–50%; intermediate level of RH 70% and high RH≈85% (maximum limit)). Under these conditions, the percent germination of inoculum was estimated at 85–92.1% for all tested isolates. Results are shown in Fig. 5 and confirmed the previous observations with the effect of RH on the variation of the pathogenicity of the tested isolates of *L. maculans*. No significant effect of the temperature was apparent. The observed effect in the combination temperature × RH was mainly due to RH. Interestingly, higher levels of RH combined with either low (20/16°C) or high temperatures (30/25°C) negatively impacted on the performance of *L. maculans* isolates more than it did on *L. biglobosa*. The growth cabinets used were limited to a maximum 85% RH, which did not allow for testing the combination of various temperatures with the saturated atmosphere.

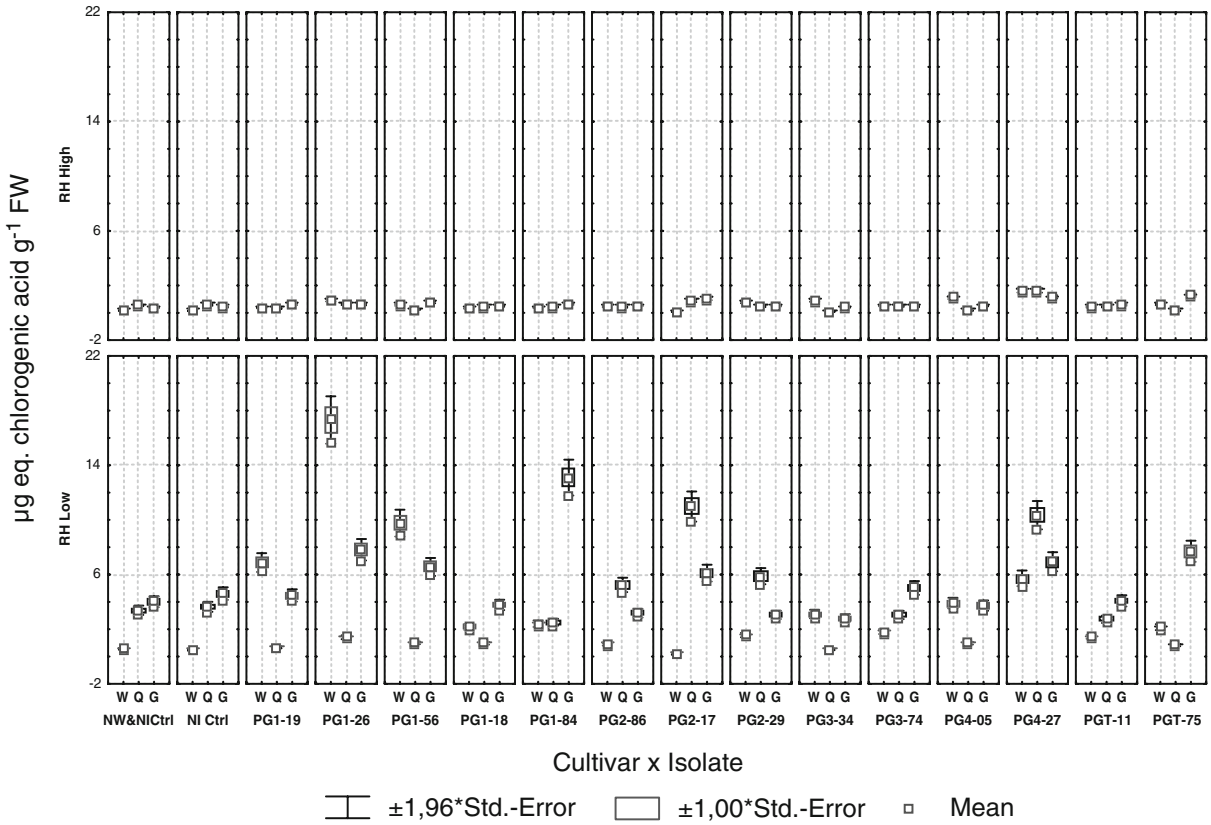
Soluble phenolics analysis

Soluble phenolics were extracted and analysed by HPLC from plants kept as controls or inoculated with either *L. biglobosa* or *L. maculans* isolates and incubated under various temperature or RH conditions. Several differences were observed among cultivars in the type and the quantity of soluble phenolics accumulated in response to various isolates (Fig. 6). The major induced compounds have been identified as hydroxycinnamates (i.e. derivatives of chlorogenic, ferulic and coumaric acids; Fig. 7). These derivatives accumulated in all treatments as



	SS	df	MS	F	P-value
1: Tm&RH	537.3	8	67.2	*531.0*	*0.000*
2: Cultivar	3421.0	2	1710.5	*13523.9*	*0.000*
3: Isolate	5568.2	14	397.7	*3144.6*	*0.000*
1*2	191.7	16	12	*94.7*	*0.000*
1*3	1126.7	112	10.1	*79.5*	*0.000*
2*3	1345.7	28	48.1	*379.9*	*0.000*
1*2*3	470.4	224	2.1	*16.6*	*0.000*
Error	153.7	1215	0.1		

Fig. 5 Disease scores on three canola cultivars (Glacier, Quinta and Westar) after inoculation with *Leptosphaeria* spp. isolates (reported by their former pathogenicity grouping PG1; PG2; PG3; PG4 and PGT) and incubation under various combinations of RH and temperature conditions (20/16; 25/23; and 30/27°C day/night) × (continuous 45–50% RH; 70% RH; ≈ 85% RH) i.e. 20 & 50 reported on the graph represents incubation at 20/16°C day/night under continuous 45–50% RH. The ANOVA table summarises the significance of each tested effect and their interactions at $P<0.05$. SS: sum of squares; df: degree of freedom; MS: mean square



	SS	df	MS	F	P-value
1: Cultivar	43.3	2	21.7	543.5	0.000
2: Isolate	215.3	15	14.4	360.0	0.000
3: RH	628.9	1	628.9	15777.2	0.000
1*2	412.9	30	13.8	345.3	0.000
1*3	33.1	2	16.6	415.4	0.000
2*3	153.7	15	10.2	257.1	0.000
1*2*3	349.4	30	11.6	292.2	0.000
Error	3.8	96	0.04		

Fig. 6 Variation in amount of soluble phenolics (µg eq. chlorogenic acid g^{-1} FW) in the cotyledon tissues of three canola cultivars (Westar (W), Quinta (Q) and Glacier (G)) in response to wounding and/or to inoculation with *Leptosphaeria* spp. isolates after incubation under low (constant 45–50%) or high RH regimes (3 days at saturated atmosphere followed by a constant 70% RH until the appearance of the lesions). Each data point represents the mean of two individual trials ± 1.96 standard-errors. The ANOVA table summarises the significance of each tested effect and their interactions at $P<0.05$. SS: sum of squares; df: degree of freedom; MS: mean square

early as 2 d.a.i. However, the rate by which their accumulation dropped overtime was differential. A faster decrease in their accumulation was observed in treatments where plants were inoculated with *L. biglobosa* isolates compared to the non-inoculated controls and to plants inoculated with *L. maculans* isolates.

Soluble phenolics accumulation also varied, both qualitatively and quantitatively, with the temperature and RH conditions tested. Their concentration de-

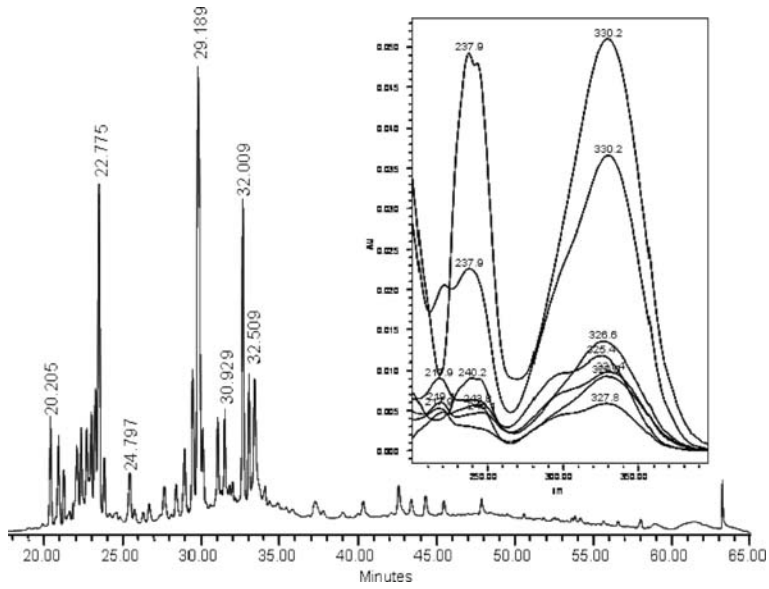


Fig. 7 HPLC profile of soluble phenolics from canola cv. Westar inoculated with *L. biglobosa*. The spectra represent the major hydroxycinnamates detected in the extract. Their UV-absorbance corresponds to their relative abundance in the chromatogram

creased in all tested cultivars when RH conditions increased (Figs. 8 & 9).

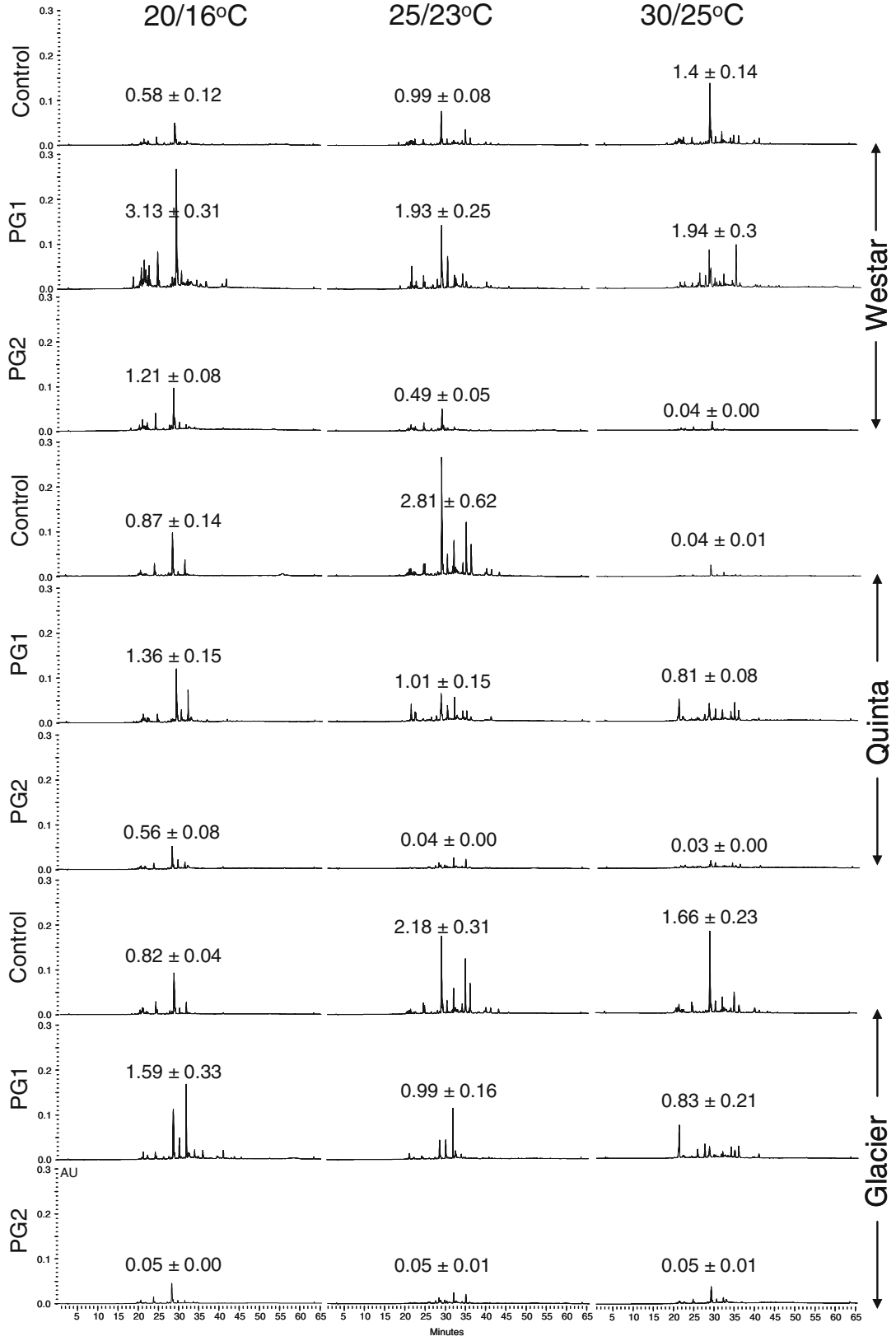
Histological observations

Cotyledons maintained as controls or subjected to inoculation with *L. biglobosa* or other *L. maculans* isolates were stained with phloroglucinol-HCl which recognises the cinnamic aldehyde groups in the lignin matrix. Cotyledons inoculated with *L. biglobosa* isolates showed a thick multi-layer of lignified material around the infection site, as compared to the wounded non-inoculated controls (Fig. 10). Cotyledons inoculated with *L. maculans* isolates displayed diffused lesions, where less lignified material accumulated (Fig. 10). Similar diffused lesions with less lignified material around were also observed in response to *L. biglobosa* isolates inoculated onto the three tested cultivars and incubated under specific high RH conditions (saturated atmosphere followed by constant 70% RH) (Fig. 10).

Discussion

Blackleg is the most important disease of canola, oilseed rape and other Brassicae. Resistance in cultivated germplasm comprises partial resistance (quantitative resistance) and in many cases this is supplemented with one or more major resistance genes (*AvrLm1-9* and *LepR1-3*; Delourme et al. 2004, 2006) such as those controlling callose deposit (Li and Cowling 2003; Li et al. 2003, 2004, 2005). Although deploying cultivars with major resistance gene seems to be efficient in reducing the disease severity in certain countries (i.e., Canada), such resistance is frequently overcome within a few seasons (i.e., cv. Surpass 400 carrying *LepR3* from *B. rapa* ssp. *sylvestris* in Australia) (Li and Cowling 2003; Li et al. 2003, 2004, 2005; Huang et al. 2006). Such a breakdown of resistance may be in part related to the pathogen's diversity and variability, which is often not tested in early screens of breeding material that uses only one or a few isolates, and which may increase as a result of selection pressure on the pathogen due to intensive cultivation of resistant cultivars (Rouxel et al. 2003).

Fig. 8 Variation of the soluble phenolics profile in cvs Westar, Quinta and Glacier, either healthy or inoculated with different *Leptosphaeria* spp. isolates, under three temperature regimes of incubation (20/16; 25/23; 30/25°C day/night). For space convenience only results from one *L. biglobosa* and one *L. maculans* isolate are shown here. Similar patterns were observed with other isolates. The major accumulated compounds are deemed to be hydroxycinnamates as shown in Fig. 6. The numbers reported on each chromatogram represent amount of soluble phenolics (μg eq. chlorogenic acid/g FW ± standard-error)


20/16°C
25/23°C
30/25°C
Control
PG1
PG2
Control
PG1
PG2
Control
PG1
PG2
0.58 ± 0.12
0.99 ± 0.08
1.4 ± 0.14
3.13 ± 0.31
1.93 ± 0.25
1.94 ± 0.3
1.21 ± 0.08
0.49 ± 0.05
0.04 ± 0.00
0.87 ± 0.14
2.81 ± 0.62
0.04 ± 0.01
1.36 ± 0.15
1.01 ± 0.15
0.81 ± 0.08
0.56 ± 0.08
0.04 ± 0.00
0.03 ± 0.00
0.82 ± 0.04
2.18 ± 0.31
1.66 ± 0.23
1.59 ± 0.33
0.99 ± 0.16
0.83 ± 0.21
0.05 ± 0.00
0.05 ± 0.01
0.05 ± 0.01
AU
Minutes
Westar
Quinta
Glacier

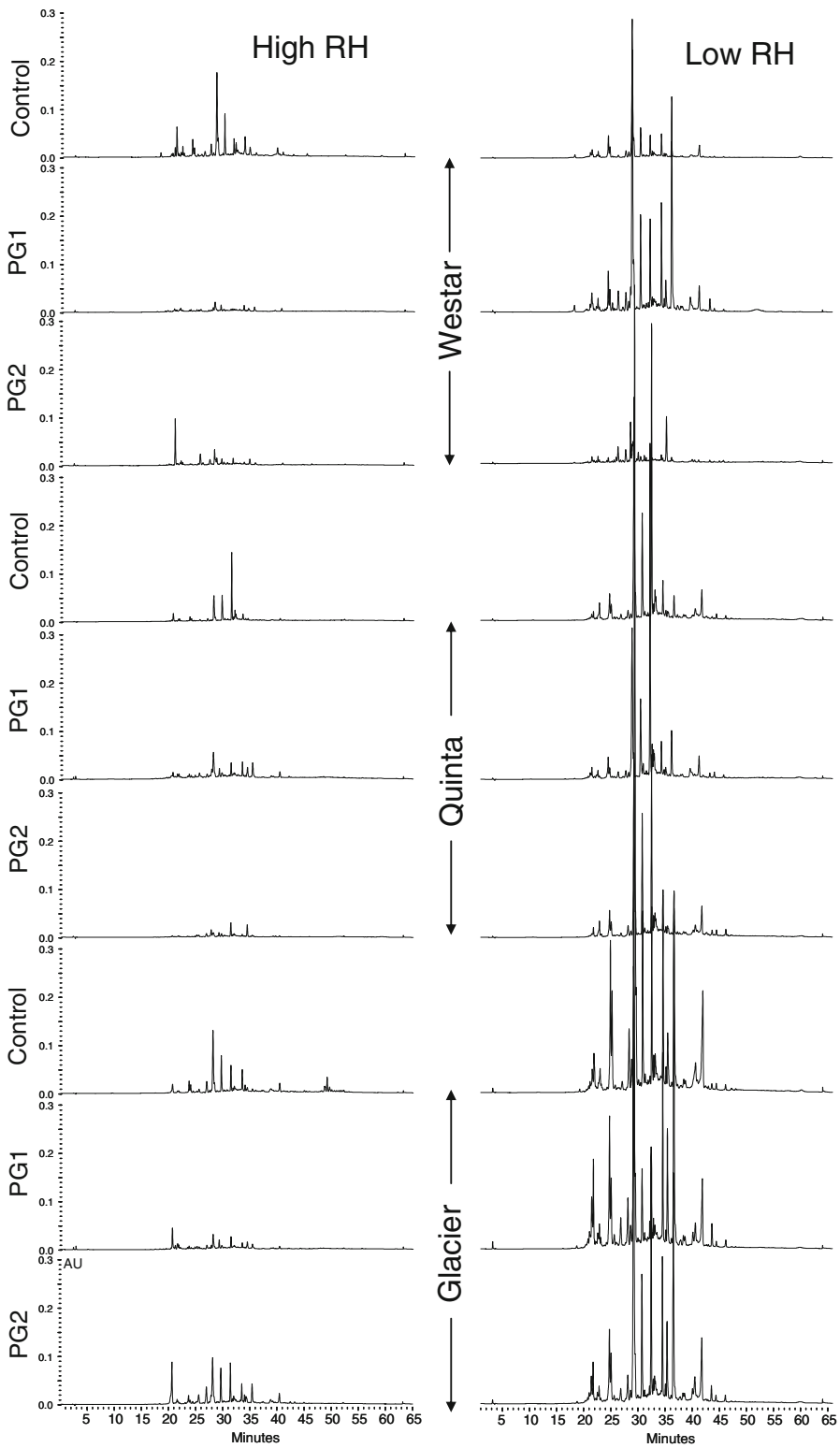


Fig. 9 Variation in the profile of soluble phenolics of cvs Westar, Quinta and Glacier, either healthy or inoculated with different *Leptosphaeria* spp. isolates, under three RH regimes of incubation (low RH: constant 45–50% RH; high RH: 3 days at saturated atmosphere followed by and incubation at 70% RH). The temperatures of these experiments were set at 20/16°C day/night. For space convenience, only results for one *L. biglobosa* (former PG1) and one *L. maculans* (former PG2) isolate are shown here. Similar patterns were observed with the other tested isolates. The major accumulated compounds are deemed to be hydroxycinnamates as shown in Fig. 6

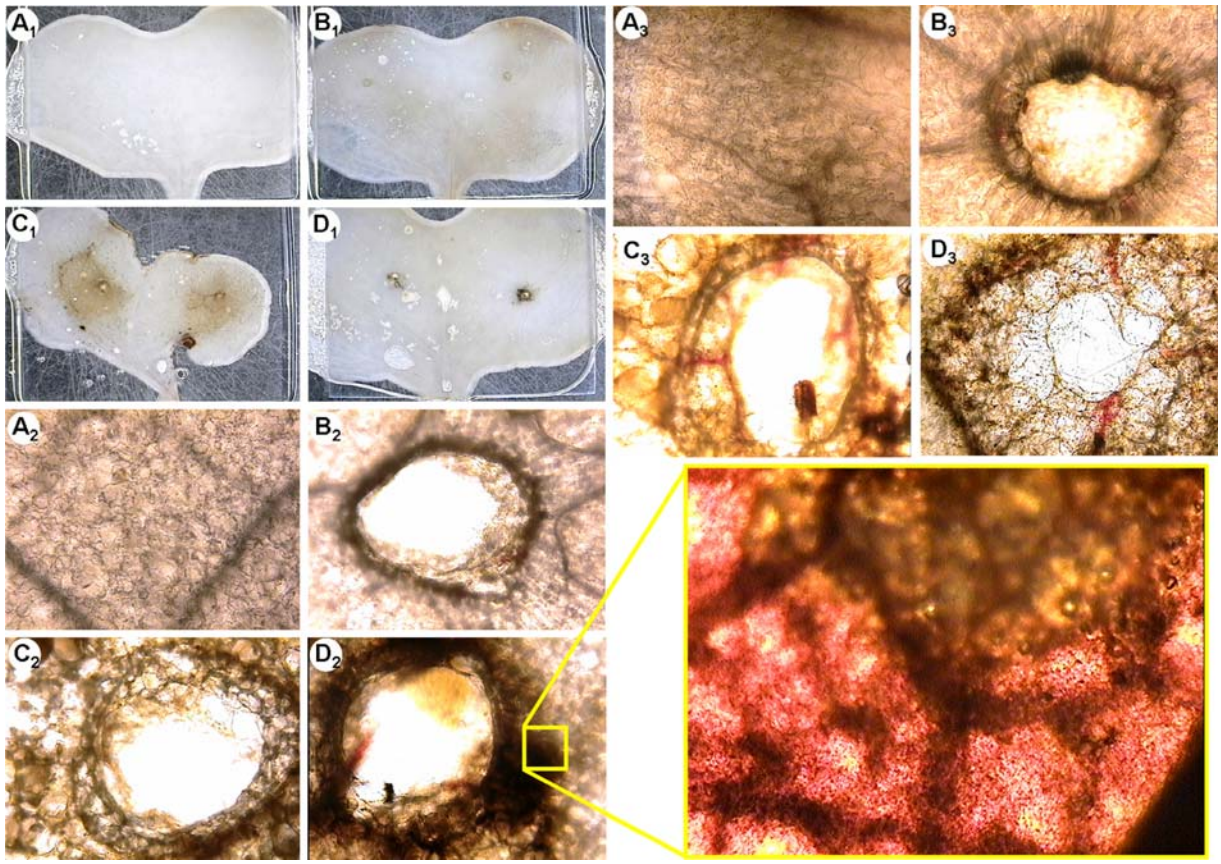


Fig. 10 Clarified canola cotyledons before (*1*) and after (*2*, *3*) phloroglucinol-HCl staining. (*2*) The cotyledons were incubated at regular RH conditions (45–50%). (*3*) The cotyledons were incubated at high RH conditions (saturation for 3 dai and 70% until rating). *A*: non-wounded and non-inoculated control. *B*: wounded and non-inoculated control. *C*: inoculated with *L. maculans* (former PG2). *D*: inoculated with *L. biglobosa* (former PG1)

In the present study, we investigated the impact of RH and temperature on the pathogenicity of several *Leptosphaeria* spp. isolates on the canola cvs Westar, Glacier and Quinta that previously served as differentials for the former PGs of *L. maculans senso lato*. Pathogenicity tests corroborated the expected disease ratings on the susceptible cv. Westar, with higher disease scores in response to *L. maculans* isolates (formerly known as PG2, PG3, PG4 and PGT) and lower scores in response to *L. biglobosa* isolates. Cultivars Quinta and Glacier displayed differential responses to *L. maculans* isolates and exhibited, in the case of resistance, restrained infections recalling a hypersensitive reaction (HR). These responses were not affected by the temperature conditions tested. High RH, on the other hand, enhanced the aggressiveness of isolates, on all tested cultivars, as well as the virulence of the *L. biglobosa* isolates (i.e., on cvs Quinta and Glacier, supposedly resistant to these isolates). This effect of RH is not thought to be due to enhanced pycnidiospore germination and penetration, since germination in droplets of inoculum remained high under all conditions tested. The effect can be explained by a gain in pathogenicity, or by suppression of plant HR-like reactions and other defence mechanisms (i.e., lignin accumulation; Ramos et al. 1997; El Hadrami et al. 2009).

Another possible explanation of these findings would be that the pathogen growth on the surface of the leaves was enhanced under high RH allowing it to either keep ahead of the host defence responses or to bypass them, as reported for *L. maculans* (Hammond et al. 1985; Hammond and Lewis 1986) and other pathogens (Wang et al. 2004, 2008) that are able to

overcome plant defence mechanisms. Others have also shown the impact of temperature, RH and rainfall on the progression of blackleg epidemics in the field (West et al. 2001). However, few have investigated the effect of RH on the expression of the HR in *B. napus* to *Leptosphaeria* spp. For instance Huang et al. (2006) and Khangura et al. (2007) found *Rlm6*- and *Rlm4*-dependent resistance were overcome under high RH and temperature conditions. However, the mechanisms by which this had occurred and the ways RH interferes with the HR, are not well understood. In pathosystems where RH interfered with resistance expression, no specific RH-regulated factor that could affect the expression of the HR has been identified (Hammond-Kosack et al. 1996; Weymann et al. 1995; Jambunathan et al. 2001; Yoshioka et al. 2001; Wang et al. 2005). Yet, the suppression of the HR, expected from the interaction of *Cf/Avr* gene products, was ascribed to a direct or an indirect functional blocking of key steps in the *Cf* downstream pathway.

Wang et al. (2005) indicated that transcript accumulation of over 60 HR-, signalling- and defence-related genes was reversed in *Cf/Avr* seedlings incubated under HR-inhibiting high RH conditions, compared to those incubated under low RH conditions. May et al. (1996) and Hammond-Kosack et al. (1996) have also reported on the dramatic repression of induction of the oxidative burst, including lipid peroxidation and glutathione accumulation, as well as ethylene and salicylic acid synthesis and accumulation under similar RH conditions. Altogether, along with the study of Zhou et al. (2004) on *ssi*-mediated HR-like cell death, these results demonstrated that the high RH-sensing factors, as well as the HR-inhibiting factors should be located upstream in the resistance gene signalling pathways. Alternatively, suppression of the HR-like reaction under high RH conditions, as observed in the present study, could be the result of a misrecognition between the R genes from cvs Quinta or Glacier and their cognate *L. maculans* effector proteins, due to a shortage in functional R proteins and/or their guardees (van der Biezen and Jones 1998; Dangl and Jones 2001). The activation of an inhibitor of the recognition complex under high RH conditions could not be ruled out as this has been shown using the *A. thaliana* RPW8 gene, which is required for the development of an HR-like cell death (Xiao et al. 2003). In this case, high levels of RH lowered the expression HR-like response and suppressed cell death.

Besides the effect on the expression/suppression of the HR-like reaction, RH seems to be affecting the pathogenicity of *Leptosphaeria* spp. isolates. In the present study, we showed that under high levels of RH, isolates of *L. biglobosa* became virulent and highly aggressive, in terms of symptom development on cotyledons on the three tested cultivars. Concurrently, under high RH conditions, canola plants synthesised less hydroxycinnamates and ultimately accumulated less lignin in their cell walls, compared to plants incubated under ambient RH conditions. Obviously, a plant incubated under high RH conditions grows faster and taller than under normal conditions. Elongating cells require less lignin deposition in their cell walls. Although this is an advantage for growth, it constitutes a disadvantage when the plant is infected by a pathogen. The scarcity of lignin in the cell walls may lead to larger lesions and extensive development of pathogens. This way, RH could be affecting canola defences, such as the amount and quality of accumulated lignin in response to infection.

The findings of the present study shed light on the importance of RH on the interaction of canola cultivars with various isolates of *Leptosphaeria* spp. They also suggest a difference in sensitivity to RH between *L. maculans* and *L. biglobosa*, allowing *L. maculans* to infect at relatively low RH. This clearly demonstrates that RH has a significant effect on the pathogenicity of *L. biglobosa* and on subsequent plant defence responses. This should be taken into consideration if isolates of *L. biglobosa* were to be tested as a resistance inducer against blackleg in canola, and in pathogenicity testing that is part of breeding programmes.

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