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Presence in *Leptosphaeria maculans* populations of isolates virulent on resistance introgressed into *Brassica napus* from the *B. nigra* B genome

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The resistance carried by chromosome 4 of the *Brassica* B genome confers on the *Brassica napus–B. nigra* addition line 'LA4+' a high level of resistance to *Leptosphaeria maculans* isolates at the cotyledon stage under controlled conditions and at all growth stages under field conditions. This resistance has never been used commercially in *B. napus* varieties. To determine the potential durability of this new resistance, it is necessary to investigate whether it exerts selective pressure on fungus populations. A study of the pathogenicity of 57 single ascospore isolates of *L. maculans* on this line at the cotyledon stage was carried out to examine possible race specificity of the resistance. Two isolates were clearly virulent and three had intermediate aggressiveness on the 'LA4+' line. Two B-group isolates and 53 A-group isolates, including isolates from darkening tissue of resistant plants of the 'LA4+' line and cv. Junius (*B. nigra*), were virulent on the susceptible isogenic 'LA–' line. These results demonstrate that: (i) the resistance of the 'LA4+' oilseed rape line, conferred on *B. napus* by *B. nigra* chromosome 4, is race specific at the cotyledon stage; and (ii) the resistant material can act as a reservoir of virulent isolates for susceptible oilseed rape lines.

Keywords: blackleg, mustard, oilseed rape, *Phoma lingam*, race-specific resistance, stem canker

Introduction

Leptosphaeria maculans (anamorph *Phoma lingam*) is the causal agent of stem canker in oilseed rape (*Brassica napus*, AACC). This fungus is responsible for severe disease outbreaks in most rape-producing regions worldwide (Gugel & Petrie, 1992). Populations of the fungus are assigned to two main groups of isolates that may represent two species, A- and B-group isolates (Johnson & Lewis, 1990; Williams & Fitt, 1999). These groups can be distinguished on the basis of their pathogenicity (Cunningham, 1927; Johnson & Lewis, 1994), isozyme banding patterns (Balesdent *et al*., 1992; Hall *et al*., 1993; Somda *et al*., 1996; Brun *et al*., 1997) and molecular markers (Johnson & Lewis, 1990; Koch *et al*., 1991; Taylor *et al*., 1991). A-group isolates cause stem canker, the most damaging symptom of the disease, whereas B-group isolates induce mild symptoms and pith degradation (Johnson & Lewis, 1994). Various races have been defined within A-group populations according to the differential set of cultivars used. Mengistu *et al*. (1991) described three races (PG2, PG3 and PG4) based on phenotypic interaction with cv. Westar, Glacier and Quinta. Badawy *et al*. (1991) described five races by adding another cv., Jet Neuf. More races were identified using an extensive differential set (Kuswinanti *et al*., 1999). Isolates of either A- or B-groups produce pseudothecia under both field and *in vitro* conditions and are heterothallic (Venn, 1979; Somda *et al*., 1997). Attempts have been made to cross the two groups, but none has succeeded (Petrie & Lewis, 1985, Somda *et al*., 1997). The two groups of isolates survive as mycelium, pycnidia and pseudothecia on crop residues, which act as the primary inoculum source (Gabrielson, 1983; Hall, 1992).

Currently, the use of resistant cultivars is the most effective way to limit yield losses due to *L. maculans* (Rimmer & van den Berg, 1992), and a continuous breeding effort must be maintained to widen the genetic basis of resistance present in oilseed rape cultivars. New sources of resistance have been investigated and used by plant breeders to improve the resistance of oilseed rape to *L. maculans*. Various crucifer species have been tested. Mustards possessing the B genome (*B. juncea*, AABB; *B. nigra*, BB; *B. carinata*, BBCC) display total

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resistance to blackleg throughout the life of the plant (Rimmer & van den Berg, 1992). Interspecific crosses between *B. napus* and *B. nigra* have given rise to resistant oilseed rape lines (Chèvre *et al.*, 1996; Struss *et al.*, 1996; Plieske *et al.*, 1998; Dixelius, 1999; Dixelius & Wahlberg, 1999; Eber *et al.*, 1999). Some of these lines are highly resistant to isolates obtained from oilseed rape cultivars both at the cotyledon stage under controlled conditions and at all growth stages under field conditions (Chèvre *et al.*, 1996; Eber *et al.*, 1999). Nevertheless, as this source of resistance has never been used in commercial cultivars of *B. napus*, it is necessary to determine whether it is likely to exert selection pressure on *L. maculans* populations leading to a possible shift in fungus populations and breakdown of the resistance. Firstly, it is necessary to determine whether *L. maculans* isolates virulent on the resistant *B. napus* line containing the *B. nigra* introgression exist. This is the subject of this paper.

Materials and methods

Plant material

Interspecific crosses between *B. napus* cv. Darmor and *B. nigra* cv. Junius gave rise to a monosomic *B. napus–B. nigra* oilseed rape addition line 'LA4+' possessing chromosome 4 from the B genome (Chèvre *et al.*, 1996). The 'LA' progeny obtained by selfing from this line were not homogeneous and contained a mixture of plants with one copy of chromosome 4 and without the additional chromosome. Plants with ('LA4+') and without ('LA–') chromosome 4 were differentiated using the isoenzymatic *Pgi-2B* marker. Plants with the marker (about 20%) displayed a high level of blackleg resistance at the cotyledon stage, whereas plants without the marker were susceptible (Chèvre *et al.*, 1996).

Cultivar Darmor is a winter oilseed rape variety (*B. napus*) that is susceptible to leaf lesions under French field conditions, but partially resistant to stem canker at the adult stage (Roussel, 1999).

Brassica nigra cv. Junius is a population variety that displays no leaf lesions or stem canker under high disease pressure in the field.

Fungus material

Single ascospore isolates were obtained as described by Somda *et al.* (1997) from pseudothecia fully developed on stem base residues of plants of the three genotypes: 'LA4+', 'LA–' and Junius. The genotypes had previously been grown in the field under high disease pressure. Stem base residues were placed outside after harvest, in conditions favourable for the development of sexual organs. Inoculum for the cotyledon test was produced as described by De March *et al.* (1986) and Brun (1994).

Pathogenicity test

The pathogenicity of the isolates was tested using the progeny ('LA4+' and 'LA−' plants) from self pollination of the *B. napus–B. nigra* addition line 'LA4+' and the *B. nigra* cv. 'Junius'. Some isolates were also tested on cv. 'Westar', 'Glacier' and 'Quinta' at the cotyledon stage to determine their pathogenicity group (PG).

A cotyledon test derived from the Williams & Delwiche (1979) test was performed following the protocol described by Somda *et al.* (1999). A plastic cover was placed over inoculated plants to create an atmosphere with 100% relative humidity (RH) and the plants were incubated in the dark at 20°C for 24 h in a growth chamber. Inoculated plants were then placed in an atmosphere with 80–90% RH (without a plastic cover) with a 16-h photoperiod at 20°C. Fourteen days after inoculation, symptoms on cotyledons were scored using the 0 (no visible reaction) to 9 (total collapse of the tissue) rating scale of Williams & Delwiche (1979). Each cotyledon lobe was treated as a replicate (therefore, four inoculation sites per plant) and each isolate was used to inoculate 40 plants of the progeny from selfing of the 'LA4+' line and 16 plants of cv. Junius. A disease index (DI) was calculated according to the formula, $DI = \Sigma(N_i\ i)/N_t$, where N_i is the number of inoculation sites with score i (0–9) and N_t is the total number of inoculation sites (i.e. the number of well-developed cotyledon lobes). For each treatment, the mean DI and SD were calculated.

Pathogenicity of isolates

The following pathogenicity classes were defined. A $DI < 4$ described an incompatible interaction (avirulent isolates), $4 \leq DI < 6$ indicated virulent isolates with moderate aggressiveness (intermediate isolates) and a $DI \geq 6$ indicated that the isolate was virulent and highly aggressive. Virulence was defined as a qualitative trait of the isolates that described the compatibility of the host–pathogen interaction. Incompatibility (i.e. avirulence of the isolate and resistance in the host) in this case was expressed as a hypersensitive reaction (HR)-like response. Aggressiveness was the ability of virulent isolates to induce symptoms with various levels of severity (Andrivon, 1993).

Results

Large numbers of pseudothecia were produced on 'LA–' plant residues. In contrast, only a few sparse pseudothecia were observed on plants of 'LA4+' and of cv. Junius. Apart from a few plants of 'LA4+', the 'LA4+' and Junius genotypes did not display stem canker before harvesting, but darkening of stem base tissue was visible at harvest on some plants (H. Brun, unpublished data).

All 'LA4+' and cv. Junius isolates from Le Rheu were A-group, whilst for 'LA–' isolates, 16 out of 17 were A-group and one was B-group. Two isolates were obtained from Dr S. Poutot, Gesamthochschule Kassel, Witzenhausen, Germany; #945 (originally T19G35)

Table 1 Pathogenicity of *Leptosphaeria maculans* single ascospore isolates collected from plants of 'LA–' with stem canker evaluated on various *Brassica* genotypes at the cotyledon stage

Isolate	Group	Genotype											
		'LA4+'		'LA–'		cv. Junius		cv. Westar		cv. Glacier		cv. Quinta	
		DI[a]	SD[b]	DI	SD	DI	SD	DI	SD	DI	SD	DI	SD
945[d]	B	8·1	1·5	7·5	1·8	7·1	1·8	–[c]		–		–	
972	B	9·0	0·0	9·0	0·0	8·4	1·1	–		–		–	
940[d]	A	2·9	1·2	8·5	1·4	3·5	1·7	–		–		–	
946	A	2·4	0·9	8·6	0·5	5·4	1·5	8·4	1·1	8·8	0·2	8·4	0·4
948	A	2·8	1·0	8·5	1·0	3·3	1·1	–		–		–	
956	A	4·5	2·8	6·7	0·8	6·6	0·5	6·8	0·7	6·7	0·2	5·8	0·9
957	A	3·0	1·7	8·6	1·2	2·8	2·0	–		–		–	
959	A	3·4	0·4	6·3	0·4	5·9	0·7	8·0	0·3	7·1	1·5	7·6	0·2
963	A	3·3	0·9	8·8	0·8	3·0	1·3	–		–		–	
966	A	4·7	2·7	3·9	1·5	3·3	1·1	–		–		–	
969	A	2·3	1·0	7·3	1·7	3·2	2·0	–		–		–	
976	A	4·5	1·5	7·6	1·9	4·5	1·7	–		–		–	
980	A	2·4	0·3	7·4	0·2	4·6	0·9	7·7	1·0	7·7	0·9	5·4	0·3
981	A	3·5	0·9	7·5	1·9	3·4	1·0	–		–		–	
983	A	5·1	2·7	8·5	1·4	4·5	2·2	–		–		–	
984	A	2·7	0·7	8·7	0·2	5·5	1·1	9·0	0·0	8·9	0·1	8·0	0·5
985	A	1·5	0·2	5·8	0·5	6·3	0·6	7·1	0·3	6·5	0·4	5·2	0·8
987	A	2·2	0·5	8·3	0·5	5·3	1·9	7·3	0·9	8·6	0·3	8·7	0·2
988	A	2·6	0·5	5·6	0·5	5·7	2·6	7·3	0·5	6·3	0·3	5·9	0·8

[a] Disease index (DI) and interaction phenotypes: DI < 4 = avirulent isolate; 4 ≤ DI < 6 = virulent moderately aggressive isolate; DI ≥ 6 = virulent highly aggressive isolate.
[b] Standard deviation. [c] Not tested.
[d] Provided by Dr S. Poutot, Germany.

was pathotype NA (B-group); #940 (originally T12aD24) was pathotype A1 (A-group).

Pathogenicity tests were performed on 59 isolates: two isolates recovered from oilseed rape varieties, 17 recovered from 'LA–', 25 recovered from 'LA4+' and 15 recovered from cv. Junius.

Nineteen isolates recovered from oilseed rape and 'LA–' plants were tested for pathogenicity on the differential set of cultivars (Table 1). The two B-group isolates (#945 and #972) were virulent on all lines, irrespective of *B. nigra* resistance. All the 17 A-group isolates were virulent on cotyledons of the 'LA–' line, except two which were intermediate in aggressiveness (#985, #988) and one (#966) which was avirulent. Conversely, all isolates were avirulent on the 'LA4+' line except four (#956, #966, #976, #983) which induced mild symptoms. Seven isolates were avirulent on cv. Junius and two were virulent (#956, #985); all the others had intermediate aggressiveness. Nine isolates were tested for PG on the Westar, Glacier and Quinta differential set. Five isolates (#940 [data not shown], #946, #959, #984, #987) belonged clearly to PG4. The other four (#956, #980, #985 and #988) had intermediate behaviour on the three genotypes.

All of the 25 A-group isolates recovered from 'LA4+' plants were virulent on the 'LA–' line, irrespective of the presence or absence of stem canker on the plants of 'LA4+' from which isolates were obtained (Table 2). These isolates were mostly avirulent (or weakly aggressive) on cv. Junius and the 'LA4+' line. However, two virulent isolates (#S5 and #S7) and three with intermediate aggressiveness on the 'LA4+' line were detected amongst isolates recovered from 'LA4+' plants with canker. Isolate #S7 belonged to PG3 (data not shown).

All the isolates recovered from cv. Junius except one (#J5) were virulent on the susceptible 'LA–' line and avirulent on both the 'LA4+' line and cv. Junius (Table 3).

Discussion

This study of the pathogenicity of *L. maculans* single ascospore isolates indicates that most of the isolates studied were virulent on the oilseed rape 'LA–' line and a few were virulent on the 'LA4+' resistant line, at the cotyledon stage. Mainly A-group isolates were obtained from pseudothecia recovered from the three genotypes, as previously reported (Brun *et al*., 1995). The two B-group isolates of *L. maculans* were virulent at the cotyledon stage on all genotypes tested, confirming the results obtained by Johnson & Lewis (1994). Somda *et al*. (1999) also demonstrated the virulence of B-group isolates on *B. juncea* cv. 'Picra'.

Twenty-five single ascospore isolates were recovered from 'LA4+' residues. Two of these isolates, which were virulent on the 'LA4+' line at the cotyledon stage, were isolated from pseudothecia sampled from plants of

Table 2 Pathogenicity of *Leptosphaeria maculans* A-group single ascospore isolates collected from plants of the 'LA4+' line evaluated on various *Brassica* genotypes at the cotyledon stage

Isolates	Genotype					
	'LA4+'		'LA–'		cv. Junius	
	DI[a]	SD[b]	DI	SD	DI	SD
Isolates from plants of the 'LA4+' line with tissue darkening at the stem base but without stem canker						
R1	2·7	0·7	8·8	0·5	–[c]	–
R3	3·1	1·0	8·3	1·1	–	–
R4	4·6	1·1	8·7	0·8	–	–
R5	2·6	0·8	8·9	0·3	3·4	1·2
R6	3·2	0·8	9·0	0·1	–	–
R7	3·0	0·8	8·8	0·5	3·4	1·64
R8	2·2	0·7	8·8	0·5	–	–
R9	3·5	1·2	8·8	0·8	–	–
R10	3·4	1·8	8·8	0·7	2·9	1·0
R11	2·2	0·6	8·2	1·2	–	–
R12	4·4	0·7	8·8	0·4	–	–
R13	4·5	1·0	8·8	0·7	–	–
R15	3·0	1·0	8·6	0·9	–	–
R16	3·6	1·2	9·0	0·0	–	–
R17	4·0	0·8	8·8	0·6	–	–
R18	3·5	1·0	8·9	0·3	–	–
R19	2·9	0·6	8·9	0·3	–	–
R20	2·7	0·6	8·7	0·6	–	–
Isolates from plants of the 'LA4+' line with stem canker						
S1	2·5	0·6	9·0	0·8	2·3	0·7
S2	5·3	0·9	9·0	1·9	3·9	1·0
S5	**7·7**[d]	**1·3**	**8·8**	**0·4**	**5·2**	**1·3**
S6	5·3	2·8	8·8	0·6	2·3	0·8
S7	**7·5**	**0·7**	**7·6**	**1·6**	**1·8**	**0·4**
S9	2·2	1·1	6·7	2·0	2·9	1·3
S11	5·2	1·5	8·8	0·8	3·4	1·3

[a] Disease index (DI) and interaction phenotypes: DI $< 4 =$ avirulent isolate; $4 \leq$ DI $< 6 =$ virulent moderately aggressive isolate; DI $\geq 6 =$ virulent highly aggressive isolate.
[b] Standard deviation.
[c] Not tested.
[d] In bold, isolates virulent on the 'LA4+' line.

this line that displayed stem canker symptoms at harvest. This suggests that the resistance may be race specific and can be overcome. The many attempts to transfer resistance from related or wild species into agronomic crops have almost always focused on race-specific resistance (Lenné & Wood, 1991). The management of breeding schemes for oilseed rape resistance to *L. maculans* involving the resistance present in 'LA4+' must take this into account if durable resistance is to be created. Further studies under field conditions are required to evaluate the potential durability of such resistance, i.e. to determine whether it exerts selection pressure on fungus populations leading to the breakdown of the resistance (Brun *et al*., 2000).

Somda *et al*. (1999) demonstrated that the resistance of the 'MX' line conferred by the *Jlm1* gene introduced into *B. napus* from the B genome of *B. juncea* is race specific. Work is underway to compare the isolates virulent on 'LA4+' with those virulent against the *Jlm1* resistance gene according to their pathogenicity on a differential set of cultivars, including various sources of resistance and each of the oilseed rape lines containing resistance genes originating from mustards. In this way, it may be possible to determine whether *L. maculans* populations virulent on the two sources of resistance are different.

As cv. Junius (*B. nigra*) had full or intermediate resistance against all isolates tested, other resistance genes may be carried by the B genome of black mustard. Chèvre *et al*. (1996) showed that resistance was limited to chromosome 4, whereas Struss *et al*. (1996) demonstrated that resistance genes were present in three different regions of the *B. nigra* B genome. Black mustards are population varieties. Therefore, the donor plant of the addition lines obtained by Chèvre *et al*. (1996) may not possess all the genes present in *B. nigra* cv. Junius. Thus, there may be other resistance genes within this species. Alternatively, the expression of resistance may be more efficient in the BB genetic background than in the AACC background.

Table 3 Pathogenicity of *Leptosphaeria maculans* A-group single ascospore isolates collected from darkened tissue at the stem base of cv. Junius (*B. nigra*) evaluated on various *Brassica* genotypes at the cotyledon stage

Isolate	Genotype					
	'LA4+'		'LA−'		cv. Junius	
	DI[a]	SD[b]	DI	SD	DI	SD
J1a	2·3	0·5	8·7	0·7	2·4	1·6
J2b	1·6	0·7	8·4	1·1	3·6	1·3
J3ab	2·6	0·8	9	0	2·6	1·2
J5	3·9	0·9	2·8	0·8	2·2	1·2
J6	2·2	1·8	8·6	1·0	2·2	1·4
J7	1·9	0·2	8·6	0·6	3·0	2·0
J8	1·5	1·1	8·1	2·1	3·0	0·9
J11	2·3	1·0	8·6	0·5	2·5	0·9
J12	2·1	0·5	7·9	1·7	2·6	0·9
J13	3·6	1·1	8·7	0·6	4·1	1·8
J14	2·4	0·5	8·5	0·7	3·6	1·2
J15	2·4	0·5	9·0	0·0	2·2	1·0
J18i	3·0	0·7	8·6	0·9	2·6	1·0
J19i	2·6	0·6	8·9	1·2	3·0	1·2
20	3·1	0·2	8·8	0·5	2·2	1·0

[a] Disease index (DI) and interaction phenotypes: DI < 4 = avirulent isolate; 4 ≤ DI < 6 = virulent moderately aggressive isolate; DI ≥ 6 = virulent highly aggressive isolate.
[b] Standard deviation.

Our results demonstrate that isolates virulent at the cotyledon stage on current oilseed rape varieties are maintained in the field on susceptible material (the 'LA−' line) and, surprisingly, also on resistant material ('LA4+' and cv. Junius). The 38 isolates recovered from resistant plants of 'LA4+' and cv. Junius without typical stem canker symptoms were not virulent on these lines at the cotyledon stage but virulent on 'LA−'. Gugel & Petrie (1992) reported that mustards were resistant to blackleg but not immune to infection. Thus, these isolates may be virulent at another stage of plant growth. Ballinger & Salisbury (1996) demonstrated that several Australian isolates were avirulent at the cotyledon stage but virulent at the adult stage on oilseed rape lines, and vice versa. Alternatively, avirulent isolates may grow saprophytically on senescent organs (leaves or stems). Whatever the underlying mechanisms, this survival of virulent isolates on a resistant host, on which they are avirulent at the cotyledon stage, provides the fungus with the possibility of maintaining extensive diversity and the opportunity to contaminate susceptible hosts. This has major implications for effective control of the disease.

In conclusion, to preserve the long-term efficacy of the resistance carried by 'LA4+', it is of vital importance to determine the best way to manage it in breeding schemes before using it in commercial cultivars. Combination with adult plant resistance and/or with various other major resistance genes should be evaluated to determine the potential durability of such strategies.

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