European Journal of Plant Pathology **103:** 835–841, 1997.

Field efficiency of *Brassica napus* specific resistance correlates with *Leptosphaeria maculans* population structure

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Accepted 19 September 1997

Key words: blackleg resistance, fungal population, pathotype, *Phoma lingam*

Abstract

Field experiments were conducted in Versailles, France, to assess blackleg resistance of *Brassica napus* cultivars Quinta and Glacier under natural infection conditions. Blackleg disease severity was assessed twice during growth of *B. napus*. Quinta resistance was highly expressed as only 13% to 18% of the plants exhibited leaf symptoms in December, whereas Glacier and other cultivars displayed more than 80% of infected plants. In June (harvest), 70% (first year) to 41.5% (second year) of Quinta plants were canker-free. In contrast, Glacier was as infected as the susceptible control cultivars, with more than 88% of plants displaying canker. The *Leptosphaeria maculans* population structure was examined in parallel. Based on soluble protein patterns, 9% of the 299 fungal isolates collected were characterized as Tox^0 species, and belonged to the NA1 sub-group. All but two of Tox^0 isolates were isolated from atypical dark necrotic leaf lesions, mainly occurring on Quinta. In contrast, the Tox^+ isolates were recovered from typical leaf lesions. Following a cotyledon inoculation test on the differential set Westar, Quinta and Glacier, 92 to 95% of Tox^+ isolates collected on susceptible cultivars were characterized as PG3 isolates, i.e. avirulent on Quinta. The remaining Tox^+ isolates belong to PG4, i.e. virulent on the three cultivars. No PG2 isolate, i.e. avirulent on both Quinta and Glacier, was identified in the sampling. The present study suggests that specific resistance expressed at the cotyledon level can be efficient under field conditions where the corresponding avirulent races of the pathogen are prevalent.

Abbreviations: IC – infection class; NA – non-aggressive isolates; PG – pathogeniticy group.

Introduction

Blackleg disease of oilseed rape (*Brassica napus* L.) and other crucifers, caused by the fungal pathogen *Leptosphaeria maculans* (Desm.) Ces. & de Not. (anamorph *Phoma lingam* Tode ex Fr.), is responsible for severe yield losses world-wide (Gugel and Petrie, 1992). In Europe, as well as in Australia or Canada, the disease is mainly controlled by breeding *B. napus* cultivars displaying a high level of field resistance. However, the genetic basis of such resistances is often poorly understood (Ferreira et al., 1995), although specific resistances within *B. napus* species have been described (Thurling and Venn, 1977; Delwiche, 1980). This lack of knowledge is mainly due to the fact that *L. maculans* is considered as a species complex where the two sub-groups of 'Aggressive' (or Tox^+) and 'Non-Aggressive' (or NA or Tox^0) isolates in fact represent major distinct species (Koch et al., 1991; Balesdent et al., 1992; Morales et al., 1993; Rouxel et al., 1994). Based on molecular data, the NA isolates are also further divided into three distinct genetic sub-groups, namely NA1, NA2 and NA3 (Koch et al., 1991; Gall et al., 1995).

The *B. napus* cultivars Westar, Quinta and Glacier have been used as differentials for the Tox^+ group since 1980 (Delwiche, 1980; Badawy et al., 1991; Mengistu et al., 1991; Koch et al., 1991). They discriminate three

pathotypes or PG, namely PG2, PG3 and PG4 (Mengistu et al., 1991). PG4 isolates are virulent on Quinta, Glacier and Westar, i.e. they cause grey-green tissue collapse following cotyledon inoculation. PG3 isolates are virulent on Westar and Glacier but induce resistance reactions on Quinta. PG2 isolates are avirulent on Quinta and Glacier but virulent on Westar. Genetic analysis recently demonstrated that single avirulence genes are present in *L. maculans* and confer specificity of interaction toward Quinta and Glacier (Ansan-Melayah et al., 1995; Ansan-Melayah, 1996). On the plant side, QTL studies showed that one major resistance locus, LEM1, is responsible for specific resistance of cultivar Major to PG2 isolates (Ferreira et al., 1995). The efficiency of Major resistance under Canadian field conditions is correlated with the predominance of PG2 isolates in the natural fungal populations (Mengistu et al., 1991). Single major resistance genes to PG3 isolates in Quinta and PG2 isolates in Glacier are also reported (Rimmer and Van den Berg, 1992; Ansan-Melayah, 1996). However, until now little information on the field behaviour of these cultivars is available. Furthermore, there are little data on *L. maculans* population structure in France, with regard to both the species complex and the PG structure.

Our objective was to assess the field efficiency of Quinta and Glacier specific resistances under natural infection conditions in France, and to analyse in parallel the population structure of *L. maculans* at a local level.

Materials and methods

Field assay. The experiment was conducted at Versailles, France, in 1994–1995 and 1995–1996, in fields were oilseed rape had not been cultivated since 1988. *B. napus* cultivars Samouraï, Tapidor, Quinta, Primor and Glacier were sown in the experimental field (18 m $\times$ 10 m) as sub-plots comprising 40 plants per m^2 each, in rows spaced at 35 cm. Plants were sown in early September, 1994 and maintained under natural inoculum conditions. Fertilisers were provided at sowing. Herbicide treatment (Kerbs 50, 2.5 kg ha^{-1}) was applied in early October, 1994. The field assay was repeated in 1995 at the same location. The experimental scheme comprised 2 major sub-plots of Quinta (197 m^2 each), separated and bordered with sub-plots of Synergy (98 m^2 and 2 $\times$ 66 m^2, respectively). Additional inoculum, i.e. infected stubble from Quinta harvested in July 1995, was provided to increase disease pressure.

Occurrence of blackleg disease was investigated twice during each growing season, in December for the presence of leaf symptoms and at the end of June for the development of crown cankers. In Autumn, the disease was assessed by examination of all plants in the experimental plot (first year) or in randomly selected rows (second year). Percentages of infected plants, i.e. exhibiting leaf lesions, were calculated. Severity of the attack per plant was assessed according to a 0–2 scale. Infection Class (IC) 0 represents the symptomless plants. IC 1 indicates plants displaying less than 5 leaf lesions per plant and IC 2 indicates 5 or more leaf symptoms per plants. In June, one plant out of every ten was uprooted and scored for occurrence of Blackleg typical crown canker and premature ripening. Stems were also longitudinally split and the pith was observed. Finally, occurrence of pycnidia and/or pseudothecia was recorded by examination of tissues under 40 $\times$ magnification. Identification of mature pseudothecia was confirmed by microscope examination of the presence of asci at 60 $\times$ magnification.

Fungal isolation. Fungal isolates were collected from leaf lesions in December 1994 and 1995. Infected leaves randomly collected from each cultivar were rinsed under tap water and dried between two sheets of paper. The pieces of leaves displaying symptoms were cut out and incubated at room temperature under 100% humidity for 1–2 days. Oozing pycnidia were collected with a sterile toothpick and spread out on V8-agar plates supplemented with streptomycin (200 μg ml^{-1}) and penicillin G (125 μg ml^{-1}) (V8-AB). After 24–48 h at room temperature, individual hyphal tips were cut out and transferred on V8-AB. Random ascospores were also collected in June 1994, from pseudothecia differentiated on stubble as previously described (Gall et al., 1994).

Fungal culture and inoculum production. The cultures were maintained on V8-agar plates. Highly sporulating cultures were obtained according to Ansan-Melayah et al. (1995).

Fungal characterization. Tox^+/Tox^0 discrimination. The isolates were grown in liquid Fries medium as previously described (Gall et al., 1994). Mycelial soluble protein patterns (SPP) were determined following isoelectric focusing (Balesdent et al., 1992; Gall et al., 1994). NA1, NA2 and NA3 sub-groups of the Tox^0 isolates (Koch et al., 1991) were identified by comparing with reference isolates profiles (Gall et al., 1995).

Table 1. Blackleg disease severity on *B. napus* cultivars scored in experimental plots in 1994–1995 and 1995–1996

	Growing season						
	1994–1995					1995–1996	
Cultivars	Samouraï	Tapidor	Quinta	Primor	Glacier	Synergy	Quinta
Number of plants scored	729	643	1229	156	466	930	1758
Leaf lesions[a]	85.3%	83.7%	17.8%	88.5%	91.6%	91.4%	13.3%
class 1	81.0%	63.3%	76.2%	75.8%	25.9%	42.7%	88.0%
class 2	19.0%	36.7%	23.8%	24.2%	74.1%	57.3%	12.0%
Number of plants analysed	63	55	125	14	43	185	354[f]
Crown canker[b]	76.2%	89.1%	26.4%	100%	88.4%	95.7%	58.5%
Premature ripening[c]	11.1%	7.3%	3.2%	0%	9.3%	0.5%	3.3%
Pith necrosis[d]	11.1%	3.6%	25.6%	0%	2.3%	1.6%	16.7%
Uninfected plants[e]	1.6%	0%	44.8%	0%	0%	2.2%	21.5%

[a] Percentage of plants displaying leaf symptoms in December. The relative proportions of infected plants displaying few (lower than 5, i.e. class 1) or numerous (5 or more, i.e. class 2) leaf spots per plants are indicated.
[b] Percentage of uprooted plants displaying typical crown cankers in June 95 or early July 1996. All the crown cankers observed were associated with internal pith necrosis.
[c] Percentage of uprooted plants displaying premature ripening.
[d] Percentage of uprooted plants displaying internal pith necrosis without any external symptom.
[e] Percentage of uprooted plants displaying neither internal nor external symptom.
[f] Including both 'resistant' and 'susceptible' Quinta plants.

Fungal characterization. Inoculation tests. Isolates were inoculated on cotyledons using the differentials Westar, Quinta and Glacier as previously described (Ansan-Melayah et al., 1995). Symptoms were scored 14–15 days after inoculation according to the Williams & Delwiche rating scale (1979), comprising of 9 infection classes where the 1–3 infection classes represented a resistance response, 4–6 an intermediate resistance response and 7–9 susceptibility classes.

Results

Heterogeneity of Quinta plants. Under field conditions, most Quinta plants displayed no or only a few leaf spots on a few leaves (IC 0 or 1), whereas some Quinta plants displayed as many leaf symptoms (IC 2) as the susceptible control cultivars (Table 1). It has already been observed that Quinta is heterogeneous for *L. maculans* resistance to PG3 isolates (Koch et al., 1991). Within our Quinta seed lot, we also observed that cotyledon inoculation tests with PG3 isolates always resulted in 7–10% of plants being susceptible (Ansan-Melayah, 1996). In order to increase the uniformity of the cultivar, seeds from self-pollinated resistant Quinta plants were used for the second year of field experiment. In addition, in the second year of sampling of isolates, the leaf infection class of Quinta plants from which each isolate originated was recorded, thus allowing a comparison of *L. maculans* populations occurring on 'resistant' (IC 1) and 'susceptible' (IC 2) Quinta plants.

Blackleg resistance under field conditions. A high level of natural inoculum was present early in Autumn 1994 since 83.7% to 91.6% of plants from Tapidor, Samouraï, Primor and Glacier had leaf lesions in December (Table 1). In contrast, only 17.8% of Quinta plants exhibited sporulating leaf lesions. When considering only infected plants, the percentage of highly infected plants (IC 2) was comparable for Quinta, Tapidor, Primor and Samouraï (19–37%), whereas up to 74% of Glacier plants were highly infected (Table 1). In June 1995, 76 to 100% of Tapidor, Samouraï, Primor and Glacier plants exhibited typical blackleg crown canker (Table 1). All cankered plants

also showed internal pith lesions, i.e. brown to dark pith coloration. This browning sometimes extended to stem or roots. Pycnidia as well as differentiated pseudothecia were observed on infected stems. Finally, up to 11% of plants showed premature ripening symptoms. In contrast to Tapidor, Samouraï, Primor and Glacier, only 29.6% of Quinta stubble showed either typical canker (26.4%) or premature ripening (3.2%) (Table 1). The remaining 70.4% of Quinta stems were externally symptomless at harvest. Among them, 63.6% were not infected whereas 36.4% developed dark internal pith coloration of the stem tissues. However, pycnidia or pseudothecia never differentiated in these latter plants.

As in the first year, a high level of infection was observed in Autumn 1995, as 91.4% of the susceptible cultivar Synergy exhibited leaf symptoms in December (Table 1). In contrast, only 13.3% of Quinta plants displayed leaf symptoms. Among these, only 12.0% exhibited 5 leaf lesions or more per plant, in contrast to 57.3% for Synergy, and were considered as 'susceptible' Quinta plants (Table 1). In early July, 95.7% of uprooted Synergy plants exhibited typical blackleg symptoms. Cankers were generally restricted to the crown and numerous pycnidia differentiated on them. Pseudothecia were observed on 16.2% of these plants. In contrast to Synergy, 21.5% of Quinta plants were not infected at all, and 16.7% exhibited pith necrosis with no external symptoms (Table 1). All the uninfected Quinta plants and 88% of the plants exhibiting pith necrosis neither displayed pycnidia nor pseudothecia. In addition, only 1.7% of cankered Quinta plants exhibited pseudothecia whereas pycnidia often developed but were usually localized on leaf scars (data not shown).

L. maculans *population structure. Tox$^+$/Tox^0 discrimination.* No Tox^0 isolate was observed in December 1994 within the 75 isolates collected from typical sporulating leaf lesions (Table 2). Twelve percent of the 224 isolates collected in December 1995 were characterized as *L. maculans* Tox^0 (Table 3). Two of them originated from typical sporulating leaf lesions occurring on Synergy. The remaining 25 Tox^0 were isolated from leaf symptoms on Quinta, and in this latter case, the lesions were dark necrotic leaf spots surrounded by a chlorotic margin, resembling *Alternaria brassicae* leaf spots, and displaying small pycnidia ejecting white or pale pink ooze. Except for one isolate, all Tox^0 isolates showed a high level of sporulation and *in vitro* pigment production (Table 3). According to SPP, all Tox^0 isolates collected belonged to the NA1 sub-group.

Table 2. Pathogenicity grouping of *L. maculans* isolates collected from leaves in December 1994

	Isolated from cv.				Total
	Samouraï	Tapidor	Quinta	Glacier	
Number of isolates	16	21	18	20	75
Tox^0 or PG1[a]	0	0	0	0	0
PG2	0	0	0	0	0
PG3	16	21	15	19	71
PG4	0	0	3	1	4

[a] PG1 isolates, or Tox^0 species (Balesdent et al., 1992) are 'non-pathogenic' on Westar, Quinta or Glacier. PG2 isolates are avirulent on Glacier and Quinta but virulent on Westar. PG3 isolates are avirulent on Quinta but virulent on both Westar and Glacier. PG4 isolates are virulent on the 3 cultivars (Mengistu et al., 1991; Ansan-Melayah et al., 1995).

L. maculans *population structure. PG characterization of Tox$^+$ isolates.* PG2 isolates were never recovered within the sampling in 1994 and 1995 (Tables 2 and 3). A total of 94.7% of Tox$^+$ isolates collected in December 1994 were characterized as PG3 (Table 2). The predominance of PG3 isolates was confirmed for the second year of sampling where 61% of the 197 Tox$^+$ isolates collected were PG3. Only PG3 isolates were recovered from pseudothecia occurring on stubble in June 1995 (data not shown).

Discussion

This paper reports on the efficiency of a specific resistance gene present in Quinta and expressed under field conditions. The field resistance of Quinta correlated with the predominance of the corresponding avirulent pathotype, i.e. PG3, in the local fungal populations.

Incidence of blackleg disease in 1993–1994 led to severe damage in France, with average yield losses due to the disease being 1 t per ha (Lagarde, 1995). Consequently, a high level of inoculum was present in the Autumn of 1994, as evidenced by the high rate of plants displaying leaf lesions in December 1994. Since *Brassica* spp. has not been cultivated in the experimental plots since 1988, airborne ascospores originating from infected stubble of nearby oilseed rape crops were probably responsible for primary infection, as ascospores can easily be dispersed by wind up to

Table 3. Characterization of *L. maculans* isolates collected from leaves in December 1995

Fungal species[a] isolated from cv.	Tox^0		Total	Tox^+			Total
	Synergy	Quinta		Synergy	Quinta		
					'resistant'[b]	'susceptible'	
Number of isolates	2	25	27	78	77	42	197
Leaf lesion aspect[c]							
typical	2	2	4	78	75	42	195
necrotic	0	20	20	0	2	0	2
nd	–	3	3	–	–	–	–
Sporulation level/*in vitro* pigment production							
high/numerous	2	24	26	0	1	0	1
low/no pigment	0	1	1	78	56	37	171
nd	–	–	–	–	20	5	25
Pathogenicity groups							
PG1	2	25	27	0	0	0	0
PG2	0	0	0	0	0	0	0
PG3	0	0	0	71	10	39	120
PG4	0	0	0	7	67	3	77

[a] Tox^+/Tox^0 according to their soluble protein profiles (SPP).
[b] Quinta plants displaying a number of leaf lesions comparable to that of the susceptible control (IC 2) are termed 'susceptible' Quinta. Plants displaying a low number of leaf lesions (IC 1), are termed 'resistant' Quinta.
[c] Typical leaf lesions are grey-green tissue collapses on which pycnidia differentiate. Necrotic lesions are atypical dark spots rounded by a yellowing halo bearing few small pycnidia ejecting a pale-pink ooze.
nd: not determined.

several kilometers (Alabouvette and Brunin, 1970). Rains in September, 1994 and 1995 probably promoted ejection of ascospores early in the growing season (Gladders and Musa, 1980), which germinated to cause leaf lesions on which pycnidia could differentiate and increase the inoculum pressure (Alabouvette et al., 1974). In addition, cold temperatures occurring in spring could be also responsible for the premature differentiation of pseudothecia in June 1995. In Western European countries, pseudothecia usually differentiate on stubble at the beginning of autumn (Alabouvette and Brunin, 1970). As far as we know, such an early differentiation of pseudothecia has only been reported once (Gladders and Musa, 1980).

In these optimal infection conditions, numerous leaf symptoms were observed in autumn 1994 and 1995. Analysis of about 300 isolates collected from leaf lesions allowed us to distinguish different leaf lesion phenotypes. Typical leaf lesions were linked with the presence of Tox^+ isolates in 99.3% of the cases. Atypical necrotic leaf spots were always associated with the presence of the Tox^0 species. Using artificial inoculation, Johnson and Lewis (1994) reported that Tox^0 isolates produced smaller leaf lesions with lesser sporulation than the Tox^+ isolates. In accordance with these observations, our study is the first to report that the two groups of isolates develop specific lesion phenotypes under field conditions. Atypical Tox^0 symptoms were more frequently observed on resistant Quinta plants, when typical leaf lesions provoked by Tox^+ isolates were unfrequent. This suggests that leaf infections with Tox^0 isolates are probably under-estimated on cultivars heavily infected by Tox^+ isolates. This can also explain the lack of Tox^0 in the first year of sampling, where only a few isolates were collected from Quinta.

At the end of the growing season, two main types of symptoms were observed on the stems : typical crown canker and pith necrosis. Stem cankers are usually attributed to Tox^+ isolates whereas pith necrosis was suggested to be caused by Tox^0 isolates (Johnson and Lewis, 1994). As Tox^+ and Tox^0 isolates are present in the experimental field and can be isolated from the same infected leaf (Gall et al., 1995; Mahuku et al., 1996; this study), one would expect four symptom classes to be observed at the end of the growing season: (1) uninfected plants, (2) typical crown cankers without internal pith necrosis, (3) pith necrosis without external crown canker and (4) crown cankers associated with pith necrosis. However, symptom class (2)

was never observed in our sampling. In addition, Quinta plants always displayed less pith necrosis than the susceptible controls. One or more of three hypotheses can explain these observations: 1/ Quinta is resistant to both PG3 and Tox^0 isolates; 2/ Tox^+ isolates can cause pith necrosis in addition to stem canker or 3/ there is a synergistic interaction between Tox^+ and Tox^0 isolates at the stem level. At the cotyledon level, we have already observed that Quinta is as susceptible to Tox^0 isolates as the susceptible cv. Westar (Gall et al., 1995; unpublished data). However, the lack of study reporting on similar field experiment does not enable us to convincingly support one hypothesis over another.

Our first year of sampling using a limited number of isolates suggested the absence of PG2 and the predominance of PG3 in the local Tox^+ population. To confirm this observation, a more extensive sampling was performed in December 1995, with each isolate being identified as originating from either a susceptible or a resistant plant. This enabled us to demonstrate that resistant genotypes of Quinta actually selected the natural inoculum at the leaf level, since 87% of isolates from 'resistant' Quinta were PG4, whereas only 7% of PG4 were recovered from 'susceptible' Quinta plants. As a consequence, the structure of field population present in Autumn 1995 can only be estimated by the analysis of isolates originating from plants that do not exert any selection pressure, i.e. susceptible to all PGs (Synergy or 'susceptible' Quinta plants). The PG3/PG4 ratio was comparable on both Synergy and 'susceptible' Quinta plants (respectively 91.1/8.9 and 92.9/7.1), thus confirming the predominance of PG3 (92%) in the local population.

The absence of PG2 and the prevalence of PG3 in the local Tox^+ population is representative of a specific population structure, as compared to what is described in Canada (Mengistu et al., 1991; Ferreira et al., 1995) and Germany (Kuswinanti et al., 1995). PG2 isolates are prevalent in Canada (Mengistu et al., 1991; Ferreira et al., 1995). In accordance with the present study, Kuswinanti et al. (1995) did not find any PG2 among the 341 isolates collected from different regions of Germany, suggesting that the absence of PG2 could be a specificity of the European situation nowadays. In contrast, the German situation differs from the French situation in that PG4 isolates represent up to 86% of the Tox^+ population in Germany (Kuswinanti et al., 1995).

In the *L. maculans/B. napus* pathosystem, it is still a matter of debate whether resistance expressed using inoculation tests in controlled environment correlates with resistance expressed under field conditions (Newman, 1984; Newman and Bailey, 1987; McNabb et al., 1993; Bansal et al., 1994). As 1/ we previously demonstrated that PG3 isolates displayed a single avirulence gene expressed on cotyledons or leaves of Quinta in controlled conditions (Ansan-Melayah et al., 1995), and 2/ PG3 pathotype is prevalent in the local Tox^+ population (this study), the field experiment reported here was ideally suited to assess whether a specific resistance observed in growth chamber conditions can also be expressed under field conditions. During our two-years survey, the percentage of infected Quinta plants was always lower than that observed for susceptible control cultivars, at both the leaf and stem levels. In contrast, Glacier was highly susceptible under field conditions in 1994–1995, which is consistent with the lack of PG2 isolates in the local inoculum. These results suggest that a single-gene resistance expressed at the cotyledon and leaf level can provide a good control of the blackleg disease whenever the corresponding avirulent populations are prevalent. Characterization of *L. maculans* populations must now be intensified to confirm the prevalence of PG3 isolates in France. If such is the case, specific resistances to PG3 such as those occurring in newly released cvs Capitol and Columbus (Ansan-Melayah et al., 1997) will have to be carefully managed to ensure their long-term survival. Both shifts in population structure and migration of virulent pathotypes are significant threats for the long-term efficiency of major-gene-based resistances.

Acknowledgements

This research was supported by grants from CETIOM and the Ministère de l'Education Nationale, de l'Enseignement Supérieur, de la Recherche et de l'Insertion Professionnelle (ACCSV7). Delphine Ansan-Melayah was funded by a grant from the Ministère de la Recherche et de l'Enseignement Supérieur. The authors wish to thank R. Khetarpal (INRA, Versailles, Pathologie Végétale) for review of the manuscript.

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