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# A large-scale survey of races of *Leptosphaeria maculans* occurring on oilseed rape in France

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**Abstract**

Nine avirulence genes (*AvrLm1–AvrLm9*) were identified in *Leptosphaeria maculans*, the causal agent of stem canker of oilseed rape (OSR), combinations of which could theoretically generate up to 512 different races of the fungus. *L. maculans* displays a high evolutionary potential to adapt to novel resistance genes as illustrated by the *Rlm1* breakdown in France, where virulent populations became prevalent within three growing seasons. An improved knowledge of the race structure of the fungal population is therefore needed to ensure a better use of available major resistance genes. The objective of this study was to characterise the *L. maculans* population structure in France using a large-scale, rationalised sample of isolates. Experimental fields, planted with ''trap plants'' harbouring no major resistance gene, were sown at 20 locations. Single-pycnidium isolates were collected from leaf lesions that developed in early autumn and 1797 isolates were genotyped at Avr loci. The frequency of *AvrLm6* and *AvrLm7* was higher than 99%, whereas *avrLm2* and *avrLm9* alleles were fixed in the population. *AvrLm1*, *AvrLm4*, *AvrLm5* and *AvrLm8* were polymorphic. *AvrLm3* isolates were detected at a very low frequency (less than 1%). Only 11 races were identified in France, with one race prevalent, namely Av5-6-7-(8) (i.e. virulent on *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4* and *Rlm9*), representing around 65% of the population. Disparities between the locations sampled were evident at all scales analysed. Some virulent races, such as those harbouring *avrLm5*, were present before the introduction of the corresponding resistance gene in the commercial OSR crop.

*Abbreviations:* Avr – avirulence; cv – cultivar; OSR – oilseed rape; R – resistance

## Introduction

The dothideomycete *Leptosphaeria maculans* (anamorph *Phoma lingam*), the causal agent of phoma stem canker disease of oilseed rape (OSR) (winter *Brassica napus*) and canola (spring *B. napus* and *B. rapa*), is the most economically important disease of oilseed Brassicas worldwide. Breeding for ''field resistance'' to *L. maculans* in the past often gave rise to cvs harbouring single major gene resistance (*Rlm* genes) that were selected unintentionally due to their complete effectiveness when challenged by populations of the pathogen harbouring the corresponding avirulence (*AvrLm*) allele (Ansan-Melayah et al., 1997; Pinochet et al., 2003; Rouxel et al., 2003a). Most currently registered winter OSR cvs in France harbour at least one *Rlm* gene (Pinochet et al., 2003, 2004; Balesdent and Pinochet unpublished data). The life cycle of the pathogen involves annual sexual recombination and large-scale dispersal of ascospores (West et al., 2001), which predisposes *L. maculans* to have a high evolutionary potential to adapt to novel resistance genes (McDonald and Linde, 2002). This

was illustrated by the case of *Rlm1* in France, where cvs harbouring this gene became popular and accounted for more than 40% of the French OSR production area (Rouxel et al., 2003a). This was associated with a concurrent increase in the proportion of virulent individuals within the population of *L. maculans* within three growing seasons (Rouxel et al., 2003a). This study, along with experimental data which indicate that the novel *Rlm6* gene (not commercially used to date in Europe) can also be overcome within three growing seasons when subjected to artificially increased inoculum pressure (Brun et al., 2000), and the current breakdown of the newly-introduced "Surpass 400" resistance in Australia (Li et al., 2003; Sprague et al., 2006), illustrate the need to monitor the race structure of *L. maculans* populations. Such information will allow more efficient use of available sources of major gene resistance (R) to prevent pathogen adaptation and the spread of novel virulent isolates to regions where novel resistances have not been used.

Although based on a large collection (1011) of French isolates, the study by Rouxel et al. (2003a) was insufficient to provide an extensive survey of the current structure of *L. maculans* populations in France since (i) it included samples taken between 1994 and 2000 at only three sampling locations, (ii) only three Avr genes were analysed, *AvrLm1*, *AvrLm2* and Av*rlm4*, whereas nine Avr genes have currently been identified (Balesdent et al., 2002, 2005; Delourme et al., 2004), (iii) part of the collection originated from unknown (or uncharacterised in terms of R genes) plant genotypes, which might exclude some races avirulent on these genotypes, resulting in a sampling bias caused by selection of the corresponding virulent alleles. For example, the *Rlm2* gene was extremely popular in French cvs (Rouxel et al., 2003a, b) and it was impossible to ascertain that the corresponding avirulent allele, *AvrLm2*, was absent in *L. maculans* populations in France.

A recent analysis of the race composition of an international *L. maculans* isolate collection for all nine identified Avr genes indicated that there was a low diversity of races in Europe, with only 8 out of 512 possible races identified, and important differences between continents in race structure (Balesdent et al., 2005). However, only historical isolates were present in this collection, and the information on the plant genotype from which the isolates originated was often absent.

In this context, the objectives of the present study were (i) to extensively investigate the *L. maculans* population structure in France, including all nine *AvrLm* genes known to date, based on a rational sampling scheme, (ii) to evaluate the putative regional differences in race structure and (iii) to relate them to the history of OSR production in the surrounding region. It was also envisaged that such a large survey would allow us to evaluate which (and where) *Rlm* genes were still effective against a significant part of the *L. maculans* population, and if some virulent races were already present before the introduction of unused novel resistance genes. For this purpose, large-scale sampling of the *L. maculans* population of France was done, on a country-wide basis from a network of similar experimental fields planted with "trap plants" (known to harbour no major resistance genes). These crops were grown by a network of OSR breeding companies, public research institutes, cultivar registration bodies and advisory bodies. The resulting collection of isolates was analysed for the occurrence of *AvrLm* alleles corresponding to *Rlm* genes which had been used commercially in the past (*Rlm2*, *Rlm3*, *Rlm4*, *Rlm9*), more recently introduced commercially (*Rlm1*) or not yet been used commercially [*Rlm7* from *B. napus*, *Rlm5* and *Rlm6* from *B. juncea* and *Rlm8* from *B. rapa* (Balesdent et al., 2002)]. Finally, control plant genotypes, each having different known specific resistance genes, were also included in each trial, to relate the *L. maculans* population structure to effectiveness of specific resistance genes in winter OSR crops.

## Materials and methods

### *Experimental fields*

Seventeen experiments were sown in August–September 2000, and three experiments were sown in August–September 2001 to cover important OSR production areas that were not included in the 2000/2001 survey (Figure 1). In 2000/2001, the experimental design comprised 10 cvs sown in four replicates at each site (Table 1). Each plot (one replicate of one cv. on one site) consisted of four lines of minimum length 2 m. Cultivar Drakkar, a

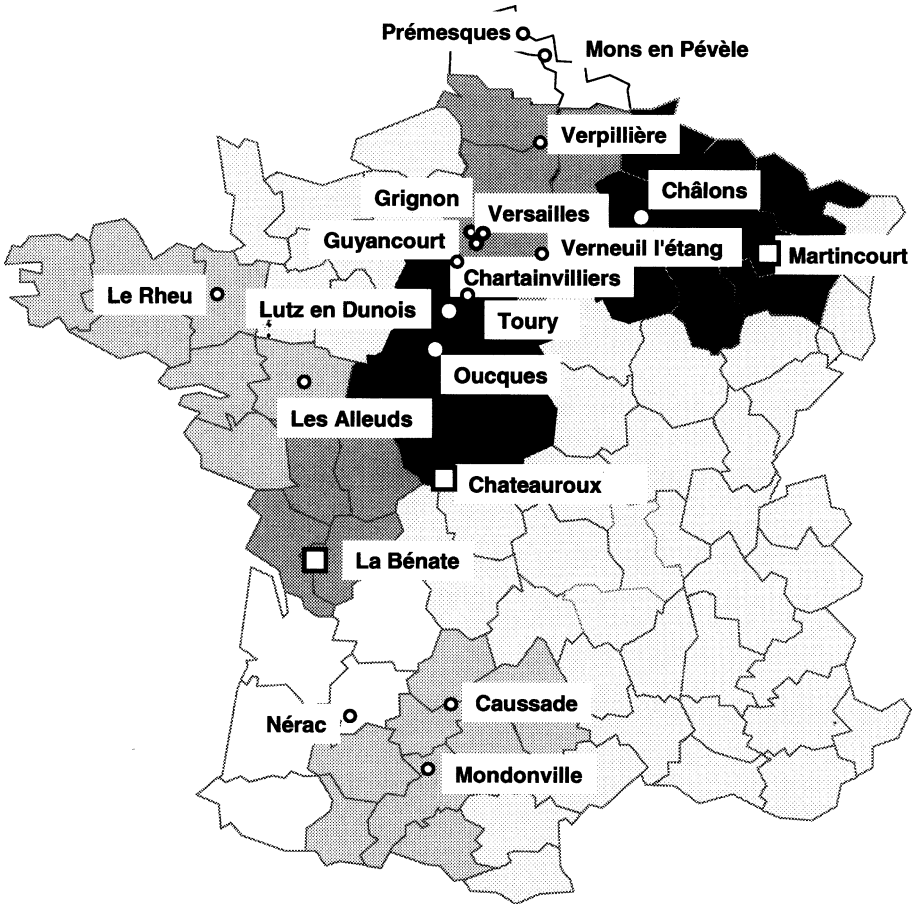


*Figure 1.* Location of the 20 OSR experimental sites sampled across France (○, sites sampled in Autumn 2000 □, sites sampled in Autumn; 2001). Regions in white, less than 20,000 ha of OSR cultivated in this region; regions in pale grey, between 20,000 and 50,000 ha; regions in medium grey, between 50,000 and 100,000 ha; regions in black, from 100,000 to 250,000 ha. Other regions were not sampled.

spring genotype known to possess no *Rlm* gene (provided by INRA-APBV, Le Rheu) was used as a "trap plant", because it exerted no direct selection on the *L. maculans* population. Cv. Surpass 400, possessing a *B. rapa* subsp. *sylvestris* major gene termed *LepR3* (Li and Cowling, 2003; Yu et al., 2004) and line 23-1-1 (*Rlm7*) were assessed as potential novel resistance sources for the French conditions, as neither had been previously used commercially in France. The other cvs chosen were commercial cultivars with or without *Rlm1*, *Rlm2*, *Rlm4* or *Rlm9* (Table 1). Jet Neuf (*Rlm4*) was also grown as a reference cultivar. In 2001/2002, the experiment included only cv. Drakkar, with four replicates at each site. No additional *L. maculans* inoculum (e.g. infected debris) was used at the beginning of the growing season. Sowing date and agricultural practices were as close as possible to what was usual in that particular region.

The incidence and severity of phoma leaf spots and stem canker were recorded in 2000/2001. Incidence (i.e. percentage of plants with at least one leaf lesion) and severity of phoma leaf spots were recorded in autumn at 11 sites by observing 60 plants per block (6 row-lengths of 10 plants each). Severity of the leaf spotting was recorded on the same set of plants by placing each plant into one of four classes: 0, no leaf symptom; 1, <5 spots per plant; 2, 5–10 leaf spots per plant; 3, >10 leaf spots per plant. These data were used to calculate a leaf disease index (LDI) according to the formula: $\mathrm{LDI} = \Sigma_i\ (n_i c_i)/N$, where $n_i$ is the number of plants in class $i$, $c_i$ is a correction coefficient score for class $i$ (1 for class 1; 3 for class 2; 5 for

*Table 1*. Characteristics of the OSR genotypes sown at 17 experimental sites across France in 2000/2001

| Cultivar/line | Resistance gene(s) | Provider | Type of cv. | Phoma leaf spot index[a] | Stem canker index[b] |
|---|---|---|---|---|---|
| Drakkar | None | INRA-APBV | Spring | $2.00 \pm 1.50$[c] | nd |
| Surpass 400 | *LepR3* | Advanta seeds | Spring | 1.01 B | nd |
| Pollen | *Rlm4* | Momont Hennette | Winter | 1.24 B | 2.72 D |
| Bristol | *Rlm2*, *Rlm9* | Monsanto SAS | Winter | 1.27 B | 4.53 A |
| Columbus | *Rlm1*, *Rlm3* | Monsanto SAS | Winter | 1.26 B | 2.26 E |
| Vivol | *Rlm1*, *Rlm3* | Monsanto SAS | Winter | 1.12 B | 3.54 C |
| Mohican | *Rlm9* | Rustica prograin génétique | Winter | 1.38 AB | 3.97 B |
| Jet Neuf | *Rlm4* | INRA-PMDV | Winter | 1.32 B | 1.76 F |
| Goéland | *Rlm9* | Momont Hennette | Winter | 1.84 A | 3.21 C |
| 23-1-1 | *Rlm7* | INRA-PMDV | Winter | 0.45 C | 0.58 G |

[a]Values are mean indices (0–5 scale) from 11 experimental sites for 2000–2001, for which complete phoma leaf spot incidence and severity data were available. Cvs followed by the same uppercase letter are not significantly different at the 5% level (Bonferroni test).
[b]Values are mean indices (0–9 scale) from 14 sites with complete stem canker (G2 index) ratings (2000–2001). Cvs followed by the same uppercase letter are not significantly different at the 5% level (Bonferroni test). nd, not determined (spring-type cvs).
[c]Cv. Drakkar (only assessed at a few sites) was not included in the statistical analysis.

class 3), and $N$ is the total number of plants. LDI values therefore ranged between 0 (no disease) and 5 (all plants severely affected). At the Versailles site, this index correlated well with the number of phoma leaf spots ($R^2 = 0.98$, data not shown). Severity of stem canker was assessed in mid-June to early July using the G2 stem canker index, ranging from 0 (no disease) to 9 (all plants lodged) (Pierre and Regnault, 1982; Aubertot et al., 2004).

### *Isolation and culture of* L. maculans

When phoma leaf spot symptoms appeared (between October and December), 25 leaves with at least one leaf lesion each were collected per block of cv. Drakkar, washed, dried, and sent to INRA-PMDV for immediate isolation of *L. maculans*. Single-pycnidium isolates were collected from one lesion per affected leaf (West et al., 2002). All fungal cultures, for sporulation or long-term storage, were processed as described by Ansan-Melayah et al. (1995).

### *The plant differential set*

A differential set, comprising genetically fixed cultivars or lines possessing only a few *Rlm* genes, was used to identify races of *L. maculans* (Balesdent et al., 2005). The differential set consisted of cvs/lines Westar (no R genes, susceptible control), Columbus (*Rlm1*, *Rlm3*), Bristol (*Rlm2*, *Rlm9*), 22-1-1 (*Rlm3*), Jet Neuf (*Rlm4*), 150-2-1 (*B. juncea* line, *Rlm5*, not characterised at the *Rlm9* locus), Falcon-MX (*Rlm4*, *Rlm6*, not characterised at the *Rlm9* locus) or Samouraï-MX (*Rlm1*, *Rlm6*, not characterised at the *Rlm9* locus) or Darmor-MX (*Rlm6*, not characterised at the *Rlm9* locus), 23-1-1 (*Rlm7*), 156-2-1 (*B. rapa* line, *Rlm8*, not characterised at the *Rlm9* locus) and Goéland (*Rlm9*). The commercial cvs Columbus and Bristol were provided by Monsanto SAS (Boissay, France) and cv. Goéland by Momont Hennette (Mons-en-Pévèle, France). Samouraï-MX, Darmor-MX and Falcon-MX were provided by M. Renard (INRA-APBV, Le Rheu, France). All other genotypes were maintained as described by Balesdent et al. (2002).

### *Pathogenicity tests and race terminology*

Isolates were inoculated onto cotyledons of the plant differential set (Balesdent et al., 2001, 2005). Each isolate/line interaction was recorded as compatible (>80% susceptibility symptoms, the isolate is virulent [*avrLm*]) or incompatible (>80% resistance responses, the isolate is avirulent [*AvrLm*]) (Balesdent et al., 2001). Combining the results of all interaction phenotypes for a given isolate on the whole differential set made it possible to identify each Avr allele for all Avr loci. For instance, an isolate which was virulent on the line 22-1-1 (*Rlm3*) but avirulent on Columbus (*Rlm1*, *Rlm3*) was classified as being avirulent at the AvrLm1 locus and virulent at the AvrLm3 locus. The isolates were then classified into races, based on their pattern of avirulence alleles as described previously (Balesdent et al., 2005). For instance, the race

Av1-2-4-7 is composed of isolates possessing the Avr alleles *AvrLm1*, *AvrLm2*, *AvrLm4* and *AvrLm7*. Figures within brackets correspond to Avr loci for which genotyping was not possible.

*Data analysis*

Phoma leaf spot incidence, phoma leaf spot severity, stem canker severity (G2 rating), and frequencies of each Avr allele were analysed by analysis of variance, with the sites and/or the plant genotypes as sources of variation, and blocks as replicates. The plant genotypes or the sites were classified using the Bonferroni test ($\alpha = 0.05$). Statistical analyses were performed using XLStat 7.5 software. Populations (isolates obtained from a given site) were compared for frequency of each avirulent allele and for complexity of the isolates (number of virulences per isolate). In addition, two indices were used to analyse populations diversity (Pinon and Frey, 1997): the Margalef index, which measures the richness in species (here, in races) of a population, and the Simpson index of richness, that also takes into account the evenness of races within each population:

Margalef index:
$D_{Mg} = (S-1)/\log_e N$, with $S$ = number of races and $N$ = total number of isolates, at a site.

Simpson index:
$D_S = \Sigma\ [(n_i(n_i-1))/N(N-1)]$, with $n_i$ = number of isolates of race $i$ and $N$ = number of isolates, at a site. The Simpson diversity index was calculated as $SD = 1-D_S$.

## Results

*Disease severity in the 2000–2001 growing season*

Incidence and severity of phoma leaf spot and stem canker data indicated that *L. maculans* inoculum concentration differed between sites in the 2000–2001 growing season. Incidence (% plants affected) of phoma leaf spot ranged from 12% (Mons) to 99% (Grignon), whereas phoma leaf spot severity ranged from 0.31 (Versailles) to 3.5 (Grignon) on a 0–5 scale. Analysis of variance revealed significant differences between cultivars or lines and between sites for both disease incidence and severity, but no correlation was observed between the variable measured and either the date of assessment or the geographical location (data not shown). For example, the two sites with the lowest and highest values for disease severity were located within 20 km of each other, with symptoms assessed on dates only 15 days apart. For both phoma leaf spot incidence and severity, whatever the experimental site, the line possessing *Rlm7* (23-1-1) was significantly less affected than other cvs (Table 1, and data not shown). Surpass 400 developed some leaf spotting in autumn. Although Surpass 400 had the second lowest leaf disease indices, it did not differ significantly from most winter-type cvs (Table 1).

Stem canker severity ranged from 0.6 to 4.5 on a 0–9 scale, depending on the cv (Table 1). Although the stem canker severity was low by comparison with other seasons, there were significant differences between sites ($F = 66.3$, df = 13, $p < 0.0001$) and between lines ($F = 189.7$, df = 7, $p < 0.0001$). Line 23-1-1 was always the most resistant line, whatever the experimental site (Table 1 and data not shown). Cultivars Jet Neuf and Columbus were usually ranked as the most resistant after 23-1-1, whereas Bristol and Mohican were always ranked as most susceptible (Table 1 and data not shown). Bristol was the most susceptible cv in 8 out of 14 sites. Stem canker resistance of cv Surpass 400 could not be evaluated, as most of the plants of this spring cv. did not survive the winter.

*Establishment of the collection of isolates*

Affected leaves of cv Drakkar collected between October and December 2000 (17 sites), or October and December 2001 (3 sites), were sampled to collect a total of 1988 single-pycnidium isolates. Except for two sites, a total of 73–120 isolates was obtained from each site. Of these 1988 isolates, 1787 were characterised for their pattern of Avr alleles (no more than 100 isolates per site were analysed) (Table 2). Only eight out of the nine Avr loci could be characterised for all sites, due to problems in multiplying the *B. rapa* genotype harbouring *Rlm8* (156-2-1). The occurrence of *AvrLm8* could therefore be fully characterised for only one site (Châteauroux, in the Central area), and some data were obtained for a second site (Le Rheu).

*Table 2*. Characteristics of the race structure of *Leptosphaeria maculans* populations at 20 experimental sites in France

| Site | Region[a] | No. isolates analysed | No. races[b] | Margalef Index | No. virulence alleles per isolate | % isolates avirulent for polymorphic Avr loci | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | *AvrLm1* | *AvrLm4* | *AvrLm5* |
| Chartainvilliers | C | 100 | 7 | 1.30 | 4.76 | 12.00 | 19.00 | 93.00 |
| Chateauroux | C | 93 | 6 | 1.10 | 5.00 | 11.83 | 4.30 | 89.25 |
| Lutz | C | 100 | 8 | 1.52 | 4.73 | 30.00 | 5.00 | 91.00 |
| Oucques | C | 100 | 8 | 1.52 | 4.88 | 20.00 | 11.00 | 76.00 |
| Toury | C | 100 | 8 | 1.52 | 4.75 | 16.00 | 13.00 | 97.00 |
| *Mean for Central region* | | | *7.40* | *1.39* | *4.82* | *17.97* | *10.46* | *89.25* |
| Châlons | E | 89 | 8 | 1.56 | 5.06 | 31.46 | 5.62 | 56.18 |
| Martincourt | E | 75 | 5 | 0.93 | 5.03 | 2.67 | 5.33 | 89.33 |
| *Mean for Eastern region* | | | *6.50* | *1.24* | *5.04* | *17.06* | *5.48* | *72.76* |
| Mons en P. | N | 43 | 4 | 0.80 | 4.72 | 18.60 | 9.30 | 100.00 |
| Prémesques | N | 98 | 8 | 1.53 | 4.79 | 33.67 | 10.20 | 77.55 |
| *Mean for North region* | | | *6.00* | *1.16* | *4.75* | *26.14* | *9.75* | *88.78* |
| Grignon | NC | 100 | 5 | 0.87 | 4.88 | 6.00 | 11.00 | 95.00 |
| Guyancourt | NC | 50 | 6 | 1.28 | 5.08 | 4.00 | 14.00 | 74.00 |
| Verneuil | NC | 100 | 5 | 0.87 | 4.68 | 25.00 | 8.00 | 99.00 |
| Verpilliere | NC | 100 | 7 | 1.30 | 4.88 | 21.00 | 13.00 | 78.00 |
| Versailles | NC | 87 | 6 | 1.12 | 4.84 | 13.79 | 19.54 | 82.76 |
| *Mean for North-Central region* | | | *5.80* | *1.09* | *4.87* | *13.96* | *13.11* | *85.75* |
| La Bénate | W | 100 | 3 | 0.43 | 4.87 | 6.00 | 7.00 | 100.00 |
| Le Rheu | W | 100 | 8 | 1.52 | 4.62 | 39.00 | 8.00 | 91.00 |
| Les Alleuds | W | 73 | 6 | 1.16 | 4.85 | 27.40 | 6.85 | 80.82 |
| *Mean for Western region* | | | *5.67* | *1.04* | *4.78* | *24.13* | *7.28* | *90.61* |
| Caussade | S | 79 | 4 | 0.69 | 5.04 | 22.78 | 0.00 | 73.42 |
| Mondonville | S | 100 | 5 | 0.87 | 4.87 | 21.00 | 2.00 | 90.00 |
| Nérac | S | 100 | 4 | 0.65 | 5.37 | 20.00 | 0.00 | 43.00 |
| *Mean for South region* | | | *4.33* | *0.74* | *5.09* | *21.27* | *0.67* | *68.81* |
| *Mean for all sites*[c] | | | | *1.13* (±0.16) | *4.88* (±0.52) | *19.1* (±4.79) | *8.6* (±2.54) | *83.8* (±6.89) |

[a]C, central region; E, eastern region; NC, north central region; W, western region; S, south region; for a guide to the location of the sites, see Figure 1.
[b]The number of races is calculated on the basis of Avr combinations for *AvrLm1–AvrLm7* and *AvrLm9*; data for *AvrLm8* are excluded because there were too few data available at this locus.
[c]Values between parentheses indicate the confidence interval at a 5 % level (Student test).

*Avirulence alleles occurring in France*

Two avirulence alleles, *AvrLm6* and *AvrLm7*, were present in more than 99% of the French *L. maculans* population (Figure 2). Only one isolate with *avrLm6* and one isolate with *avrLm7* were identified out of the total of 1787 isolates. These two isolates came from an experimental field located close to a stem canker nursery at one of the oldest OSR breeding sites in France. Two virulence alleles were fixed in the populations, as all isolates were virulent at the AvrLm2 and AvrLm9 loci. Only three isolates, from three different sites, were avirulent at the AvrLm3 locus (Figure 2). *AvrLm1* and *AvrLm4* avirulence alleles were harboured by a small proportion of the population (19.6 and 8.6%, respectively). In contrast, the majority of the isolates were avirulent at the AvrLm5 and AvrLm8 loci (83.8 and 68.4%, respectively) (Figure 2).

*Regional differences for individual avirulence alleles*

Site to site differences were observed for all three avirulence alleles which were polymorphic in the French population, i.e. *AvrLm1*, *AvrLm4* and *AvrLm5*, as *AvrLm8* could not be investigated nationally (Table 2). Depending on the site, *AvrLm1* was present in 2.7–39% of the isolates, with significant differences between sites ($F = 6.54$, $p < 0.0001$). This Avr allele was present at high frequencies in the Northern and Western areas where it was present at frequencies of up to 39% of the isolates. Regions where OSR is not one of the

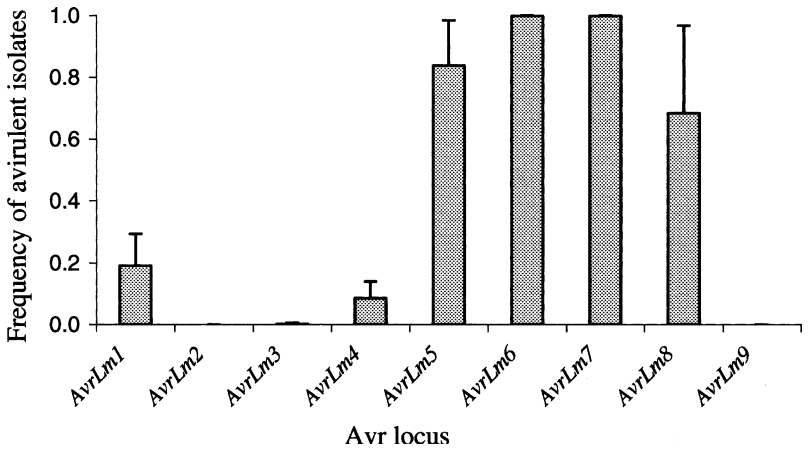


*Figure 2.* Frequency of avirulent (*AvrLm*) alleles for the nine Avr loci AvrLm1–AvrLm9 for isolates of *L. maculans* sampled at 20 experimental sites across France. Values are means over the 20 sites in Figure 1. Vertical lines are the standard deviation. A total of 1787 *Leptosphaeria maculans* isolates were analysed at each locus, except for *AvrLm8* (129 isolates).

main crops (Southern, Western and Northern areas, Figure 1) had the highest frequencies of isolates harbouring *AvrLm1* (Table 2). Within the main OSR growing areas, there were large differences between individual sites, with a few sites where the *AvrLm1* allele was almost absent (e.g. Martincourt in the Eastern area) (Table 2). Significant differences between sites ($F = 7.283$, $p < 0.0001$) were also observed within a region. For example, for the seven sites within a 100-km radius south of Paris (i.e. Guyancourt, Versailles, Grignon, Verneuil, Lutz, Toury and Chartainvilliers; Figure 1), the frequency of *AvrLm1* allele in the populations ranged between 4 and 30%. Due to this variation between sites within a region, no significant differences between regions were observed in frequency of *AvrLm1* ($F = 0.569$, $p = 0.723$).

Significant differences between sites were also observed for *AvrLm4* ($F = 3.225$, $p < 0.0001$). Depending on the site, the *AvrLm4* allele was present in 0–19.5% of the isolates (Table 2). The range of variation between sites within a region was small. In the Southern area, the allele was rare, and it was absent from two sites. In the North-Central area and three sites located in the North of the Central area (Oucques, Toury and Chartainvilliers) the frequency was >10% of the population (Table 2). A significant difference ($p = 0.0012$) between these populations and those from the Southern region was observed. The frequency of *AvrLm5* varied between sites, ranging from 43 to 100%, with significant differences between individual sites ($F = 8.1$, $p < 0.0001$), even when comparing the seven neighbouring sites around Paris ($F = 3.831$, $p = 0.01$). Consequently no evidence for regional differences was observed ($F = 1.255$, $p = 0.336$).

### *Race structure at the national and regional scales*

As most *AvrLm8* data were missing, race structure was analysed only for the combination of eight Avr genes; *AvrLm1–AvrLm7* and *AvrLm9*. Since only six of these eight genes were polymorphic in the current study, 64 (i.e. $2^6$) possible combinations of Avr alleles could be expected to occur in the collection of isolates. However, only 11 distinct races of *L. maculans* were identified (Table 3). This small number of races is, however, consistent with the fact that, for three Avr loci (i.e. AvrLm3, AvrLm6 and AvrLm7), one of the two alleles was at a very low frequency. Considering only the three loci with a frequency >5% for each allele (i.e. *AvrLm1*, *AvrLm4* and *AvrLm5*), all possible combinations of the avirulent and virulent alleles were identified in the collection. Only two races, Av5-6-7-(8) and Av1-5-6-7-(8), were found at all 20 sites sampled, and these two races represented more than 75% of the whole population (Table 3). In contrast, four races were each at a frequency <1%.

*Table 3*. *Leptosphaeria maculans* races identified in the large-scale French survey of experimental OSR sites

| Race[a] | Frequency (%) | No. sites[b] |
|---|---|---|
| Av5-6-7-(8) | 64.52 | 20 |
| Av1-5-6-7-(8) | 13.20 | 20 |
| Av6-7-(8) | 8.11 | 16 |
| Avl-6-7-(8) | 5.20 | 16 |
| Av4-5-6-7-(8) | 5.15 | 18 |
| Av4-6-7-(8) | 2.52 | 13 |
| Av1-4-5-6-7-(8) | 0.56 | 9 |
| Avl-4-6-7-(8) | 0.50 | 5 |
| Avl-3-5-6-7-(8) | 0.11 | 2 |
| Av3-5-6-(8) | 0.05 | 1 |
| Av5-7-(8) | 0.05 | 1 |

[a]Race nomenclature according to Balesdent et al. (2005): the figures indicate the Avr loci for which the isolate is avirulent. Figures between brackets indicate that the corresponding Avr locus has not been characterised.
[b]Number of sites where the race was found.

The number of races identified at each individual site ranged from 3 to 8 (Table 2). No significant differences between regions were observed for the richness in races, as estimated by the Margalef index ($F = 1.716$, $p = 0.196$) or the Simpson diversity index ($F = 0.09$, $p = 0.993$), or for the number of virulences per isolate ($F = 2.444$, $p = 0.086$). In contrast, sites from the same region showed both simple and complex race structures (Table 2, Figure 3). The lowest richness (Margalef Index) and the greatest number of virulences per isolate were both detected at the three sites in the south of France, where there was a low frequency of *AvrLm4* isolates.

*Race structure and disease severity*

The multi-site assessment of *B. napus* cultivars or lines for resistance to stem canker included a range of *B. napus* cvs differing in their R gene composition. There was a correlation between the good resistance of the *Rlm7* line (always ranked as "least diseased" or even immune) and the absence of isolates virulent for this R gene in the population. To correlate the frequencies of *AvrLm1* and *AvrLm4* and the stem canker severity at harvest, but take into account differences between sites in the severity of epidemics, the mean G2 ratings were first converted into a "relative G2 index" for each site and each cv. This relative index was calculated by normalising the G2 rating against that of the most susceptible cv. (i.e. Bristol). Bristol was also chosen as the reference cultivar because it did not contain resistance genes that could recognise any isolate in the *L. maculans* populations. This "relative G2 index" was correlated to the frequency of *AvrLm4* in populations for one cv. with *Rlm4* (Pollen, $R^2 = 0.523$, df = 13, $p < 0.05$) but not for Jet Neuf (also with *Rlm4*, $R^2 = 0.028$, NS) (Figure 4). In contrast, no correlation could be identified between the frequency of *AvrLm1* and the severity of stem canker on *Rlm1* cvs (either Columbus or Vivol, data not shown).

## Discussion

In this paper, we report data from a large-scale collection of French *L. maculans* isolates, that

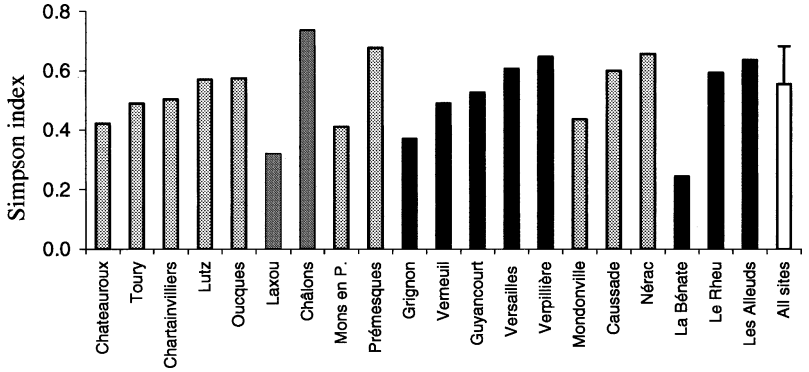


*Figure 3*. Values of Simpson indices of richness for isolates of *L. maculans* sampled at 20 experimental sites across France. Sites are ordered by region (alternate grey bars or black bars) as described in Table 2. White bar, mean over all sites + standard deviation.

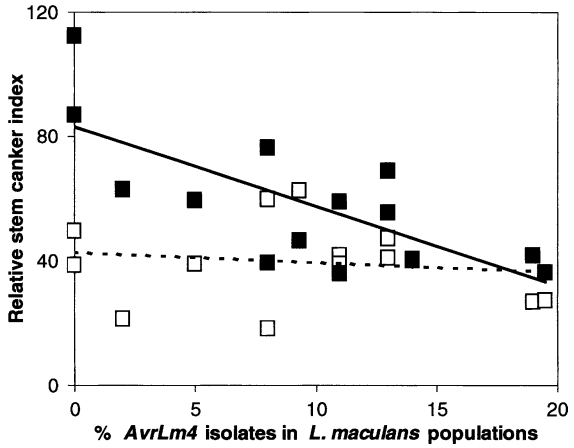


*Figure 4*. Relationship between the frequency (%) of *AvrLm4* isolates and the relative stem canker index on two cvs with the corresponding resistance gene *Rlm4*, Pollen (■) and Jet Neuf (□) at 14 sites across France. Regression lines: Pollen (—), Jet Neuf (- - - -). Stem canker index was evaluated using the G2 index (0, healthy, 9, severely diseased) and the data were then expressed as a percentage of the G2 index of Bristol, the most susceptible cv over all sites.

were characterised at eight Avr loci. Studies that aim to analyse the race structure or pathogenicity grouping of *L. maculans* populations often lack information on the host genotypes from which isolates originate or the R genes they harbour is often not known (Koch et al., 1991; Kutcher et al., 1993; Mahuku et al., 1997; Pongam et al., 1999; Barrins et al., 2004; Balesdent et al., 2005). In the present study, a spring-type cultivar (Drakkar) that was characterised for its lack of any known resistance gene was chosen as a trap cultivar on which ascospores from any race of *L. maculans* could produce a leaf lesion. Previous work had demonstrated that winter-type cvs harboured R genes more frequently than spring-type cvs (Rouxel et al., 2003b). Additional screening of more than 60 French commercial winter-type OSR cvs demonstrated that they all possess at least one gene for resistance to *L. maculans* (Balesdent and Pinochet, unpublished data). Therefore, although spring cvs are never sown in autumn under French climatic conditions, only a spring-type cv sown alongside the winter-type cvs could be used to trap *L. maculans* isolates without introducing a sampling bias. Leaf disease indexes measured on both Drakkar and winter-type cvs during autumn 2000 (Table 1) indicated that Drakkar was effectively infected by *L. maculans* in autumn, in a proportion similar to that of winter-type cvs. Leaves were sampled within a few days after the first phoma leaf spots were visible, to ensure that the collection was representative of the initial release of ascospores deposited on a given crop. Molecular analyses of a subset of this collection of isolates from three sites, and of isolates originating from different lesions on the same leaves, demonstrated that nearly all these isolates possessed distinct haplotypes and therefore did not result from a secondary cycle of conidial infection (L. Gout, pers. comm.).

In the present study, 1787 isolates originating from 20 fields were genotyped at 8–9 Avr loci. Only such a large-scale analysis could allow us to identify some rare alleles or races. For instance, there was only one isolate identified that could overwhelm either of the new resistance genes *Rlm6* or *Rlm7*. This information is relevant to the future management of these resistance sources in commercial cvs, as (i) these two R sources are effective against the current French *L. maculans* populations, since nearly 100% of the population is avirulent at these two loci, but (ii) the virulent alleles are already present, albeit at a very low frequency, in natural populations, even before the resistances have been introduced commercially.

One of the objectives of this work was to investigate the relationships between the frequency of a given Avr allele in a population and the effectiveness of the corresponding R gene for control of stem canker. This objective was only partly achieved, for two reasons. Firstly, the multi-site analysis of the relationships between phoma leaf spot incidence and severity in autumn, stem canker severity at harvest, frequencies of each Avr allele in the local pathogen population and susceptibility of cultivars was confounded by many uncontrolled factors that differed from one site to another, such as sowing date, time at which phoma leaf spot was assessed, accuracy of the phoma leaf spot assessment, along with epidemics which were not severe at some sites or loss of one experimental trial. Secondly, only extreme values were obtained for the frequencies of all Avr alleles, whatever the sites analysed, with either no or little polymorphism, or polymorphism within a limited range of values of Avr frequencies, such as 0–19% of *AvrLm4* isolates, or 2.7–39% of *AvrLm1* isolates. Nevertheless, a positive correlation was observed between the frequency of *AvrLm4* isolates and the

severity of stem canker on cv. Pollen, possessing *Rlm4*. There may have been no such correlation for cv. Jet Neuf (also possessing *Rlm4*) because it possesses a high level of polygenic, general resistance, which explains why the severity of stem canker was less on Jet Neuf than all other cvs except the *Rlm7* line in the field experiment. In such a genetic background, the role of a specific, major R gene facing only a small proportion of the pathogen population (maximum 20% of the isolates) may be insignificant.

All field experiments demonstrated the complete resistance of *Rlm7*, which resulted in little leaf spotting or stem canker development at all experimental sites. This result clearly correlates with the very high frequency of *AvrLm7* isolates, and demonstrates, for a second pair of *AvrLm*/*Rlm* gene pairs, that a specific resistance expressed at the leaf stage is sufficient to control this monocyclic disease when the corresponding avirulence allele is prevalent in the local population. The first evidence of such a correlation was found for the gene pair *AvrLm1*/*Rlm1* (Ansan-Melayah et al., 1997), with *Rlm1* effectively controlling stem canker in France at a time when the frequency of *AvrLm1* in *L. maculans* populations was >80%. However, the commercial success of *Rlm1* rapidly provoked a shift in the population (Rouxel et al., 2003a), confirmed during the present study, with the frequency of *AvrLm1* <20% nationally in 2000 and 2001. Knowing that commercial cultivars harbouring *Rlm7* have recently been released in France (Pinochet et al., 2004) and that virulent isolates are already present at a low, but detectable, frequency, a new selection pressure will be exerted in favour of this virulent population. One can expect a shift in the frequency of *AvrLm7*, accompanied by a progressive loss in effectiveness of this resistance source, over the next few years, except if a significant fitness cost is associated with the gain of virulence to *Rlm7*, which is not yet known. Although the frequency of *avrLm1* was much higher, when *Rlm1* was released on a significant area (4.9% isolates *avrLm1* in 1994, Rouxel et al., 2003a), than that observed now for *avrLm7* isolates (<0.1% isolates *avrLm7* in 2000–2001), the *Rlm7* gene will have to be managed carefully through information to advisory bodies and breeding companies, surveys of virulence in *L. maculans* populations and close monitoring of the resistance of *Rlm7*-cultivars. Even though the appropriate durable resistance management strategy is not yet known, advisory bodies like CETIOM have started to recommend a diversification in use of the specific resistance genes (Pinochet et al., 2004; http://www.cetiom.fr/CTMSite/page/technique/sommairef.htm).

Taking into account the eight Avr loci for which complete information was available, only 11 distinct races of *L. maculans* were identified in the large-scale survey. This relatively low diversity is explained by the fact that only three Avr alleles, *AvrLm1*, *AvrLm4* and *AvrLm5*, displayed a significant level of polymorphism. That all possible combinations of alleles at these three loci were identified in the collection supports the conclusion that the three loci are genetically independent (Balesdent et al., 2002) and the annual occurrence of genetic recombination in the life cycle of *L. maculans*. For example, the frequency of isolates with *AvrLm1* or *AvrLm4* in the whole population was 19.5 or 8.5%, respectively. It is noteworthy that the observed frequency of races combining these two Avr alleles (i.e. races Av1-4-5-6-7-(8) plus race Av1-4-6-7-(8), 1.06% in total, Table 3) is not very different from the calculated probability of finding these two Avr alleles in the same isolate (i.e. frequency of *AvrLm1* multiplied by frequency of *AvrLm4* = 1.65%). Similarly, the observed frequency of races combining *AvrLm4* and *AvrLm5* (5.71%, Table 3) is close to the calculated probability of finding them in the same isolate (7.21%). This illustrates the importance of recombination in this fungus and questions the efficiency of R-gene pyramiding as a breeding strategy.

In spite of the limited diversity in *L. maculans* races at the national scale, this large-scale survey revealed considerable variability between neighbouring sites for different parameters assessed (i.e. disease severity, frequency of a given Avr allele, or richness in races). For example, the two sites in the North region which were only 35 km apart differed greatly in the richness in races; the Margalef index value was high at Prémesques (1.53) and differed significantly from that at Mons-en Pévèle (0.8). The site with the lowest richness, La Benate (Margalef index = 0.43, only three races identified) was in a region of intensive OSR cultivation. Similarly, the large differences in *AvrLm1* frequencies between sites (i.e. from 2.7 to 39% isolates avirulent) may illustrate different stages in

the evolution of *L. maculans* population, linked to differences in selection pressure from site to site. For example, the site with the highest frequency of *AvrLm1* (Le Rheu) is located in a region where OSR is not a major crop, whereas sites with very low frequencies of *AvrLm1* are often in regions of intensive OSR cropping (Grignon, Martincourt, La Benate). Finally, the race which was most frequent, Av5-6-7-(8) (64.5% of the population) is representative of what would be expected following selection by the sequential use of R genes *Rlm2*, *Rlm4* and *Rlm1* in France (Rouxel et al., 2003b), along with the very frequent occurrence of *Rlm9* in winter OSR cvs. (Balesdent et al., 2005; Balesdent and Pinochet, unpublished data). All these data suggest that, for Avr loci, the structure of populations of *L. maculans* may be determined by the local cropping history of OSR with different R genes, in spite of the lack of population structure revealed by neutral molecular markers (L. Gout, pers. comm.). Analyses of *L. maculans* populations with AFLP markers showed not only a high level of genetic diversity within a field but also significant, although lower, differences in isolate diversity between fields, both in Canada (Mahuku et al., 1997), and Australia (Barrins et al., 2004). Our study revealed that a significant proportion of the population was virulent at the AvrLm5 and AvrLm8 loci, although *Rlm5* and *Rlm8* have never been used in commercial OSR crops. This suggests that these R sources may be present in some *B. rapa*, *B. juncea* or other mustard cultivars, used on a small scale as green manure or vegetable crops, which could have selected for the virulent alleles. Also, the more ancient agricultural use of *B. rapa* and *B. juncea*, compared to the recent history of OSR cropping, may account for the presence of these virulence alleles in the population present now on OSR.

A preliminary characterisation of *L. maculans* avirulence genes in French populations was previously done through the characterisation of the isolates in the IBCN collection (Balesdent et al., 2005). Although based on a very limited number of isolates (only 13 French isolates mostly sampled in 1990 and 1992), some of the conclusions based on this limited sample were consistent with the large-scale survey done during the current study. Both the high frequency of *AvrLm6* and *AvrLm7* (100% each in the IBCN French isolates) and the high frequencies of *avrLm2*, *avrLm3* and *avrLm9* (92% for *avrLm2*, 100% for *avrLm3* and *avrLm9* in the French IBCN isolates) were confirmed by the present study. The analysis of collections of intermediate sample size (i.e. 100–200 isolates) can allow a first description of the race structure in a given country, should the information be completely lacking. Such a survey is currently being done in additional European countries (Stachowiak et al., 2004, 2006) and in Australia (M. Barbetti, pers. comm.). In these studies, the use of the sampling procedure and host plant differentials described here will facilitate the comparison of *L. maculans* population structures in different countries or continents. In addition, starting from the very detailed analysis of the *L. maculans* French population in 2000–2001 described in the current study, it also seems feasible, with a reduced work load, to up-date the information on an annual basis by analysing around 100 isolates from 2 to 3 sites in turn. The collection of isolates gathered and characterised during such studies will be a useful resource for further population genetic analysis, or for the validation of molecular markers for a given race or virulent allele (Attard et al., 2002; L. Gout, pers comm.). Such molecular markers will greatly facilitate future population surveys by combining the use of trap cultivars with the possibility to extract DNA from individual leaf lesions, as developed for large-scale PCR analysis of the *L. maculans* mini-satellite *MinLm1* (Attard et al., 2001).

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