Genetics

Tetrad Analysis of Acid Phosphatase, Soluble Protein Patterns, and Mating Type in *Leptosphaeria maculans*

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ABSTRACT

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We investigated the utility of mating type, soluble protein profiles obtained after isoelectric focusing, and acid phosphatase (ACP) patterns as genetic markers for the blackleg fungus, *Leptosphaeria maculans*. Fertile matings were obtained in vitro by pairing single-ascospore isolates. A method for dissecting asci and recovering the eight component ascospores was developed that allowed tetrad analysis. Protein electrophoresis revealed limited polymorphism among the 73 single-ascospore field isolates. Only one isolate (H5) displayed a different soluble protein profile (SPP), termed SPP$^-$, characterized by one missing band and one additional band. We found three ACP patterns in our collection. Protein profiles and ACP patterns were mitotically and meiotically stable. A 4:4 segregation ratio in asci was obtained for mating type, SPP, and ACP patterns, suggesting that each marker was controlled by a single gene. Mating type and ACP genes seemed to be independent. The reliability of the crossing protocol and the Mendelian segregation of the markers following in vitro meiosis mean that this fungus is now amenable to genetic analysis of pathogenicity.

Additional keywords: *Brassica napus*, in vitro sexual mating, *Phoma lingam*.

Leptosphaeria maculans (Desmaz.) Ces. & De Not. (anamorph *Phoma lingam* (Tode:Fr.) Desmaz.) is a filamentous ascomycete that causes blackleg of crucifers. The fungus is present throughout the *Brassica*-growing areas of the world (6). The sexual stage is of vital importance in the disease epidemiology, since primary inoculum mainly consists of sexual spores (ascospores) (5) that can be spread over several kilometers (1,21). The sexual stage of the life cycle takes place on crucifer stubbles where numerous sexual fruiting bodies, termed pseudothecia, are produced.

L. maculans isolates belonging to the "virulent" group can be differentiated on a set of *Brassica napus* L. cultivars as a function of their pathogenicity grouping (14). Genetic analysis of *B. napus* resistance to *L. maculans* suggested that a gene-for-gene interaction occurs in this plant-pathogen system (25). Moreover, the importance of the sexual stage of *L. maculans* suggests that recombination of pathogenicity genes may occur at high frequency under field conditions. Several investigators have tried to genetically analyze the fungus under laboratory conditions and have succeeded in obtaining the sexual stage in vitro (3,8,11,22,28). However, only recently a reliable method allowing a reproducible obtainment of fertile crosses in vitro has been developed (15).

Asci of *L. maculans* contain eight ascospores, which can be recovered as a complete tetrad. The eight ascospores represent the four meiotic products followed by a mitotic division. Cultures derived from ascospores are haploid; therefore, direct inference of genotypes from phenotypes and direct observation of the segregation of characters can be obtained by analyzing tetrads. Two examples of tetrad analysis in *L. maculans* were previously described using pseudothecia isolated from diseased stubbles in the field. Boudart (3) determined the mating type of four complete tetrads and Plummer and Howlett (23) determined the electrophoretic karyotypes of two tetrads.

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As a prerequisite for genetic analysis of pathogenicity following in vitro crosses, reliable genetic markers are needed. Isozyme banding patterns (for a review see 16) and genes determining mating type frequently have been used to examine segregation among ascospore progeny. This paper considers three potential markers: mating type, a specific soluble protein pattern following isoelectric focusing (IEF), and acid phosphatase (ACP) electrophoretic types. The objectives of our study were to: 1) develop methods allowing tetrad recovery in *L. maculans* and 2) establish the genetic control of the markers using in vitro crosses and tetrad analysis.

MATERIALS AND METHODS

Fungal strains and culture maintenance. *Random single-ascospore field isolates.* Field pseudothecia from two locations in France were collected from *B. napus* stubbles. Single-ascospore isolates were dissected according to Koch et al (9). Single pseudothecia were washed in tap water, rinsed twice in sterile water, then dried with filter paper and fixed onto the inside of the lid of a petri plate inverted over 2% water agar. Single ascospores ejected from pseudothecia were examined with a microscope and transferred to V8 agar (17) plates. Other single-ascospore isolates were provided to us by H. Brun, P. H. Williams, and H. H. Hoppe (Table 1).

Tetrads sampled from stubbles obtained in the field. A few tetrads from asci recovered from field pseudothecia (Table 1) were dissected according to the protocol described below.

Single-conidium isolates. To verify the mitotic stability of the markers, 10–20 single-conidium isolates were obtained from a few selected isolates. Conidia suspensions (10^3 to 10^4 conidia ml^{-1}), in 100-μl amounts, were plated on 2% water agar containing 200 μg ml^{-1} of streptomycin and 125 μg ml^{-1} of penicillin (WA-AB). Petri plates were maintained 4 days at room temperature, and single germinating spores were transferred to V8 agar.

TABLE 1. Geographic origin and phenotypes of isolates used in this study

Isolate number	Geographic origin[a]	Provider	Mating type	ACP pattern[b]	SPP pattern[c]
Single-ascospore isolates					
A1	France (35)	C. Gall (1990)	−	2	+
A2	France (35)	C. Gall (1990)	−	2	+
A3	France (35)	C. Gall (1990)	−	2	+
C1	France (35)	C. Gall (1990)	+	2	+
C2	France (35)	C. Gall (1990)	+	2	+
C5	France (35)	C. Gall (1990)	−	2	+
C7	France (35)	C. Gall (1990)	+	2	+
D1	France (77)	C. Gall (1990)	−	1	+
D2	France (77)	C. Gall (1990)	−	1	+
D3	France (77)	C. Gall (1990)	−	1	+
D4	France (77)	C. Gall (1990)	+	2	+
E1	France (77)	C. Gall (1990)	−	2	+
E2	France (77)	C. Gall (1990)	+	2	+
E3	France (77)	C. Gall (1990)	+	2	+
E4	France (77)	C. Gall (1990)	−	2	+
F1	France (77)	C. Gall (1990)	−	2	+
F2	France (77)	C. Gall (1990)	+	2	+
F3	France (77)	C. Gall (1990)	+	2	+
F4	France (77)	C. Gall (1990)	+	2	+
H1	France (77)	C. Gall (1990)	+	2	+
H2	France (77)	C. Gall (1990)	+	2	+
H3	France (77)	C. Gall (1990)	+	2	+
H4	France (77)	C. Gall (1990)	+	2	+
H5	France (77)	C. Gall (1990)	−	2	−
1243	France	P. H. Williams	+	2	+
1245	France	P. H. Williams	+	2	+
1276	Western Australia	P. H. Williams	−	2	+
1275	Western Australia	P. H. Williams	+	2	+
IIa1	Germany	H. H. Hoppe	ND[d]	2	+
HB759	France (57)	H. Brun (1990)	ND	2	+
HB789	France (45)	H. Brun (1990)	−	2	+
HB801	France (55)	H. Brun (1990)	+	2	+
HB813	France (35)	H. Brun (1990)	+	2	+
HB826	France (31)	H. Brun (1990)	+	2	+
HB827	France (31)	H. Brun (1990)	+	2	+
HB829	France (55)	H. Brun (1990)	ND	2	+
HB290	France (35)	H. Brun (1985)	−	2	+
Field tetrads					
a.1	France	C. Gall (1992)	+	3	+
a.2	France	C. Gall (1992)	+	2	+
a.4	France	C. Gall (1992)	−	2	+
a.5	France	C. Gall (1992)	−	3	+
a.6	France	C. Gall (1992)	+	2	+
a.8	France	C. Gall (1992)	−	3	+
b.1	France	C. Gall (1992)	+	2	+
b.2	France	C. Gall (1992)	+	3	+
b.3	France	C. Gall (1992)	+	3	+
b.4	France	C. Gall (1992)	+	2	+
b.5	France	C. Gall (1992)	−	2	+
f.1	France	C. Gall (1992)	+	2	+
f.2	France	C. Gall (1992)	+	2	+
f.3	France	C. Gall (1992)	+	2	+
f.6	France	C. Gall (1992)	−	2	+
f.7	France	C. Gall (1992)	−	2	+
f.8	France	C. Gall (1992)	−	2	+
g.1	France	C. Gall (1992)	+	2	+
g.2	France	C. Gall (1992)	−	2	+
g.3	France	C. Gall (1992)	+	2	+
g.5	France	C. Gall (1992)	−	2	+
g.6	France	C. Gall (1992)	−	2	+
g.7	France	C. Gall (1992)	+	2	+
i.1	France	C. Gall (1992)	+	2	+
i.2	France	C. Gall (1992)	−	2	+
i.3	France	C. Gall (1992)	−	2	+
i.4	France	C. Gall (1992)	+	2	+
i.5	France	C. Gall (1992)	+	2	+
i.7	France	C. Gall (1992)	−	2	+
j.1	France	C. Gall (1992)	+	2	+
j.2	France	C. Gall (1992)	+	2	+
j.3	France	C. Gall (1992)	+	2	+
j.4	France	C. Gall (1992)	+	2	+
j.5	France	C. Gall (1992)	−	2	+
j.6	France	C. Gall (1992)	−	2	+
j.7	France	C. Gall (1992)	−	2	+

[a] Number in parentheses indicates French department: 35 = West, 31 = South, 55 and 57 = East, and 77 and 45 = Center.
[b] Electrophoretic type revealed after isoelectric focusing.
[c] + = SPP^+ phenotype, − = SPP^- phenotype.
[d] ND = not done.

Maintenance of cultures. Cultures were maintained on V8-agar medium as described previously (27).

In vitro crossing and mating type determination. Crosses were made by pairing single-ascospore isolates according to Mengistu et al (15) with the following modifications. Isolates were paired on V8 agar and maintained at 20 C under continuous fluorescent light for 7 days. Cultures were then flooded with 1.5% water agar cooled at 50 C. Cultures were maintained at 16 ± 1 C under black light (Osram 38W tubes) with a 12-h photoperiod. Under these conditions, pseudothecia were obtained 4–5 wk after the pairing. For each cross, some pseudothecia were crushed and observed microscopically to confirm that they contained mature asci and ascospores. The mating type (Mat) of our collection of field isolates was determined in crosses with two mating type testers, 1275 (Mat^+) and 1276 (Mat^-), and three selected isolates, E1 (Mat^-), F2 (Mat^+), and F1 (Mat^-) (Table 1). When determining the mating type of isolates belonging to a tetrad, all isolates within the tetrad were paired in all combinations. Moreover, each isolate was paired with four isolates, g.1 and g.7 (Mat^+) and g.2 and g.5 (Mat^-), selected from our collection for their fertility. Pairings were performed in duplicate, with the repeats kept at either 17 or 25 cm from the lights.

Dissection of asci and tetrad recovery. The pseudothecia produced in vitro were viewed with a binocular microscope (15×) and collected with a sterile needle. Care was taken to select pseudothecia that were isolated from surrounding pycnidia, to avoid contamination with parental conidia (vegetative spores). The ascospores in each ascus were separated from each other by micromanipulation with the "oil chamber" technique (7). Pseudothecia were crushed with a sterile blade on a sterile microscope slide and covered with 1 μl of sterile water. Ten 0.8-μl water droplets were deposited on the slide, along with two 1.2-μl droplets of 1% Helicase (IBF) in water (sterilized by filtration through a 0.22-μm low-protein binding Millipore filter). The slide was rapidly covered with sterile paraffin oil and turned upside down over the oil chamber filled with sterile paraffin oil. A De Fonbrune microforge was used to produce glass microtools (mainly loops). Mature asci were selected and transferred to the enzyme solution by means of the De Fonbrune hydraulic micromanipulator. After the ascus wall dissolved, the ascospores were transferred twice in water droplets to remove enzyme solution. Finally, the ascospores were lassoed individually with a microloop and transferred to a microscope slide covered with WA-AB. All micromanipulation steps were performed under 30 or 100× magnification. Ascospores were allowed to germinate overnight under saturant humidity. Germinated ascospores were transferred separately to V8 agar plates containing the same antibiotics. As an alternative, ascospores were allowed to germinate for a few hours in the water droplets, within the oil chamber prior to the transfer to V8 agar. In many cases, incomplete tetrads were obtained, due to loss of ascospores during the micromanipulation and/or poor germination.

Isolate identification. Random isolates were assigned a capital letter and a number (Table 1). All isolates sharing the same letter were isolated from the same stubble. Tetrads were assigned lowercase letters and isolates within a tetrad shared the same letter (Tables 1 and 2). Within a tetrad, isolates could usually not be recovered as an ordered set; therefore, they were identified by a randomly assigned number (Table 1).

Protein extraction. Static cultures were incubated for 10 days in modified Fries liquid medium as described by Balesdent et al (2). Mycelia were harvested by filtration, rinsed with deionized water, frozen at −20 C, and then freeze-dried. Proteins were extracted by grinding freeze-dried mycelia in TE (10 mM Tris HCl, pH7; 1 mM EDTA) as described previously (2). The amount of protein was estimated by the method of Bradford (4) and extracts were stored at −80 C. Before electrophoresis, samples were centrifuged for 5 min at 4 C and 10,000 g (r_{av} 80 mm).

IEF of soluble proteins. Proteins were separated on 4-6.5 PhastGels using PhastSystem (Pharmacia-LKB) as previously described (2), except that protein concentration was adjusted to 1.5 mg ml^{-1} in TE. Silver nitrate staining was performed according to the PhastSystem Development Technique File No. 210 (1986) with the following modifications: 1) Gels were fixed for 5 min in 20% trichloracetic acid at room temperature immediately following electrophoresis; 2) for sensitization of the proteins, the glutaraldehyde concentration was 2.5% instead of 8.3%; 3) the silver nitrate concentration was 0.25% instead of 0.5%; and 4) the developer contained 0.015% formaldehyde.

TABLE 2. Characterization of the progeny of the H5 (Mat^-, SPP^-, ET2) × a.2 (Mat^+, SSP^+, ET2) cross[a]

Tetrad type / Isolate number	Mating type	Protein marker[b]	ACP pattern[c]	Twin ascospores[d]
Tetratype				
v.1	−	−	2	a
v.2	−	+	2	b
v.3	+	+	2	c
v.4	−	+	2	b
v.5	+	+	2	c
v.6	+	−	2	d
v.7	+	−	2	d
v.8	−	−	2	a
Parental ditype				
w.1	−	−	2	?
w.2	+	+	2	?
w.3	−	−	2	?
w.4	+	+	2	?
w.5	+	+	2	?
w.6	−	−	2	?
w.7	+	+	2	?
w.8	−	−	2	?
Parental ditype				
s.1	−	−	2	?
s.2	−	−	2	?
s.3	+	+	2	?
s.4	−	−	2	?
s.5	−	−	2	?
s.6	+	+	2	?
Tetratype				
t.1	−	+	2	a
t.2	+	−	2	b
t.4	−	−	2	c
t.5	+	+	2	d
t.6	+	+	2	d

[a] Isolates with the same letter originated from the same ascus. The four tetrads were isolated from a single pseudothecium.
[b] + = SPP^+ phenotype, − = SPP^- phenotype.
[c] Electrophoretic type revealed after isoelectric focusing.
[d] The same letter is given to twin ascospores within a tetrad as determined by the combination of mating type and SPP phenotypes. ? = Twin ascospores could not be identified using the two markers.

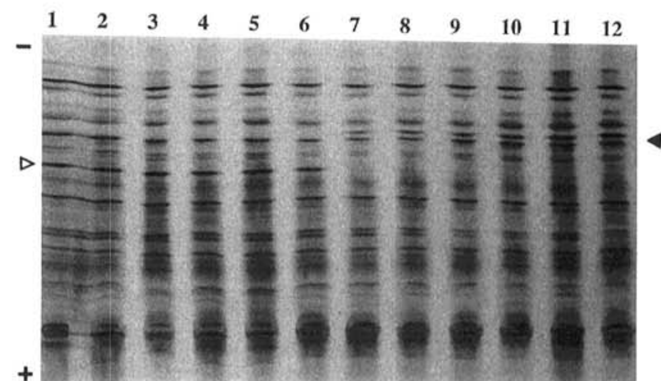

Fig. 1. Soluble protein profiles of *Leptosphaeria maculans* following isoelectric focusing and silver nitrate staining. Lanes 1–6 are single-conidium cultures derived from the single-ascospore isolate w.4, and lanes 7–12 are single-conidium cultures derived from the single-ascospore isolate w.3. The black arrow indicates the additional band characterizing the SPP^- phenotype, and the white arrow indicates the band missing in the SPP^- phenotype.

Electrophoresis and detection of acid phosphatase. ACP isozymes were resolved on 2.5–6.5 pH gradient gels. The gels (245 × 110 × 0.5 mm) contained 5% (w/v) acrylamide, including 3% bis-acrylamide; 12% (v/v) glycerol; 3.125% (v/v) Pharmalytes 4-6.5; and 3.125% (v/v) Pharmalytes 2.5-5. Samples (8 μl) of crude extracts were loaded at the anode using a 52 × 20 μl applicator strip. The electrophoresis took place at 25W constant power, the voltage limit was 2,000 V, and the intensity limit was 25 mA. Samples were loaded following a 300-AVh prefocusing step, and runs were stopped after 3,000 AVh. Gels were stained for enzyme activity according to Roux and Roux (26).

RESULTS

Occurrence of markers in natural populations of *L. maculans*. When paired with the two mating type tester strains 1275 and 1276, as well as with a few selected isolates of the collection, field isolates could be unambiguously characterized as either Mat^+ or Mat^- (Table 1). Among the 24 random single-ascospore isolates that we collected in France, 13 were Mat^+ (54.17%) and 11 were Mat^- (45.83%).

As compared to our previous analyses (2), the modification in the silver nitrate staining protocol enabled us to obtain an improved resolution of protein patterns. Under these conditions, only one of the 73 isolates analyzed (i.e., isolate H5) consistently showed a different soluble protein profile (SPP). This profile was characterized by the lack of one major band and the addition of another major band as compared to the other 72 isolates (Fig. 1). According to Balesdent et al (2), the pI of these bands could be estimated at pI 5.2 for the missing band and at pI 5.4 for the additional band. The phenotype corresponding to this specific profile was referred to as SPP^-.

Three ACP electrophoretic types (ET) were observed among isolates (Table 1). Each ET was characterized by six to eight isozyme bands (Fig. 2). The ET displaying the most basic isozymes, termed ET1, was observed for the three isolates D1, D2, and D3 that originated from the same rapeseed stubble (Table 1). The ET displaying the most acidic isozymes was termed ET3. This ET was found for five of the 73 tested isolates (Table 1). However, these five isolates were obtained from only two field tetrads. Finally, ET2 was the most frequently observed ACP pattern and displayed isozymes focusing at intermediate pH values (Fig. 2). It was found in isolates sampled from different regions in France as well as from Germany and Australia.

Reproducibility of ACP and specific protein profiles. To assess the reproducibility of the three ACP patterns and that of the H5 specific protein profile, single-conidium progenies were obtained from selected isolates displaying ET1, ET2, or ET3, as well as SPP^- or SPP^+ phenotypes. As illustrated in Figure 1, the protein profiles characterizing SPP^- or SPP^+ isolates were highly stable through subculturing or asexual multiplication. The 78 single-conidium progenies from SPP^+ isolates always displayed SPP^+ patterns. Similarly, the 58 single-conidium isolates derived from SPP^- isolates could all be characterized as SPP^- isolates. ACP patterns were also unaffected by asexual multiplication (data not shown). Eighty-eight single-conidium isolates were analyzed, representing 50 single-conidium cultures from ET2 isolates, 10 single-conidium cultures from ET1 isolates, and 28 single-conidium cultures from ET3 isolates. All of them displayed ACP profiles identical to the isolate from which they were derived.

Segregation of mating type, ACP, and protein profiles. To determine the genetic control of mating type, ACP, and protein profiles, in vitro crosses were performed between isolates differing for one or two of these three markers (Fig. 3). Some variants were rare, so all pairings between the different phenotypes could not be made in the first generation of crosses. For instance, H5 was the only SPP^- isolate of our collection. Since H5 is Mat^-, it could only be crossed with Mat^+ isolates and consequently could not be crossed with the ET1 isolates D1, D2, and D3, which were all Mat^-.

In the four complete tetrads obtained in this study, the segregation ratio of mating type within each tetrad was 4:4 (tetrads v and w in Table 2 and z and y in Fig. 2). The four tetrads from which seven ascospores were recovered all displayed a 3:4 segregation ratio for mating type. Finally, only six ascospores were recovered from nine other tetrads (Tables 1 and 2, Fig. 3). In these, the segregation ratio for mating type was either 2:4 or 3:3. These data are consistent with the 4:4 ratio observed with complete tetrads, thus suggesting that mating type is controlled by a single genetic locus with two alleles.

The segregation of the SPP phenotypes was mainly analyzed in the cross between H5 (SPP^-) and a.2 (SPP^+) (Fig. 3). The two resulting complete tetrads, w and v, displayed a 4:4 ratio for SPP^-:SPP^+ phenotypes (Table 2). In all crosses between two SPP^+ isolates, the resulting progenies displayed the SPP^+ phenotype. In addition, the cross between H5 and a.2 allowed us to obtain SPP^-, Mat^+ isolates (Table 2) that could be crossed with SPP^-, Mat^- isolates. When t.2 (SPP^-, Mat^+) was crossed to t.4 (SPP^-, Mat^-) (Fig. 3), only the SPP^- phenotype was recovered in the two tetrads resulting from this cross. These data suggest that the SPP phenotype is controlled by a single genetic locus at which we have detected two alleles termed SPP1 (SPP^+ phenotype) and SPP2 (SPP^- phenotype).

The segregation of ACP profiles was analyzed in the progeny of crosses between ET2 × ET2 isolates, ET2 × ET1 isolates, and ET3 × ET1 isolates (Fig. 3). The eight independent crosses performed between ET2 isolates led to progenies displaying the ET2 phenotype (see Fig. 2). One complete tetrad, z, was obtained from one cross between ET1 and ET2 isolates, in which these two phenotypes segregated in a 4:4 ratio (Fig. 2). Three independent crosses between ET1 and ET3 isolates enabled us to obtain tetrads y, m, and n. All of them displayed a 4:4 segregation

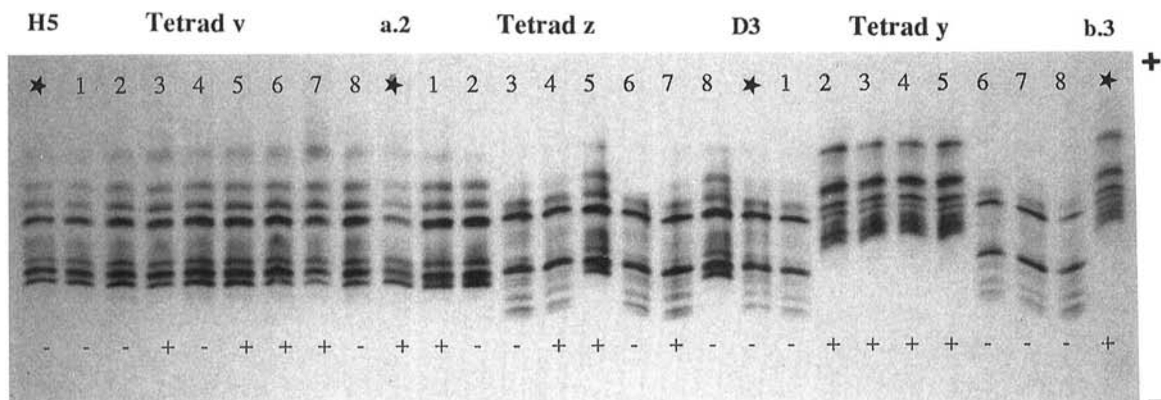

Fig. 2. Tetrad analysis of acid phosphatase electrophoretic types (ET) segregation. H5 (ET2), a.2 (ET2), D3 (ET1), and b.3 (ET3) were used as parents for in vitro crosses. Tetrads v (v.1–v.8), z (z.1–z.8), and y (y.1–y.8) resulted from the crosses H5 × a.2, a.2 × D3, and D3 × b.3, respectively. Parent isolates are indicated by a star. The mating type of each isolate is displayed as a minus or plus sign at the bottom of the figure.

ratio for the ACP phenotypes (Fig. 2). No crosses were attempted between ET2 and ET3 isolates. However, the ET2 and ET3 phenotypes segregated 3:2 and 3:3 within the two natural incomplete tetrads b and a, respectively. From these data, it can be suggested that ACP ET are controlled by a single genetic locus with at least three alleles.

Recombination between markers. When analyzing the segregation of both mating type and SPP phenotypes, two types of segregation were observed within the tetrads. Both parental ditypes (PD) and tetratypes occurred in tetrads obtained from a single pseudothecium (Table 2). Among the seven tetrads recovered from crosses between SPP^+ and SPP^- isolates, a 3:0 ratio was observed for PD:NPD (nonparental ditype) (Table 3). These data are insufficient to conclude as regards linkage between Mat and SPP genes, since the smallest PD:NPD ratio capable of providing significant indication of linkage is 5:0 (18).

When analyzing the segregation of both mating type and ACP ET, the three possible combinations of characters were obtained, i.e., tetratypes (tetrads a, b, and z), parental ditype (tetrad y), and nonparental ditypes (tetrads m and n) (Tables 1 and 3, Fig. 2). Mat and ACP genes therefore are likely to map to different linkage groups.

In the case of tetratypes, two markers were sufficient to identify four pairs of twin ascospores within a tetrad (see tetrad v in Table 2 or tetrad z in Fig. 2). When incomplete tetrads were isolated, the characterization of isolates according to the two markers allowed us to verify whether the four genotypes resulting from the meiosis were present (see tetrad t in Table 2) or not (see tetrad x in Table 2).

Aspect of the confrontation line. When isolates were grown alone in petri plates, pycnidia were randomly scattered through the culture. When isolates belonging to the same tetrad and displaying the same mating type were paired, pycnidia were either mostly produced along the confrontation line between the two isolates or randomly scattered through the petri plate. The former event was often accompanied by a deposition of a dark brown layer of pigment. The latter event was observed for self pairings and when ascospore twins were paired. Four pairs of isolates were identified by this criterion in each of the complete tetrads v, w, and z. These pairs were the same as those identified in tetrads v and z using the soluble protein and ACP markers. In the case of complete tetrads, the absence of pycnidia-rich confrontation line could therefore be used, in combination with the mating type, as an additional criterion for identifying twin ascospores.

DISCUSSION

The numerous crosses reported in this study ensure the reproducibility and reliability of the protocols that were used to obtain fertile in vitro crosses of *L. maculans* (15). All of the pairings that were expected to produce fertile crosses, i.e., crosses performed between isolates of opposite mating types, led to the production of pseudothecia and ascospores, in contrast to previous reports that used other methods (e.g., 22,28). For a cross between any given pair of compatible isolates we also noted, as did Mengistu et al (15), that the production of pseudothecia was not observed in all plates and that the number of pseudothecia per plate was highly variable. Maturity of pseudothecia, as determined by the time when asci containing ascospores were ejected, also varied from plate to plate.

Our data on mating type allele frequency in natural populations are in accordance with previous reports suggesting that this fungus is heterothallic (3,28). This result is further supported by the numerous data showing that self-matings of single-ascospore isolates fail to produce pseudothecia (this study; 15,22,28). In addition, we confirmed the 4:4 segregation ratio of mating type within an ascus previously noted by Boudart (3). These data clearly establish that a single genetic locus with two functional alleles controls sexual compatibility in *L. maculans*. Our data also suggest that the *L. maculans* mating type alleles are very stable, as observed for *Neurospora crassa*, for example (24). Actually, interconversion or unidirectional reversal of mating type has never been observed in *L. maculans*, in contrast to what was described for a few other filamentous ascomycetes (19).

In addition to mating type, two potential protein markers were characterized and their heritability studied. These markers, SPP phenotypes and ACP electrophoretic types, were stable through vegetative subculturing, protein extraction, and analysis. Segregation patterns in the meiotic progeny provide further arguments for the stability of these polymorphic markers through sexual reproduction. Based upon a 4:4 segregation ratio of SPP^-:SPP^+, the presence of the additional band at pI 5.4 characterizing the SPP^- phenotype appears to be controlled by a single gene. The

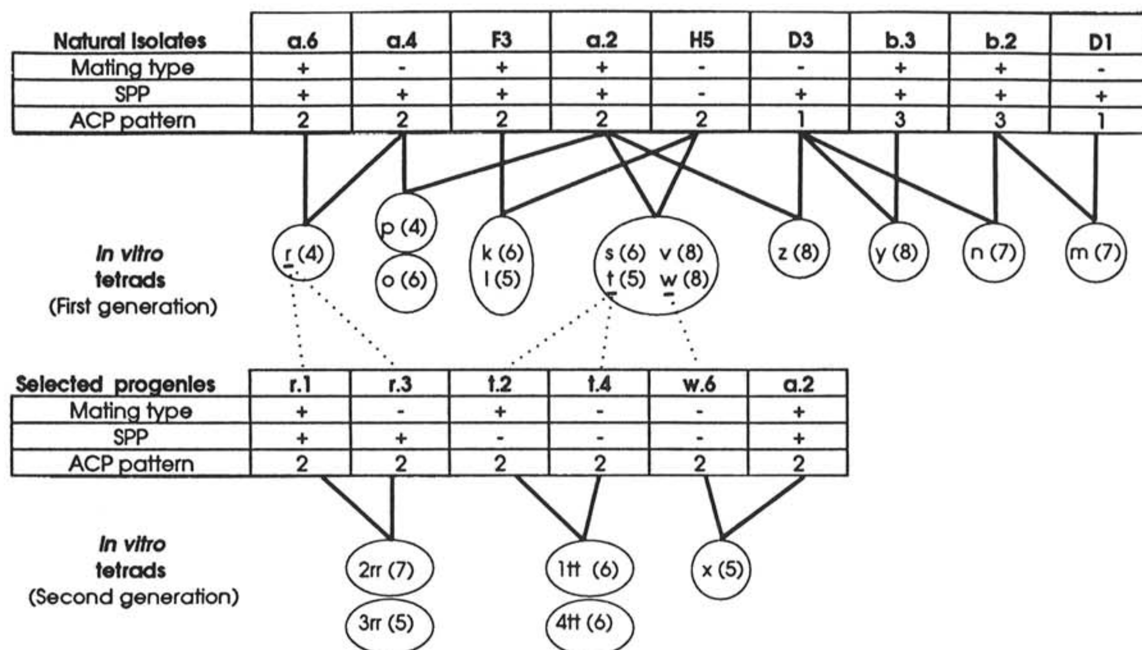

Fig. 3. Diagrammatic representation of in vitro crosses performed for progeny analysis. The phenotypes (mating type, acid phosphatase electrophoretic type, and soluble protein phenotype) of the parental isolates are indicated. The numbers in parentheses represent the number of isolates recovered from each ascus. Tetrads within the same circle were derived from the same pseudothecium.

SPP⁻ phenotype is also characterized by one missing major band at pI 5.2 as compared to the average profile. In the seven heterozygous meiosis that we observed, these two characters always cosegregated, i.e., each isolate displayed either the pI 5.2 or the pI 5.4 protein band. One hypothesis to explain these data is that a mutation in the gene controlling the pI 5.2 band, leading to a modification of the pI of the protein, is responsible for the SPP⁻ phenotype. IEF of ACP in plant or fungal systems usually reveals complex profiles (e.g., 10,26). In contrast, starch gel electrophoresis of ACP usually reveals only a few bands, thus allowing an easy genetic interpretation of the data (10,13). The complex ACP ET obtained following IEF in *L. maculans* all contained six to eight bands. However, allelism tests revealed that all isozymes of a given ET always cosegregate. Furthermore, a 4:4 segregation ratio for each ET was always observed when parents displaying different ET were crossed. These data suggest that the observed ACP patterns are controlled by a single genetic locus with at least three alleles. It is unclear, however, whether this gene encodes for ACP or controls posttraductional modifications of the enzyme.

Boudart (3) reported both first-division segregation (prereduction) and second-division segregation (postreduction) of mating type alleles when analyzing mating type and the serial order of the ascospores from two tetrads. The order of the ascospores within the asci is difficult to determine in *L. maculans* because ascospores intermingle and because the three ascospores located at the ascus top still adhere tightly to one another after ascus wall dissolution. As a consequence, it was not attempted in this study to determine the ascospore order. Our data regarding the segregation of mating type and one of the two protein markers confirmed that both prereduction and postreduction separation of allelic markers occurs in *L. maculans* tetrads.

As an alternative to protein markers and mating type, the lack of a pycnidia-rich confrontation line between two isolates in culture can be used to establish pairs of ascospores. This confrontation line was observed in tetrads v, w, and z and could be a manifestation of vegetative incompatibility (for a review see 12). Mutant complementation experiments suggested that parasexuality may occur in *L. maculans* (20). However, no data are available on genes governing vegetative compatibility in this fungus. In model ascomycetes *Aspergillus* and *Neurospora*, eight and 10 vic (vegetative incompatibility) loci have been described, respectively (12). If such a complex control occurs in *L. maculans*, it could be expected that field isolates from different stubbles will be heterozygous at many vic loci. Actually, tetrads v, w, and z all originated from crosses between parents from different stubbles. As a consequence, it can be hypothesized that only twin isolates are homozygous at all vic loci in these tetrads. However, only three tetrads were analyzed according to this criterion and more data are needed to ensure the reliability of the criterion as a marker and to confirm the involvement of vegetative incompatibility in this phenomenon.

At present, only the three markers described here are available for progeny analysis of *L. maculans*. Mating type, even though it is a highly reliable marker, is quite inconvenient since it requires a 5-wk culture period and numerous combinations of pairings to be definitive. Soluble protein and ACP data are easily scored by visual observation of IEF patterns. In addition, pulsed-field gel electrophoresis has revealed significant chromosome length polymorphism in *L. maculans* within two tetrads collected on *B. napus* stubbles (23). These tetrads displayed a 2:2:2:2 ratio of identical karyotypes, thus suggesting that twin ascospores can be discriminated according to their electrokaryotypes.

The reliability of the crossing protocol, the feasibility of tetrad separation, and the identification of three markers displaying Mendelian segregation following in vitro meiosis now enable us to further study the genetics of *L. maculans* pathogenicity.

TABLE 3. Tetrad types as determined by segregation of both mating type and either SPP phenotype or ACP electrophoretic type

Marker combination	Parent isolates	No. of tetrads of each type: Parental ditype	Non-parental ditype	Tetratype
Mat × ACP ET	a.2 × D3	...	...	1
	D3 × b.3	1	...	...
	b.2 × D1	...	1	...
	b.2 × D3	...	1	...
	? (field tetrads)	...	...	2
Mat × SPP	a.2 × H5	2	...	2
	F3 × H5	1	...	1
	a.2 × w.6	...	...	1

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