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Mating behaviour and vegetative compatibility in Spanish populations of *Botryotinia fuckeliana*

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Abstract

Mating behaviour and vegetative compatibility were studied in Spanish populations of *Botryotinia fuckeliana*. Fifty-seven isolates out of the 61 tested were sexually fertile with one or more of the reference strains of known mating type (MAT1-1 or MAT1-2). Thirty-nine isolates were heterothallic, giving fertile crosses when mated with the MAT1-1 (24 isolates) or the MAT1-2 (15 isolates) reference strain. Eighteen isolates crossed successfully with both MAT1-1 and MAT1-2 reference strains, and were referred to as homothallic or MAT1-1/2. Both mating types were widespread, being represented in isolates from two regions, from the same and different greenhouses, from different hosts, and from different years of isolation. Isolates were paired on Malt Extract Agar + NaCl to evaluate vegetative compatibility. Most of the paired isolates were unable to fuse and showed a different reaction of incompatibility. Nitrate-non-utilising (nit) mutants were selected by growth on a medium amended with 30–50 gl^{-1} potassium chlorate. Over 600 chlorate-resistant sectors were recovered from 40 isolates at a mean frequency of 0.15–2.39 sectors per colony, but only 11% were identified as nit mutants by their thin growth with no aerial mycelium on minimal medium. However, most of these nit mutants reverted to wild type during six months of storage on chlorate-amended medium. Genetic complementation between nit mutants occurred only in two cases between mutants from the same isolate.

Introduction

Grey mould, caused by *Botryotinia fuckeliana* (de Bary) Whetzel (*Botrytis cinerea* Pers.), is an economically important disease on protected crops grown in Almería, a region in South-eastern Spain where unheated plastic greenhouses cover an area of ca. 30,000 ha. (Anonymous, 1993; Belda et al., 1994). Epidemics occur every year causing severe losses as crops grown in winter are mainly vegetables susceptible to grey mould and weather conditions are usually conducive to the disease. Almería is comprised of two districts, 50 km apart, namely Poniente and Levante, which are located to the west and east of Almería city, respectively. Growers protect their crops during November to March by application of benzimidazole and dicarboximide fungicides. In 1992, strains resistant to these fungicides were found at high frequencies in populations of *B. fuckeliana* (Raposo et al., 1996).

Knowledge of the population genetics of fungal plant pathogens may eventually contribute to the development of more durable disease management strategies. Previous studies carried out in populations of *B. fuckeliana* from Almería using RAPD markers showed that this pathogen is highly heterogeneous (Alfonso et al., 2000). However, the relative importance of the different evolutionary forces, such as mutation, recombination, migration, selection, and random drift were not evaluated.

Sexual reproduction is not regarded as a cause of this genetic diversity because apothecia have never been found in Almería (Raposo et al., 2001). Previous

studies with *B. fuckeliana* showed that the species is basically heterothallic, with mating type determined by two alternative alleles of the mating-type gene *MAT1* (*MAT1-1* and *MAT1-2*). Some strains are heterokaryotic for mating type and thus self fertile (secondarily homothallic) (Faretra and Pollastro, 1993).

Heterokaryosis is another important source of genetic diversity in fungal populations. Although heterokaryosis is widely accepted as an important source of variation in *B. fuckeliana* (Lorbeer, 1980), its role is still not well understood. Recently, Weeds et al. (1998) demonstrated complementation between selenate-resistant (selR) mutants and nit mutants from the same parent. These results provide support for the suggestion of Beever and Parkes (1993) that a vegetative incompatibility system may be operating in *B. fuckeliana*. However, further work is needed to evaluate vegetative compatibility amongst field strains.

The present paper reports results of observations on sexual behaviour, mating system, and vegetative compatibility in *B. fuckeliana* isolates from Almería with the aim of explaining the source of genetic variability in the fungus and the mechanism of gene flow in its natural populations.

Materials and methods

Isolates of B. fuckeliana

In a first survey, 55 isolates were collected in December 1992 (at the beginning of a grey mould epidemic) from 28 commercial greenhouses of common winter crops (cucumber, bean, tomato, squash, eggplant, and pepper) located within Levante and Poniente (Table 1). Isolations were made from single lesions on stems, leaves, flowers, and fruits randomly in selected greenhouses. Small tissue pieces taken from the edges of lesions were placed on potato dextrose agar (PDA, Difco) amended with 0.5 g l^{-1} of streptomycin sulphate and kept at 20–25 °C. Growing colonies were singly transferred to PDA slants.

A second survey in the Poniente district was made three months later, in March 1993, after many fungicide applications (Table 1). In this survey, spores were collected from air on 9 cm Petri dishes containing a selective medium (Kerssies, 1990). These traps were placed in greenhouses for at least 4 h and then incubated in the laboratory until colonies of *B. fuckeliana* grew. Growing colonies were transferred singly to PDA slants. Twenty-five isolates were obtained from seven greenhouses.

Additional isolates came from Italy (GU.1, GU.2, GU.3, GU.4), and Israel (TO95.261, TO95.267, TO95.270, TO95.275, CU95.41, CU95.298, and CU95.301) together with various strains from random-ascospore progeny of different crosses: three isolates from cross 112 × SAR11008 (112-4, 112-5, and 112-19); and eight isolates from cross 5.4 × SAR11004 (5.4-2, 5.4-5, 5.4-8, 5.4-21, 5.5-36, 5.4-43, 5.4-65, and 5.4-80) (Table 1). The mating type reference strains were SAR11008 (genotype *MAT1-1*) and SAR11004 (genotype *MAT1-2*).

The collection was stored in glass tubes containing sand: 3% v/v oat flakes, at 4 °C; isolates were grown on PDA at 25 °C in the dark before being used. A selection of the strains (1.7, 4.2, 5.4, 7.5, 11.1, 30.1, 40.1, 43.2, 44.4, 45.4, 1208, and 1298) was deposited in the Spanish Type Culture Collection.

Sexual crosses

Isolates were mated (Faretra et al., 1988a). Individual isolates were grown on 2% malt agar (Difco, Detroit, Mich.) plates at 15 °C in the dark for 4 weeks to obtain sclerotia. Plates were incubated for 4 weeks at 0 °C in the dark for production of microconidia. Sclerotia were transferred aseptically to glass tubes (6.5 cm × 1.5 cm) (4–5 sclerotia per tube). Mass spermatization of the sclerotia was carried out by flooding them immediately with 2 ml of a mixed suspension of microconidia, macroconidia, and mycelial fragments prepared by scraping the surface of colonies of the donor partner into 10 ml of sterile tap water. Vials were closed with cotton wool and incubated on their sides under fluorescent light at 11 °C on a 12-h photoperiod for five months. Carpogenic germination of sclerotia was checked weekly and care was taken to keep sclerotia moist by periodical addition of sterile tap water. Sixty-one field isolates (Table 1) were individually crossed with the two reference strains of each mating type and with themselves either as sclerotial partner or as donor partner in the cross. Four replicate vials were made for each mating. In addition, eight strains from random-ascospore progeny of the cross 5.4 × SAR11004 were backcrossed with both parental strains to determine their mating type (Table 1).

Vegetative incompatibility

Vegetative incompatibility was tested (Beever and Parkes, 1993) by observing the interaction zone between paired colonies. Actively growing mycelial

Table 1. Characteristics of isolates of *Botryotinia fuckeliana*

Isolate	GH[z]	Region	Year	Host	Mating type[y] MAT1-
1.1♦/1.7♦	1	Levante	1992	Bean	1/2
1.5♦	1	Levante	1992	Bean	2
4.2♦*	4	Levante	1992	Bean	1
5.4	5	Levante	1992	Bean	1
6.3♦	6	Levante	1992	Bean	2
7.5♦	7	Levante	1992	Squash	NT
8.4♦	8	Levante	1992	Bean	1/2
11.1*/11.2*/11.3/11.12*	11	Levante	1992	Tomato	1
11.4*/11.6/11.8/11.13/11.14	11	Levante	1992	Tomato	2
11.5*/11.9*	11	Levante	1992	Tomato	NF
11.7/11.10/11.11	11	Levante	1992	Tomato	1/2
12.7	12	Levante	1992	Tomato	2
16.5♦	16	Levante	1992	Cucumber	NT
18.2	18	Poniente	1992	Squash	1/2
21.1♦/21.4♦*	21	Poniente	1992	Squash	2
22.4	22	Poniente	1992	Tomato	1
23.2♦	23	Poniente	1992	Tomato	NT
30.1♦*	30	Poniente	1992	Cucumber	1
30.3*	30	Poniente	1992	Cucumber	NT
31.1*/31.3*/31.4*/31.5*	31	Poniente	1992	Pepper	NT
31.2♦*	31	Poniente	1992	Pepper	NF
31.5	31	Poniente	1992	Pepper	NT
32.1*/32.2*/32.3*/32.4*	32	Poniente	1992	Pepper	NT
34.4♦	34	Poniente	1992	Eggplant	NT
40.1♦/40.2♦	40	Poniente	1992	Squash	1/2
43.2♦*	43	Poniente	1992	Cucumber	1/2
44.4♦	44	Poniente	1992	Pepper	1
45.3♦*	45	Poniente	1992	Pepper	1/2
45.4♦*	45	Poniente	1992	Pepper	2
46.1♦	46	Poniente	1992	Tomato	1
50.1♦	50	Poniente	1992	Eggplant	1
52.5♦	52	Poniente	1992	Cucumber	NT
62.4	62	Poniente	1992	Tomato	1
65.1♦*	65	Poniente	1992	Tomato	1
65.4♦	65	Poniente	1992	Tomato	1/2
67.4♦	67	Poniente	1992	Cucumber	1/2
68.4♦	68	Poniente	1992	Eggplant	1
112♦*	B	Poniente	1993	Air	1/2
172♦*	E	Poniente	1993	Air	1
264♦/265♦	G	Poniente	1993	Air	2
602*/612/640*/683*/684*/746*	H	Poniente	1993	Air	NT
603/732♦*/142*/1258*/624♦*/662♦*	H	Poniente	1993	Air	1
636*/676♦*	H	Poniente	1993	Air	1/2
884	I	Poniente	1993	Air	1
911♦*	I	Poniente	1993	Air	1/2
948	J	Poniente	1993	Air	2
951/1298	J	Poniente	1993	Air	1
1294	J	Poniente	1993	Air	1/2
1208*	K	Poniente	1993	Air	2
GU.1*		Italy	1994	Basil	NT
GU.2*		Italy	1989	Poinsettia	NT
GU.3*/GU.4*		Italy	1995	Grapevine	NT
TO95.261*/TO95.267*/TO95.270*/TO95.275*		Israel	1995	Tomato	NT
CU95.41*/CU95.298*/CU95.301*		Israel	1995	Cucumber	NT
5.4-2*/5.4-8*/5.4-43*/65*			1996	5.4 × SAR11004	2

Table 1. (*Continued*)

Isolate	GH[z]	Region	Year	Host	Mating type[y] MAT1-
5.4-5*/5.4-36*			1996	5.4 × SAR11004	1
5.4-21*			1996	5.4 × SAR11004	1/2
5.4-80			1996	5.4 × SAR11004	NF
112.4♦			1996	112 × SAR11008	NT
112.5♦			1996	112 × SAR11008	NT
112.19♦			1996	112 × SAR11008	NT

[y]NF: non-fertile; NT: not tested. [z]GH: greenhouse. ♦Isolates used in tests of vegetative compatibility in Malt Extract Agar + 0.68 M NaCl. *Isolates used to produce *nit* mutants.

plugs (6 mm dia.) of inoculum were placed 3 cm apart in 9-cm Petri dishes, one pairing per dish, containing Malt Extract Agar (MEA, Pronadisa, Hispanlab S.A., Madrid, Spain) + 0.68 m NaCl and incubated at 22 °C in the dark. Field isolates shown in Table 1, SAR11008, and SAR11004 were paired against themselves and each other. Three monoascospore strains (112-4, 112-5, and 112-19) from random progeny of the cross 112 × SAR11008 were also tested in all combinations. Each pairing was performed at least twice and four replicates were made of each combination. Pairings were examined 4, 7, and 14 days after inoculation.

To study morphological features of the compatible/incompatible reactions, pairings were made on sterile slides coated with a thin layer of PDA. Slides (75 mm × 25 mm), were dipped in PDA (50 °C) and then placed in sterile glass Petri dishes containing three pieces of filter paper impregnated with water to maintain adequate moisture during incubation. The slide was inoculated with blocks of inoculum (1 mm^3) placed 1.8 cm apart, for each of the two isolates in a pairing, and incubated at 22 °C in the dark. For each pairing, four slide cultures were prepared and the experiment was repeated at least twice. The interaction zones between colonies were observed daily under light microscopy.

Nit mutants

Basal medium of Puhalla (BMM) (Puhalla, 1985) was prepared as follows (per litre of distilled H_2O): sucrose, 30 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 10 mg; agar, 20 g; and trace-element solution, 0.2 ml. The trace-element solution contained (per 95 ml of distilled H_2O): citric acid, 5 g; $ZnSO_4 \cdot 7H_2O$, 5 g; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 1 g; $CuSO_4 \cdot 5H_2O$, 0.25 g; $MnSO_4 \cdot H_2O$, 50 mg; H_3BO_4, 50 mg; and $NaMoO_4 \cdot 2H_2O$, 50 mg. Minimal medium of Puhalla (MM) (Puhalla, 1985) was BMM amended with 2 g l^{-1} of $NaNO_3$.

Rose bengal-chlorate medium (RBC) (Elias and Cotty, 1995) was prepared as follows: Czapek–Dox broth (35 g l^{-1}) (Difco, Detroit, Mich.) was amended with 25 g l^{-1} $KClO_3$ and 50 mg l^{-1} rose bengal (10 ml of 5 mg ml^{-1} stock solution in 95% ethanol), and adjusted to pH 7.0–7.2 with HCl or NaOH prior to addition of 20 g l^{-1} Bacto agar (Difco).

Actively growing mycelial plugs (5 mm dia.) of each isolate (Table 1) grown on PDA at 22 °C in the dark for 3 days were individually transferred to MM amended with 1.6 g l^{-1} L-asparagine and 30 or 50 g l^{-1} $KClO_3$ (KMM) or to PDA amended with 15, 20, 25, 55, 65, 75, 85, 95, or 105 g l^{-1} $KClO_3$ (PDC). Plates were incubated at 22 °C in the dark for 20 days and chlorate-resistant sectors were transferred to MM.

Nit mutants were also produced on RBC. Twenty-five microlitres aliquots of suspensions containing 10^3–10^4 conidia were inoculated in a well in the centre of the agar in each of 10 plates containing 25 ml of RBC. Plates were sealed with parafilm and incubated on the laboratory bench (17–22 °C). Growth was observed every 5–7 days and putative nit mutants appearing as sectors were subcultured on MM.

True nit mutants were identified by growing subcultures on BMM, MM, or PDA at 22 °C in the dark for 4 days: they showed thin growth on BMM and MM and wild-type growth on PDA. Nit mutants were kept on KMM until used.

The physiological phenotypes of nit mutants recovered from isolates of *B. fuckeliana* were interpreted on the basis of information on nitrate reduction in other fungi (Correll et al., 1987). The nit mutants were assigned to different phenotypic classes on the basis of their growth on media containing one of several

nitrogen sources: (1) BMM (without nitrate), (2) PDA (without added nitrate), (3) MM (nitrate as nitrogen source), (4) BMM + 0.5 g l^{-1} $NaNO_2$, (5) BMM + 0.2 g l^{-1} hypoxanthine, (6) BMM + 1 g l^{-1} ammonium tartrate. A mycelial plug of the nit mutant was put on each of the five media. The plates were incubated as described above, and the colony morphology was compared to the wild-type parent after 4 days. *Nit1* mutants grew thinly in BMM and MM, nit3 grew thinly in BMM, MM and BMM + nitrite; and NitM grew thinly in BMM, MM, and BMM + hypoxanthine (Correll et al., 1987).

Complementation tests

Vegetatively compatible nit mutants may complement one another when they come in contact by forming a heterokaryon on MM which forms a dense aerial growth (Puhalla, 1985). Pairings were made by placing mycelia from nit mutants 1–3 cm apart on MM. Pairings were incubated at 22 °C in the dark for 14 days and then scored for complementation. Complementation tests were made between each nit mutant from one isolate and all the nit mutants obtained from the other isolates of the same or different phenotypes.

Results

Sexuality of B. fuckeliana *isolates*

Fifty-seven field isolates out of the sixty-one tested were sexually fertile with one or more reference strains of known mating type. The apothecial primordia, arising singly or in groups, appeared one and a half months after setting up the crosses. Apothecia were formed from two to five months after crossing. Thirty-nine isolates were heterothallic, giving fertile crosses when mated with the MAT1-1 (24 isolates) or the MAT1-2 (15 isolates) reference strain. Eighteen isolates crossed successfully with both MAT1-1 and MAT1-2 reference strains and are referred to as homothallic or MAT1-1/2 (Table 1). Both *MAT1-1* and *MAT1-2* alleles of the mating-type gene were represented in isolates from the two regions (Levante and Poniente), from the same and different greenhouses, from different hosts, and from different years.

Eight monoascospore strains from the cross 5.4 × SAR11004 were backcrossed with both the reference strains SAR11004 and SAR11008. Two of the isolates were MAT1-1, four were MAT1-2, one was MAT1/2, and one was non-fertile (Table 1).

Vegetative incompatibility

Pairings were scored as compatible when two isolates merged to form one colony, with no distinct interaction zone. Pairings were scored as incompatible when a dark line, a strip of thin mycelium with no sclerotia, or a light brown line between colonies was observed.

When each field isolate and the reference strains SAR11008 or SAR11004 were paired in all combinations, only the pairing 68.4 × 911 and all self–self pairings were compatible.

In the pairings of the three monoascospore strains (112-4, 112-5, and 112-19) from the sexual cross 112 × SAR11008, all self–self pairings and pairings of monoascospore isolates against the parental isolate 112 were compatible. Pairings 112 × SAR11008, 112-4 × SAR11008, 112-5 × SAR11008, 112-19 × SAR11008, 112-4 × 112-5, 112-4 × 112-19, or 112-5 × 112-19 were incompatible as they show clear dark brown lines between colonies.

In the interaction zone between colonies of some pairings (45.4 × 65.4, 45.4 × 732, 45.4 × 911, 65.4 × 676, 65.4 × 732, 264 × 732, 1.1 × 1.7, 1.1 × 6.3, 1.1 × 21.1, and 1.7 × 43.2) a mycelium-free space was observed. These reactions were not necessarily due to incompatibility but possibly to staling products and nutrient depletion.

Microscopic studies of pairings

Intercolony and intracolony anastomosis occurred in compatible pairings, although not in all of them. Anastomosis occurred generally by direct fusion. In some cases, it was preceded by winding of one hypha around the other or by formation of a simple appressorium and a vesicle-like process in the penetrated cell (Figure 1). Deterioration of hyphae after anastomosis was not observed.

In incompatible pairings intracolony anastomosis was also frequent while very few intercolony anastomoses occurred. Anastomosis was similar to that in compatible pairings but hyphal deterioration was observed in some strains from day 6.

In both compatible and incompatible interactions, hyphal tips in many isolates became dedicated to microconidiogenesis, especially in the interaction zone,

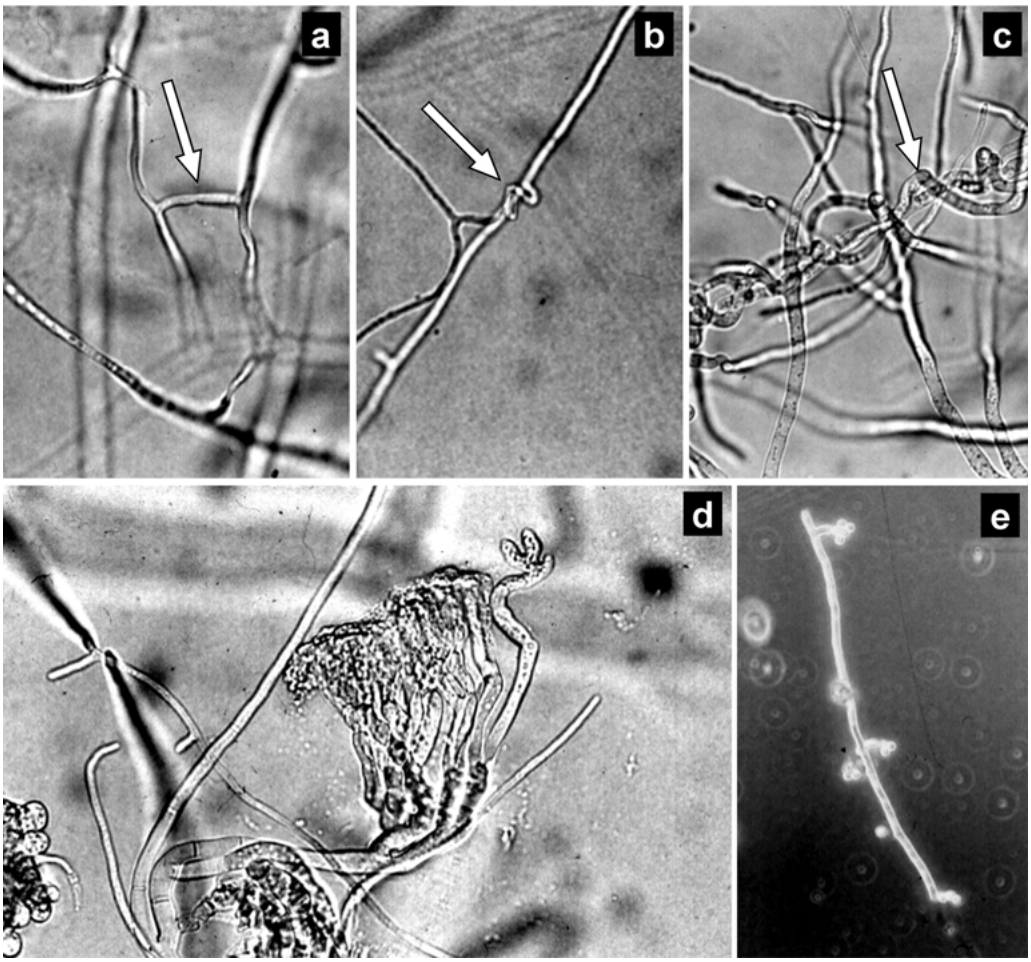

Figure 1. Photomicrographs of pairings of two *Botryotinia fuckeliana* isolates. (a) anastomosis by direct fusion of two hyphae (arrow) of isolates 68.4 × 911 in a compatible interaction; (b,c) winding of one hypha around the other (arrows) in the compatible interaction of isolates 68.4 × 68.4; (d) appressorium of isolate 1.7 in a compatible interaction of 1.7 × 1.7; (e) microconidiogenesis in the interaction zone of the confrontation of isolates 1.1 × 1.7.

visible macroscopically, and incapable of anastomosis (Figure 1). Also some isolates produced abundant loops.

Nitrate-non-utilising mutants

Different culture media were used to obtain *nit* mutants from *B. fuckeliana* isolates. Isolates of *B. fuckeliana* showed an innate low sensitivity to chlorate: they grew uniformly over all PDA plates amended with chlorate from 15 to 105 g l^{-1}, without producing any sectors. Isolates of *B. fuckeliana* also did not produce sectors in RBC medium, specifically designed to produce chlorate-resistant sectors (Elias and Cotty, 1995), even though this medium restricted colony growth in the centre of the plates. However, in KMM, isolates produced dense aerial restricted colonies which at day 3, began to produce spontaneous chlorate-resistant sectors until day 10. These sectors had two kinds of morphological characteristics: dense and aerial mycelia or thin non-aerial mycelia. Both of them were potential *nit* mutants.

Forty field isolates from Spain, four field isolates from Italy and six isolates from Israel (Table 1) were used to produce nit mutants in five repetitions in KMM, and 602 chlorate-resistant sectors were obtained (0.15–2.39 sectors per colony). Most sectors appeared in the medium amended with 30 g l^{-1} chlorate. Isolates

from Italy, Israel, and from one of the sampled greenhouses (greenhouse 11) produced sectors only in $50\,g\,l^{-1}$ chlorate.

Only 11% of the chlorate-resistant sectors were unable to utilise nitrate as a sole nitrogen source; they grew as thin expansive colonies with no aerial mycelium on BMM or MM, but showed wild-type growth on PDA; these sectors were designated nit mutants.

The phenotypes of nit mutants were determined by their morphology on media containing different nitrogen sources: 45% were *nit1*, 25% were nit3 and 11% were NitM. Some mutants had other phenotypes: (i) no growth on nitrate, (ii) no growth on nitrate and hypoxanthine, (iii) growth only on PDA + nitrate, (iv) growth only on PDA + hypoxanthine, (v) thin growth on nitrite, but not on nitrate.

Nit mutants were stored in KMM and most of them were not stable, reverting to the wild type. Only 22 *nit* mutants continued growing thinly on BMM and MM after six months of storage; 6 were *nit1*, 8 were nit3, 3 were NitM and 5 had one of the phenotypes (i)–(v) described above.

Complementation tests

Twenty-two stable nit mutants were paired in all combinations; within 12 days of incubation, relatively thick dense mycelial growth was observed along the line of contact only in two pairings between NitM × *nit1* mutants. In both the cases, the NitM and *nit1* mutants paired came from the same original isolate. Heterokaryon formation was observed after 12 days of incubation. Other NitM × *nit1* pairings, always made between NitM or *nit1* mutants derived from different original isolates, resulted in incompatible interactions.

Discussion

Our studies of the mating types of *B. fuckeliana* present in Almería confirm and extend those of Beever and Parkes (1993) and Faretra and Pollastro (1993) by showing that both the alleles, *MAT1-1* and *MAT1–2*, are common in nature. As in other populations, the percentage of isolates with the allele *MAT1-1* was slightly higher than that with the allele *MAT1-2* (Beever and Parkes, 1993; Faretra and Pollastro, 1993; Van der Vlugt-Bergmans et al., 1993).

A proportion of field isolates (18 isolates out of 61 tested) was fertile with both MAT1-1 and MAT1-2 reference strains. Strains of this phenotype have been referred to as homothallic MAT1-1/2 strains (Faretra and Grindle, 1992). Previous studies of field isolates of *B. fuckeliana* have identified homothallic strains in populations (Beever and Parkes, 1993; Faretra and Pollastro, 1993). Inclusion of two nuclei of opposite mating type in one ascospore occurs in the four-spored asci of secondarily homothallic species such as *Neurospora tetrasperma* (Dodge, 1927). Faretra et al. (1988b) demonstrated a similar mechanism in functionally homothallic field isolates of *B. fuckeliana*: heterokaryons contained both *MAT1-1* and *MAT1-2* alleles in separate nuclei.

In this study some of the monoascospore isolates obtained from a previous sexual cross also were homothallic. Faretra and Pollastro (1996) found pairs of homothallic ascospores in seven out of 105 asci of *B. fuckeliana* tested and reported that homothallism of monoascospore isolates cannot be caused by inclusion of more than one nucleus in ascospores or by simple mutation from the *MAT1-2* to the *MAT1-1* allele. Other causes, such as irregularities during meiosis that lead to irregular nuclear distribution among ascospores may account for homothallism in this case. Although mating type instability has been documented in several species of fungi (Leslie et al., 1986; Mathieson, 1952; Uhm and Fujii, 1983) the phenomenon is not well understood in *B. fuckeliana*. Faretra and Pollastro (1996) showed that the mating type change in *B. fuckeliana* always involved the *MAT1-2* allele and is apparently unidirectional.

The occurrence of the sexual stage of *B. cinerea*, *B. fuckeliana*, has never been reported in Spain (Raposo et al., 2001). In this paper, we demonstrated that both mating types, MAT1-1 and MAT1-2, were present in the population of *B. fuckeliana* of Almería. Other factors may account for the inability to find the teleomorph in Almería. Production of apothecia in the laboratory is a tedious process that requires specific conditions of temperature and light. Perhaps climatic conditions in Almería do not favour the production of apothecia. Recently, Raposo et al. (2001) reported that sclerotia were not found in Almería, neither on plant debris, nor on living material, indicating that they played a minor role in epidemic development of *B. fuckeliana* in this region.

The high ascospore viability in the crosses studied indicates a high genetic similarity between the Italian tester strains and Spanish isolates. Faretra and Pollastro (1993) demonstrated apothecial production and high ascospore viability in all crosses between

their tester strains and isolates from different countries in Europe, Israel, Japan, and Australasia. Beever and Parkes (1993) obtained the same results when crossing Italian tester strains with isolates from New Zealand. Populations of *B. fuckeliana* seem to have no problems with crossing, which may indicate similarity between populations. Analysis of *B. fuckeliana* populations from different countries (Italy, Holland, Israel, and Spain) using RAPD markers revealed that most of the genetic diversity (96%) is present within subpopulations, indicating high similarity between populations (Alfonso et al., 2000).

Most of the paired isolates of *B. fuckeliana* were unable to fuse and form one colony and only one pairing resulted in a compatible interaction. High levels of vegetative incompatibility were also observed in many ascomycetes or ascomycetous anamorphs (Kohn et al., 1990) such as *Ophiostoma ulmi* (Brasier, 1983), *Cryphonectria parasitica* (Anagnostakis, 1977), and also in *B. fuckeliana* (Weeds et al., 1998).

Results obtained when pairing progeny strains against themselves or against the parents of one sexual cross (112 × SAR11008) differed from those obtained by Beever and Parkes (1993) when testing isolates of *B. fuckeliana* from New Zealand. We obtained compatible interactions in pairings of the parent 112 against the 3 progeny strains while Beever and Parkes (1993) did not obtain any compatible interaction in pairings of a random selection of 11 progeny strains against both the parents. However, when pairing selected progeny, Beever and Parkes (1993) obtained 3 compatible interactions in a total of 144, while we did not obtain any. Fewer pairings were made in our work (only 6), but the occurrence of 50% compatible interactions (3 in 6) would suggest that the two parental strains differ at one *VC* (vegetative compatibility) gene. Vegetative incompatibility has been shown in a number of systems to be controlled by a series of genes, variously termed *VC* or *het* (heterokaryon incompatibility) genes, each of which exists in two alleles. Compatibility results when the paired strains are isogenic at all loci, incompatibility when the strains carry different alleles at one or more loci. If the strains differ by only one *VC* gene one would predict 50% compatible interactions (1 in 2), if differing by two (unlinked) genes the prediction is 25% (1 in 2^2), by three genes 12.5% (1 in 2^3), etc. (Beever and Parkes, 1993).

Microscopic characteristics of vegetative compatibility are generally complex. We tested here clearly compatible or incompatible pairings looking for differential reactions. The most important differences were that some incompatible reactions were usually followed by hyphal deterioration as described by Kohn et al. (1990) together with a frequent differentiation of appressorial pads.

Vegetative incompatibility in *B. fuckeliana* populations could act to prevent the formation of heterokaryons as has been suggested for other ascomycetes (Leslie, 1990; Perkins, 1988). However, evidence should be demonstrated. In this study, we tried to produce nit mutants of *B. fuckeliana* to test more clearly the formation of heterokaryons.

Production of nit mutants in isolates of *B. fuckeliana* was tedious because the fungus is highly tolerant to chlorate, and concentrations as high as 30–50 $g\,l^{-1}$ of chlorate were necessary to produce mutants. Weeds et al. (1998) used a concentration of 30 $g\,l^{-1}$ chlorate to produce nit mutants in *B. fuckeliana*. This low sensitivity to chlorate occurs in other fungi, such as *Aspergillus flavus* (Bayman and Cotty, 1991), *Fusarium moniliforme* (Lamondie and Elmer, 1989), *Verticillium dahliae* (Straugbaugh et al., 1992), and *Sclerotinia sclerotiorum* (Kohn et al., 1990).

The broad variability in production of chlorate-resistant sectors observed between isolates of *B. fuckeliana* and even between colonies of the same isolate was similar to that experienced with other fungi (Bowden and Leslie, 1991; Klittich and Leslie, 1988). However, the percentage of sectors that were true nit mutants was much lower in *B. fuckeliana* than in other fungi (70% in *V. dahliae* (Daayf et al., 1995), 65% in *F. graminearum* (Bowden and Leslie, 1991), or 60% in *F. oxysporum* (Puhalla, 1985)), probably due to its low sensitivity to chlorate.

Characterisation of nit mutants gave a higher proportion of *nit1*, followed by nit3 and nitM mutants. Weeds et al. (1998) only produced *nit1* mutants in *B. fuckeliana*. In *F. oxysporum,* nitM mutants readily complemented all of the *nit1* and nit3 mutants from the same strain and even each other, and Correll et al. (1987) recommended identification of a nitM tester strain for each vegetative compatibility group to be complemented with other strains. In the case of *B. fuckeliana*, as no knowledge exits about vegetative compatibility groups nit mutants were paired in all possible combinations in this study. However, crosses were made only with the 22 nit mutants that were viable and continued having nit-mutant phenotype after six months of storage (stable nit-mutants). Heterokaryons were formed only in two complementation tests between NitM × *nit1* mutants derived from the same original isolate. Reactions were

similar to those obtained with other fungi: a dense aerial growth developed where mycelia of the two nit mutant colonies came into contact, anastomosed, and formed a heterokaryon. Weeds et al. (1998) obtained complementation among *nit1* mutants in *B. fuckeliana* and suggested intragenic complementation. Although neither the frequency nor the importance of heterokaryon incompatibility in *B. fuckeliana* is known, lack of complementation between nit mutants of *B. fuckeliana* is evident. A vegetative incompatibility system may be operating in *B. fuckeliana*. This lack of complementation may be due to an isolate's inherent inability to anastomose, as occurred in other fungi such as *F. oxysporum* f. sp. *melonis* (Jacobson and Gordon, 1988) or *F. moniliforme* f. sp. *melonis* (Correll et al., 1987). If heterokaryon incompatibility is widespread in *B. fuckeliana*, then other measures of genetic diversity, such as molecular markers, should be employed to determine the relationships between isolates (Alfonso et al., 2000). Other sources of variation, such as migration or mutation, rather than hyphal anastomosis, may introduce variability into populations of *B. fuckeliana* (Alfonso et al., 2000).

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