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Use of nitrate non-utilising (Nit) mutants to determine vegetative compatibility in *Botryotinia fuckeliana (Botrytis cinerea)*

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Abstract

Nitrate non-utilising (Nit) mutants of six field strains and three single-ascospore strains of *Botryotinia fuckeliana* were selected by plating mycelial plugs onto a nitrate-containing minimal medium amended with chlorate. Mutants were characterised by growth responses on minimal medium amended with various sole nitrogen sources. For each parental strain two mutants were produced: *nit1*, defective in nitrate reductase apoenzyme; and NitM, defective in the molybdenum cofactor pathway. Complementation on nitrate minimal medium was observed between *nit1* and NitM mutants from the same parent in all instances. However, complementation was not observed between any such combinations when the mutants were derived from different parents. It is concluded that a vegetative incompatibility system operates in *B. fuckeliana* resulting in multiple vegetative compatibility groups. One of the single-ascospore strains was derived from a cross between two of the field strains, indicating that new compatibility groups can be generated during sexual reproduction. Mycelial interaction zones were formed between all parental strains when they were paired on NaCl-amended medium, indicating congruence between mycelial incompatibility and vegetative incompatibility.

Introduction

Botryotinia fuckeliana (anamorph *Botrytis cinerea*), commonly known as grey mould, is an important pathogen in temperate climates affecting a large number of economically important crops (Coley-Smith et al., 1980). It has long been recognised as a very variable species in culture (Paul, 1929; Grindle, 1979; Lorbeer, 1980), although its reputation in this regard derives in part from the use of non-standardised growth conditions. While individual studies have recognised sub-specific clusters based on morphological and physiological criteria (Lorbeer, 1980), there is little evidence of host specialisation and, until recently, little information on population structure. The application of various molecular techniques has confirmed a high degree of genetic heterogeneity across a diversity of loci (Van der Vlugt-Bergmans et al., 1993; Kerssies et al., 1997; Giraud et al., 1997; 1999; Thompson and Latorre, 1999; Alfonso et al., 2000). While in general these studies show little population structuring related to geographic source or host, Giraud et al. (1997, 1999) recognised two distinct sibling populations in Europe, a relatively parasitic form (*transposa*) and a relatively saprotrophic form (*vacuma*). In contrast, sexual crossing of strains from various parts of the world, including many from Europe, suggest *B. fuckeliana* comprises a single well-defined species (Beever and Parkes, 1993; Faretra and Pollastro, 1993).

Characterisation of groups of vegetatively (somatically) compatible individuals provides a powerful approach to subdividing a species into discrete populations in filamentous ascomycetous fungi

(Leslie, 1993; Correll and Gordon, 1999; Malik and Vilgalys, 1999; Glass et al., 2000). Members of the same vegetative compatibility group (VCG) can undergo hyphal fusion followed by transfer of nuclear and cytoplasmic elements, resulting in the formation of heterokaryons and heteroplasmons. Additionally, parasexual recombination may follow heterokaryon formation. Attempted hyphal fusion between members of different VCGs, in contrast, results in an incompatible reaction that restricts genetic interchange.

Despite the longstanding interest in heterokaryosis in *B. fuckeliana* (Hansen and Smith, 1932; Menzinger, 1966), the existence of vegetative incompatibility systems in this fungus has received little study. We have previously noted that strains of *B. fuckeliana* form interaction lines when paired on agar media (Beever and Parkes, 1993). These putative 'barrage' lines (Leslie, 1993) indicate the existence of mycelial incompatibility, and are suggestive of the existence of a vegetative compatibility system in this species. Proof of the existence of a vegetative compatibility system, however, requires demonstration that strains that are mycelially compatible are capable of fusion and heterokaryon formation. Such evidence has usually been obtained using auxotrophic mutants. The use of different classes of nitrate non-utilising (Nit) mutants has been widely used for this purpose in other fungi (Correll et al., 1987; Brooker et al., 1991). In an initial study (Weeds et al., 1998) we sought complementing Nit mutants in *B. fuckeliana*, but only recovered *nit1* mutants (defective in the nitrate reductase structural gene). Nevertheless, we were able to demonstrate complementation between these mutants and sulphate non-utilising (SelR) mutants of *B. fuckeliana* when the mutants were derived from the same parent strain and the absence of complementation when strains were from different parents. While this study provided evidence of the existence of vegetative incompatibility, it was limited because only two laboratory-derived single-ascospore strains were compared. Delcán and Melgarejo (2002) describe the recovery of Nit mutants of *B. fuckeliana*, but their isolates were mostly unstable. Nevertheless complementation was observed between *nit1* and NitM mutants (putatively defective in synthesis of the molybdenum-containing cofactor) derived from the same parent isolate in two instances. We now report the recovery of stable NitM mutants, and use these along with stable *nit1* mutants to demonstrate the existence of multiple VCGs in field strains of *B. fuckeliana*.

Materials and methods

Media and growth conditions

Malt Extract Agar (MEA, Oxoid, Basingstoke, England) was used for routine culturing. MEA + NaCl comprised MEA amended with NaCl ($40\,g\,l^{-1}$). Minimal media were based on Vogel's medium N (Vogel, 1964) with NH_4NO_3 excluded from the concentrated salts mixture and $FeSO_4$ substituted for $Fe(NH_4)_2(SO_4)_2$ in the trace element solution, and amended with sucrose ($20\,g\,l^{-1}$) and agar ($15\,g\,l^{-1}$). Nitrogen sources were added as follows: nitrate as potassium nitrate at $1.01\,g\,l^{-1}$ (MM + NO_3); nitrite as potassium nitrite at $0.85\,g\,l^{-1}$; ammonium as ammonium tartrate at $0.92\,g\,l^{-1}$; hypoxanthine at $0.1\,g\,l^{-1}$ (MM+HX); uric acid at $0.1\,g\,l^{-1}$. MM+ClO_3 medium comprised MM + NO_3 amended with potassium chlorate ($30\,g\,l^{-1}$). MM + NO_3 + Triton comprised MM + NO_3 amended with Triton X-100 ($0.5\,ml\,l^{-1}$).

Cultures were incubated in vented plastic petri dishes in the dark at 20–22 °C, except for the preparation of spore inoculum when cultures were incubated under a 12-h on/12-h off cycle of a mixture of white and near-UV fluorescent light. Mycelial plug inoculum (5-mm diameter) was taken from the actively growing margin of 3–4-day-old MEA colonies. Spore inoculum was prepared by suspending conidia from 1–3-week-old MEA light-grown cultures in sterile 0.01% Tween-80.

Fungal strains

Strains were stored as dried conidia on silica gel (Perkins, 1977). Working cultures were maintained on MEA slopes. The parental strains used are listed in Table 1; tester strains developed in this study are listed in Table 3. Strains lodged in International Collection of Micro-organisms from Plants (ICMP), are available from Landcare Research.

Nit mutants and testing for complementation and mycelial compatibility

To obtain Nit mutants, mycelial plugs were transferred mycelial side down to MM + ClO_3 using four plugs per plate and examined at intervals for up to 4 weeks. The first 15–20 sectors from each strain were 'purified' by being transferred individually to the margin of a fresh MM + ClO_3 plate and incubated for

Table 1. Sources and characteristics of *B. fuckeliana* strains

Strain[a]	Type[b]	ICMP No.	Mating type[c]	Host	Locality
REB666-1	scs	7596	*MAT1-1*	Kiwifruit	NZ, Bay of Plenty
REB658-1	scs	7577	*MAT1-2*	Kiwifriut	NZ, Auckland
REB702-1	scs	9430	*MAT1-1*	Grape	NZ, Marlborough
REB705-1	scs	9433	*MAT1-2*	Grape	NZ, Waikato
REB671-1	scs	7663	*MAT1-1*	Cucumber (protected)	NZ, Waikato
REB678-1	scs	7869	*MAT1-2*	Tomato (protected)	NZ. Mid Canterbury
A1	sas	13670	*MAT1-1*	Lab cross of REB658-1 × REB666-1	NZ
SAS56	sas	10934	*MAT1-1*	Lab cross	Italy
SAS405	sas	10935	*MAT1-2*	Lab cross	Italy

[a]Strains prefixed REB are single-spore isolates derived from mass field cultures stored in ICMP. Strains prefixed SAS are from F. Faretra (Italy).
[b]scs = single conidial strain, sas = single-ascospore strain.
[c]MAT1-1 = mating type 1, MAT1-2 = mating type 2.

Table 2. Nit mutations in *B. fuckeliana*, their designations and growth responses on various nitrogen sources (modified from Correll et al., 1987; Brooker et al., 1991)

Mutation	Designation[a]	Growth on nitrogen sources				
		Nitrate	Nitrite	Ammonium	Hypoxanthine	Uric acid
None	Wild type	+	+	+	+	+
Nitrate reductase structural gene	*nit1*	−	+	+	+	+
Major nitrogen regulatory gene	n.d.[b]	−	−	+	−	−
Pathway specific regulatory gene or nitrite reductase	Nit3	−	−	+	+	+
Molybdenum cofactor genes	NitM	−	+	+	−	+

[a]Single-locus mutations are italicised indicating genotype; phenotype designations are not italicised as more than one locus may confer these phenotypes.
[b]n.d. indicates this class of mutants has not yet been recognised, and thus not yet designated, in *B. fuckeliana*.

4 weeks. Sectors that had covered over half the plate by the end of 4 weeks were considered putative Nit mutants and were transferred from the colony margin to MEA, MM + NO_3 and MM + HX. After a further 4–5 days in the dark they were scored as showing 'minus nitrogen' growth (thin, spreading growth similar to that on nitrogen-free medium) or wild-type growth. Putative Nit mutants showing 'minus nitrogen' growth on MM + NO_3 medium and wild-type growth on MM + HX medium (putative *nit1* mutants), and mutants showing 'minus nitrogen' growth on both these media (putative NitM mutants) were subcultured from the colony margin of the MM + NO_3 plate, taking particular care to avoid areas that showed indications of prototrophic growth (putatively heterokaryotic). They were transferred to MEA plates, incubated under lights to allow sporulation, and stored on silica gel. Full phenotype tests were only conducted on the mutants chosen as testers. Strain were tested by comparing growth on media amended with 4 nitrogen sources and scored following responses recognised for other filamentous fungi (Table 2). These tests allowed our putative *nit1* mutants to be classified as either *nit1* or Nit3 mutants and our putative NitM mutants as either NitM mutants or mutants in the major nitrogen regulatory gene. Strains recovered from silica gel storage retained their designated phenotypes and showed no evidence of instability.

Complementation was tested by superimposing drops of spore suspensions (5–10 µl) of the isolates under test on MM + NO_3 + Triton. Results were scored after 6–8 days incubation, with a dense pad of growth indicating complementation and a thin faint pad indicating the absence of complementation (Figure 1). Mycelial compatibility was tested by placing mycelial plugs of the different isolates 2 cm apart, mycelium

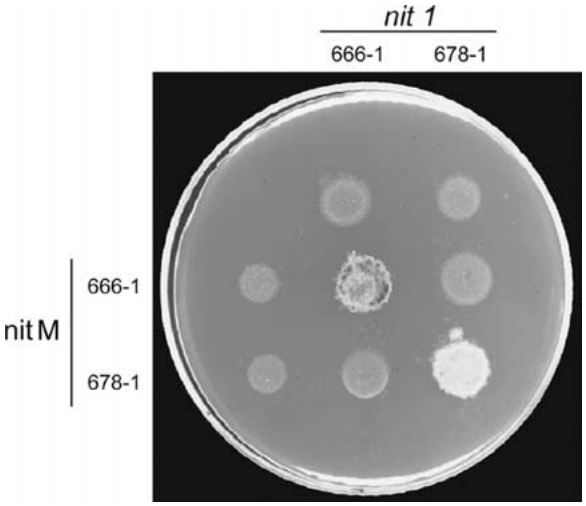

Figure 1. Complementation tests between *nit1* and NitM mutants derived from different field strains of *B. fuckeliana*. Mutants were inoculated as drops of spore suspension in 2 rows (nitM strains) and 2 columns (*nit1* strains) on MM + NO_3 + Triton and incubated for 8 days.

side down, on MEA + NaCl. They were scored after 14 days for the presence or absence of interaction lines (Beever and Parkes, 1993).

Results

Nit mutant isolation

Putative chlorate-resistant 'sectors' arose as spreading colonies with all nine parental strains. Some sectors showed strongly growing leader and secondary hyphae, resulting in a more or less uniform thin colony, and others showed poorly developed secondary hyphal growth, resulting in a spidery-like colony. Both putative *nit1* and putative NitM mutants were recovered from both morphological types and from all strains. Initially, a sample of only 10–15 sectors was checked for each strain which always gave multiple putative *nit1* strains; further sectors were checked only if putative NitM strains were not recovered in this first sample. Considering all nine strains, a total of 251 mutants was examined 81% of which were putative *nit1* mutants and 17% were putative NitM mutants (Table 3). The growth phenotypes of representative putative *nit1* and putative NitM mutants were checked on the five nitrogen sources and all behaved as expected, except one putative *nit1* strain (ICMP 14168) derived from strain SAS56. This strain showed 'minus nitrogen' growth on nitrate and nitrite indicating it was a Nit3 strain. Such mutants in filamentous fungi are typically defective in a pathway-specific regulatory gene but some are defective in nitrite reductase (Brooker et al., 1991). In this instance, we checked a further five putative *nit1* strains, all of which behaved as expected, and selected one as a *nit1* tester strain.

Complementation and incompatibility testing

Each *nit1* mutant was paired with each NitM mutant in duplicate. Complementation was observed in all *nit1* + NitM pairings from the same parent, but in no instances when pairings involved different parents (Figure 1). We conclude that each parental strain is a member of a distinct VCG. In some instances, more than one NitM mutant was recovered from the same parent. When such NitM strains were paired with each other, complementation was sometimes observed, indicating intergenic complementation. The Nit3 strain (ICMP 14168) and *nit1* tester strain (ICMP 14381) derived from parent SAS56, both complemented the NitM tester strain (ICMP 14169) from this parent. The 9 VCGs represented by each of the parental strains were allocated unique 3-figure numbers; *nit1* and NitM tester strains for each group are stored in culture collection ICMP (Table 3).

All 9 parental strains were paired on MEA + NaCl to test for incompatibility. Dark interaction zones formed in all tests except between the self pairings, indicating all 9 isolates were mycelially incompatible with each other. The zones consisted either of a single narrow interaction line or a pair of such lines.

Discussion

There have been a number of reports of Nit mutants in *B. fuckeliana* (Levis et al., 1997; Weeds et al., 1998; White et al., 1998; Delcán and Melgarejo, 2002). Of these, only Delcán and Melgarejo (2002) report the existence of NitM mutants and they found their strains were unstable. We suspect that two features of our protocol are important in obtaining stable Nit mutants. First, we use a chlorate medium in which nitrate is the sole nitrogen source. Some previous workers have used a chlorate medium containing a reduced nitrogen source such as ammonium, urea or asparagine. We suggest that the absence of reduced nitrogen and the

Table 3. Recovery of Nit mutants and designation of VCGs and tester strains of *B. fuckeliana*

Parent strain	No. of mutants screened	Percent putative *nit1* mutants recovered (%)	Percent putative NitM mutants recovered (%)	Slow growing/score inconclusive(%)	VCG	ICMP No. of *nit1* and nitM tester strains
REB666-1	10	90	10	0	001	14120 (*nit1*) 14121 (nitM)
REB658-1	49	86	14	0	002	14122 (*nit1*) 14123 (nitM)
REB702-1	50	68	28	4	003	14124 (*nit1*) 14125 (nitM)
REB705-1	10	80	20	0	004	14127 (*nit1*) 14126 (nitM)
REB671-1	12	83	17	0	005	14128 (*nit1*) 14129 (nitM)
REB678-1	12	75	25	0	006	14131 (*nit1*) 14130 (nitM)
A1	15	93	7	0	007	14166 (*nit1*) 14167 (nitM)
SAS56	51	88	10	2	008	14381 (*nit1*) 14169 (nitM)
SAS405	42	62	26	12	009	14255 (*nit1*) 14254 (nitM)

presence of nitrate results in high expression of nitrate reductase activity and hence greater inhibition by the nitrate analogue chlorate. It is generally considered that chlorate itself is not toxic to fungi but is reduced to the toxic hypochlorite by nitrate reductase (Correll et al., 1987). Second, our final subculture of putative Nit strains is from the margin of a colony expressing 'minus nitrogen' type growth when growing on nitrate as a sole nitrogen source. We propose that growth for a period on nitrate medium will favour the expression of any wild-type nuclei that may have been present in the heterokaryotic state in the putative mutant growing on the chlorate-amended medium, and allow such potentially 'unstable' strains to be avoided.

The use of complementary mutants has been widely used to define VCGs in many filamentous fungi (Leslie, 1993; Correll and Gordon, 1999; Malik and Vilgalys, 1999; Glass et al., 2000). Our results showing that all 9 strains of *B. fuckeliana* tested are in different VCGs, indicating that there are a minimum of 9 VCGs in this species, but given the fact that only 9 strains have been examined it is likely that many more VCGs occur in nature. Mycelial compatibility groups (MCGs) defined by the mycelial incompatibility tests matched those defined by the complementation tests, in that interaction zones formed between all of the 9 parental strains. However, we have found that classifying field strains into distinct groups using the mycelial compatibility test described is often difficult, with the number and intensity of dark lines varying depending on combination. Thus we do not recommend, unlike Delcán and Melgarejo (2002), that the mycelial test be used to define VCGs in *B. fuckeliana* and suggest that the distinction between MCGs and VCGs be maintained. In another member of the Sclerotiniaceae, *Sclerotinia sclerotiorum*, no direct correlation was found between groups defined by complementation and those defined by mycelial interaction (Ford et al., 1995). It is apparent from Tables 1 and 3 that there is no simple correlation between VCG and host, geographic location, or mating type. To coordinate observations among *B. fuckeliana* workers, we propose a simple numerical system to identify VCGs and recommend deposition of tester strains for each VCG in a publicly available culture collection.

The existence of multiple VCGs in the *B. fuckeliana* population is consistent with previous observations showing that dsRNA profiles, indicative of mycovirus presence, are very variable and few field strains show the same profiles (Howitt et al., 1995). Because mycoviruses are transmitted only by hyphal fusion, this suggests such fusions are not common in nature.

Recognition of VCGs is hampered in some species by the existence of heterokaryon self-incompatibility, where some strains cannot undergo hyphal fusion even with themselves (Leslie, 1993). The ready

complementation shown by the chosen *nit1*/NitM tester mutants indicates that none of the parental strains in our study is self-incompatible.

The genetic basis of vegetative incompatibility in *B. fuckeliana* is not known, but it is likely to conform to the system found in diverse genera of filamentous ascomycetous fungi including plant pathogenic species of *Fusarium*, *Colletotrichum*, and *Verticillium* (Leslie, 1993; Correll and Gordon, 1999; Malik and Vilgalys, 1999). The genetic and molecular basis of these interactions has been clarified in a number of species (Glass et al., 2000; Saup, 2000). Vegetative incompatibility has been shown to be typically determined by series of genes variously referred to as *vic* (vegetative incompatibility), *vc* (vegetative compatibility) or *het* (heterokaryon incompatibility) genes. These genes exist in two or occasionally more allelic states, and strains that carry identical alleles at all loci are compatible. Various numbers of *vic* genes have been recognised in different species, with at least 11 in *N. crassa*, 8 in *Aspergillus nidulans* and 7 in *Cryphonectria parasitica* (Glass et al., 2000). VCGs are determined by unique combinations of *vic* genes, such that if 6 *vic* loci with 2 alleles per locus are segregating in a population, 64 (2^6) VCGs are theoretically possible. Our finding that single-ascospore strain A1 belonged to a different VCG from either of its parents (Table 2), is consistent with this bi-allelic system, but further genetic studies are needed to better clarify the system in *B. fuckeliana*.

Expectations as to the actual number of VCGs in natural populations differ depending on the role of sexual recombination (Leslie, 1993; Correll and Gordon, 1999; Huang et al., 2001). If sexual recombination is common, new VCGs will be continually generated and large numbers of VCGs are expected in the population. If sexual recombination is rare, or absent as in asexual populations, it is anticipated that the number of VCGs will be relatively low. While our sample of 6 field strains is relatively small, the observation that all are in different VCGs supports the suggestion that sexual recombination plays an important role in the field (Giraud et al., 1999), despite the observation that apothecia have only been observed infrequently in nature (Grindle, 1979). This conclusion is consistent with the finding that most field strains are sexually competent in the laboratory, and that the frequency of the two alleles of the mating-type genes (*Mat1–1* and *Mat1–2*) is approximately equal in all populations (Beever and Parkes, 1993; Faretra and Pollastro, 1993; Delcán and Melgarejo, 2002).

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