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Microsatellite stability in the plant pathogen *Botrytis cinerea* after exposure to different selective pressures

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ABSTRACT

The stability of microsatellite markers was investigated in the spore-producing fungus *Botrytis cinerea* exposed to four growth conditions. This knowledge is essential in order to differentiate mutations from genetic exchanges or recombination in population genetics studies. It is also important when using strains from collections that need to be regularly propagated on medium. Successive spore generations of four isolates of the fungus were realised in plates on different agar media: a nutrient-rich medium, a nutrient-poor medium, a medium supplemented with the antibiotic pyrrolnitrin and a medium supplemented with the fungicide iprodione. The stability of nine microsatellite markers was studied by comparing the molecular pattern obtained between the wild type parent strains and the final generations obtained. The results showed that, despite the phenotypic changes observed in some generations, no changes were observed in the allele size at nine microsatellite loci whatever the selective pressure endured by the fungus. This is the first study that reveals long-term stability of microsatellite markers of a spore-producing fungus exposed to different stresses.

Introduction

During their natural life or during laboratory experiments, microorganisms undergo successive cycles of reproduction and are exposed to various environmental conditions. Some of these conditions may represent a stress to which they have to adapt. For certain fungi, stresses such as nutritive deficiency could increase mutation rates in the genome (Drake *et al.* 1998) by modifying the activity of transposable elements and inducing systems involved in the modification or repair of DNA (Mes *et al.* 2000; Ikeda *et al.* 2001).

Botrytis cinerea Pers.:Fr (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is a haploid necrotrophic fungal pathogen of numerous plants estimated to more than 200 species (Jarvis 1980). It is considered a high-risk plant pathogen due to its capacity to produce a massive amount of asexual spores (conidia) in a short time in absence of efficient control method (Nicot & Baille 1996) and to adapt to various control methods. Nine microsatellite markers were characterized in the fungus *B. cinerea* (Fournier *et al.* 2002) and have been applied to strain typing in population genetic studies (Karchani-Balma *et al.* 2008; Vaczy *et al.* 2008; Decognet *et al.* 2009). Microsatellite markers (simple sequence repeats) are short tandem repeated tracts of DNA composed of units that are 1–6 base pairs long, spread throughout the genome and used as genetic markers in genome mapping or population genetics studies because of their high level of polymorphism (Jarne & Lagoda 1996). Their mutation rates are thought to vary from 10^{-6} to 10^{-3} per locus per gamete per generation depending on organism and locus (Weber & Wong 1993; Schug *et al.* 1997). Population studies

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of *B. cinerea* using microsatellite markers reveal a high level of genotypic variation (Fournier *et al.* 2002; Karchani-Balma *et al.* 2008; Vaczy *et al.* 2008; Decognet *et al.* 2009). This high genetic variability is interpreted as an indication of genetic exchange between distant populations (Karchani-Balma *et al.* 2008; Decognet *et al.* 2009) and/or an indirect proof of recombination (Karchani-Balma *et al.* 2008; Vaczy *et al.* 2008). However, mutations in the microsatellite loci overtime may also reveal diversity and therefore misinterpret the conclusions of these studies. In order to properly apply these markers in population studies, one should estimate the mutation rate of the microsatellite markers used. Lasker & Ran (2004) demonstrated the short-term stability of microsatellite profiles after the serial culture of *Penicillium marneffei* during 7–8 weeks. More recently, Kohn *et al.* (2008) reported that no mutations were detected in alleles of seven microsatellite loci after 400 d of continuous *in vitro* hyphal growth of 12 strains of the plant pathogenic ascomycete *Sclerotinia sclerotiorum*. However, studies regarding the stability of microsatellites markers in fungi that produce spores and are exposed to different selection pressure are missing.

The main purposes of this study were (i) to test the stability overtime of the nine microsatellite markers developed for this fungus and (ii) to estimate the effect of different growth conditions on this stability in the case of the sporulating filamentous fungus *B. cinerea*.

Materials and methods

Characteristics of Botrytis cinerea *isolates*

Four single-spore isolates of *Botrytis cinerea* originating from France, with differing phenotypic and genotypic characteristics (Table 1) were used in this study. They were all isolated from tomato between 1989 and 1991. They were maintained in stock cultures stored at −20 °C in a 0.06 M phosphate buffer containing 20 % (V/V) of glycerol. Inoculum was produced in Petri dishes on Potato Dextrose Agar medium (PDA 39 g L^{-1}; Difco Laboratory, Detroit, MI, USA) incubated under cool white fluorescent light (14-h photoperiod – 65 $\mu mol\, m^{-2} s^{-1}$) in a growth chamber at 21 °C.

In vitro *conidial-transfer experiments and growth conditions*

Successive spore generations were produced in 55 mm diameter Petri plates on four agar media providing different growth conditions: a nutrient-rich medium (PDA), a nutrient-poor medium (containing 1 g L^{-1} glucose and 15 g L^{-1} agar), a medium (PDA) amended with the antibiotic pyrrolnitrin (Sigma chemical) and a medium (PDA) amended with the anti-*Botrytis* fungicide iprodione (BASF Agro SAS). At the start of the experiment, a 40 μL drop containing 40 000 spores was deposited at the centre of a Petri dish containing unamended PDA medium. A colony developed and sporulation occurred within 3–5 d. These spores were harvested in 2 mL of a 0.06 M phosphate buffer containing 20 % (V/V) glycerol and their concentration was adjusted to 10^4 spores mL^{-1}, using the same buffer. As germination did not occur on spore-bearing conidiophores, all the spores harvested from a Petri plate were considered to constitute a generation. To produce the next generation, 40 μL aliquots of this suspension were then deposited at the centre of fresh Petri plates containing the desired growth medium. After 14 or 30 d of incubation, depending on the conditions tested, the average number of spores produced on those plates was close to 10^8. The spores were harvested and suspensions were prepared as described above. From each suspension, 40 μL aliquots were used to inoculate fresh plates, thus producing the next generation, and the remaining spores were stored at −20 °C until they were used for phenotypic and genotypic characterizations.

A total of 20 successive generations were produced for each type of PDA medium. On PDA medium supplemented with pyrrolnitrin or iprodione the first ten generations were produced using a constant concentration of the compound and the ten last generations were produced with increasing doses of the compound (Table 2). For all generations produced on PDA medium, an incubation period of 14 d at 21 °C allowed sufficient conidial production of *Botrytis cinerea* to obtain the next generation. For the generations produced on the nutrient-poor medium, the incubation period was prolonged to

Table 1 – Phenotypic and genotypic characteristics of the four isolates of *Botrytis cinerea* used in this study.

Isolate of *Botrytis cinerea*	Haplotype[a]	Phenotypic characters						
		Aggressiveness[b]		Pyrrolnitrin	Chemical family (active ingredients of fungicides)			
		Tomato plant	Apple fruit		Dicarboximides (iprodione, vinclozolin)	Benzimidazoles (carbendazim)	Phenylpyrroles (fludioxonil)	Aromatic Hydrocarbons (dicloran)
BC1	H5	+++	+++	S[c]	R	S	S	R
BC25	H437	−	++	S	S	S	S	S
BC26	H829	+	++	S	R	S	S	R
H6	H830	−	++	S	S	R	S	S

a Haplotype code was based on the allele size of the nine microsatellite loci (Decognet *et al.* 2009).

b +++ = high level of aggressiveness, ++ = moderate level of aggressiveness, + = low level of aggressiveness, − = not aggressive based on results obtained by Ajouz *et al.* (2010).

c S = susceptible, R = resistant. For pyrrolnitrin, the dose used was 100 μg L^{-1} (Ajouz *et al.* 2010). For the fungicides, the discriminatory doses used were based on those proposed by Leroux *et al.* (1999).

Table 2 – Composition of the medium used for the production of the different generations of the different isolates of *Botrytis cinerea* with the four different growth conditions studied.

Growth conditions	Isolates tested	Nomenclature of generations[a]	Composition of medium for the production of the different generations[b]						
			G0	G1–G10	G11–G12	G13–G14	G15–G16	G17–G18	G19–G20
Rich medium	BC1, BC25, BC26, H6	G*n*C	PDA	PDA	PDA	PDA	PDA	PDA	PDA
Poor medium	BC1, BC25, BC26, H6	G*n*N	PDA	Glucose 1 g L^{-1}	–	–	–	–	–
Pyrrolnitrin	BC1, BC25, BC26, H6	G*n*P	PDA	10 μg L^{-1}	100 μg L^{-1}	500 μg L^{-1}	1000 μg L^{-1}	2000 μg L^{-1}	4000 μg L^{-1}
Iprodione	BC1, BC25, BC26	G*n*I	PDA	5 mg L^{-1}	10 mg L^{-1}	20 mg L^{-1}	40 mg L^{-1}	100 mg L^{-1}	200 mg L^{-1}

a *n* is the number of generation realised (from 0 to 20).

b For pyrrolnitrin and iprodione stresses, the different doses of the antibiotic or fungicide used were added to PDA medium.

30 d to adjust for a slower growth rate of the fungal strains. As a consequence, only ten generations were produced in the span of the study.

Generations were produced for each of the four strains used in this study, except for H6 on iprodione. For each isolate and set of growth conditions tested, three repetitions (lineages) of the successive generations were carried out independently. For some reasons after storage, some of the lineages produced were not able to grow anymore on PDA medium. Therefore they were not used for phenotypic and genotypic characterizations.

To avoid repeating long descriptions when referring to different generations, each one was given a code G*n*X, where *n* represented the generation rank (starting with 0 for the parental strain) and X took the values C, P, I or N if the generations were produced on, PDA, pyrrolnitrin-amended PDA, iprodione-amended PDA or on nutrient-poor medium, respectively (Table 2).

The cumulated durations of the successive cultures realised in the different conditions are equivalent to 140 d for G10P, 280 d for G20C and G20I, 294 d for G20P and 300 d for G10N.

Microsatellite marker analyses

To assess the stability of microsatellites, the microsatellite profiles of the parent isolates (G0) were compared to those of different lineages of the generations produced on PDA (G20C), on pyrrolnitrin (G10P and G20P), on iprodione (G20I) and in condition of nutritive stress (G10N).

Genomic DNA was extracted from 15 mg of lyophilized fungal material, obtained following the protocol described by Decognet *et al.* (2009), in 96-well plates according to the DNeasy Plant extraction Kit (Qiagen, Chatsworth, CA, USA). The microsatellite analysis was performed with the nine markers developed for *Botrytis cinerea* (Fournier *et al.* 2002). These nine microsatellites loci present different size ranges and various types of core repeat motifs (Fournier *et al.* 2002). Seven of the nine microsatellite markers are 2 base pair (bp) repeats, Bc4 is 3 bp repeat and Bc1 is a joint microsatellite loci with two core sequences of 2 bp and 4 bp repeats separated by about 50 bp. They are generally well distributed throughout the genome of *B. cinerea* except BC9 and BC10 which are very close to each other (Pradier, pers. comm.). The polymerase chain reactions were carried out with forward primers conjugated with fluorescent dyes IRD-700 or IRD-800 (MWG-Biotech, Courtaboeuf, France) and the PCR products were separated on a LI-COR IR^2 (LI-COR Biosciences, Lincoln, USA) sequencer (Decognet *et al.* 2009). Two internal reference strains (S1-4639 and S1-10877) were run along with samples to ensure constancy of allele sizing across the gels, as well as a 50–350 bp size ladder (ScienceTec, Les Ulis, France). Allele sizing was performed using SAGA^{GT} software (LI-COR). Each isolate was attributed a haplotype code based on the work from Decognet *et al.* (2009).

Phenotypic characterization

To assess the phenotypic stability of the isolates grown under various growth conditions, four traits were used to compare the parent isolates (G0) to different lineages of the generations produced on PDA (G20C), on pyrrolnitrin (G10P and G20P), on iprodione (G20I) and in condition of nutritive stress (G10N). The phenotypic traits included aggressiveness to tomato and apple and sensitivity to pyrrolnitrin and to fungicides.

Tests of aggressiveness on apple fruits cv. Golden and on tomato plants cv. Monalbo were carried out as described by Ajouz *et al.* (2010). For a generation of a given isolate, the aggressiveness of the three lineages was tested in a growth chamber with a photoperiod of 14 h and maintained at 21 °C with a relative humidity above 90 %. After inoculation of a calibrated spore suspension (10^6 spores mL^{-1}), the diameter of lesions on apples and the length of stem lesions on tomatoes were monitored daily for 7 d after inoculation. The experiments were all repeated three times independently per lineage, each with three replicate plants or fruits. To take into account the kinetics of disease development for each isolate, we computed the Area Under the Disease Progress Curve (AUDPC) as described by Decognet *et al.* (2009). To facilitate the comparison of aggressiveness, each generation of a given isolate was categorized as high level of aggressiveness (+++), moderate level of aggressiveness (++), low level of aggressiveness (+) and not aggressive (–) in relation to reference strain BC1.

Sensitivity to the antibiotic pyrrolnitrin was assessed as described by Ajouz *et al.* (2010) using the discriminatory inhibition dose of 100 μg L^{-1}. Sensitivity to fungicides was defined using the discriminatory inhibition concentrations (Leroux *et al.* 1999). For a generation of a given isolate, the level of resistance to pyrrolnitrin and fungicides of the three lineages was tested using a spore germination test at 21 °C. The experiments were all repeated three times independently per

Table 3 – Phenotypic characteristics of the four wild type isolates of *Botrytis cinerea* and their generations produced on various growth conditions: level of aggressiveness on tomato plant and on apple fruit, sensitivity to pyrrolnitrin and to different fungicides.

Isolate	Generation (number of lineages)[a]	Growth conditions[b]	Phenotypic characters						
			Aggressiveness[c]		Pyrrolnitrin[d]	Chemical family (active ingredients of fungicides)[d]			
			Tomato	Apple		Dicarboximides (iprodione, vinclozolin)	Benzimidazoles (carbendazim)	Phenylpyrroles (fludioxonil)	Aromatic Hydrocarbons (dicloran)
BC1	G0		+++	+++	S	R	S	S	R
	G20C (2)	Rich medium	+++	+++	S	R	S	S	R
	G10N (3)	Poor medium	+++	+++	S	R	S	S	R
	G10P (3)	Pyrrolnitrin 10	+++	+++	S	R	S	S	R
	G20P (3)	Pyrrolnitrin 4000	+	+	R	R	S	R	R
	G20I (3)	Iprodione	+	+	nd	R	S	R	R
BC25	G0		−	++	S	S	S	S	S
	G20C (3)	Rich medium	−	++	S	S	S	S	S
	G10N (3)	Poor medium	−	++	S	S	S	S	S
	G10P (3)	Pyrrolnitrin 10	−	++	S	S	S	S	S
	G20P (3)	Pyrrolnitrin 4000	−	+	R	R	S	R	R
	G20I (3)	Iprodione	−	+	nd	R	S	R	R
BC26	G0		+	++	S	R	S	S	R
	G20C (2)	Rich medium	+	++	S	R	S	S	R
	G10N (2)	Poor medium	+	++	S	R	S	S	R
	G10P (3)	Pyrrolnitrin 10	+	++	S	R	S	S	R
	G20P (2)	Pyrrolnitrin 4000	−	+	R	R	S	R	R
	G20I (3)	Iprodione	−	+	nd	R	S	R	R
H6	G0		−	++	S	S	R	S	S
	G20C (2)	Rich medium	−	++	S	S	R	S	S
	G10N (2)	Poor medium	−	++	S	S	R	S	S
	G20P (3)	Pyrrolnitrin 4000	−	+	R	R	R	R	R

a G0 is the wild type isolate and G10 and G20 are the tenth and twentieth generations obtained under the different growth conditions tested; the numbers of lineages tested are indicated between brackets.

b Rich medium = PDA, poor medium = glucose ($1\,g\,L^{-1}$) agar, pyrrolnitrin 10 = PDA + pyrrolnitrin at $10\,\mu g\,L^{-1}$, Pyrrolnitrin 4000 = PDA + increasing doses of pyrrolnitrin from 10 to $4000\,\mu g\,L^{-1}$, Iprodione = PDA + increasing doses of iprodione from 5 to $200\,mg\,L^{-1}$.

c +++ = high level of aggressiveness, ++ = moderate level of aggressiveness, + = low level of aggressiveness, − = not aggressive.

d S = susceptible, R = resistant, nd = not done. For pyrrolnitrin, the dose used was $100\,\mu g\,L^{-1}$ (Ajouz *et al.* 2010). For the fungicides, the discriminatory doses used were based on those proposed by Leroux *et al.* (1999).

lineage, each with three replicate plates. Each generation of a given isolate was categorized as susceptible (S) or resistant (R) to the given product.

Results

Phenotypic changes

Resistance to the antibiotic pyrrolnitrin was observed for all the lineages of the four isolates tested after a total of 20 successive generations on increasing doses of pyrrolnitrin (G20P) (Table 3). These results were extensively described previously (Ajouz *et al.* 2010). Modification of resistance to the fungicide fludioxonil was also observed for all the lineages of the four isolates tested after a total of 20 successive generations produced in presence of pyrrolnitrin (G20P) or iprodione (G20I) (Table 3). The two isolates sensitive to iprodione and dicloran (BC25 and H6) acquired resistance to these fungicides when grown on medium amended with pyrrolnitrin (G20P) or iprodione (G20I) (Table 3). Finally, no changes in the resistance of carbendazim were observed (Table 3). In parallel a modification in the level of aggressiveness on apple fruits and tomato plants was observed: the generations that acquired a resistance to pyrrolnitrin (G20P) were less aggressive compared to the corresponding control G0 isolates suggesting a fitness cost of resistance to pyrrolnitrin (Table 3).

Stability of the microsatellite genetic markers

The amplification products obtained with the nine microsatellites revealed that the four isolates used in this study presented different haplotypes (Table 1). For each isolate, after the generations realised on rich PDA medium (G20C), poor glucose medium (G10N), pyrrolnitrin-amended medium (G10P and G20P) or iprodione-amended medium (G20I), the

Table 4 – Allele sizes at the nine microsatellite loci for the four wild type isolates of *Botrytis cinerea* and their generations produced on various growth conditions.

Isolate	Generation (number of lineages)[a]	Growth conditions[b]	Allele sizes of the microsatellite loci[c]									Haplotype[d]
			Bc1	Bc2	Bc3	Bc4	Bc5	Bc6	Bc7	Bc9	Bc10	
			$(CTTT)_{10}$–…–$(CA)_2CG(CA)_{10}$	$(AC)_{12}AT(AC)_4$	$(GA)_{10}$	$(TTC)_6TTATC(TTC)_2$	$(AT)_{12}$	$(CA)_{10}$	$(TA)_9$	$(CT)_{11}$	$(AC)_{13}$	
BC1	G0		**235**	**159**	**217**	**125**	**157**	**116**	**123**	**148**	**187**	**H5**
	G20C (2)	Rich medium	235	159	217	125	157	116	123	148	187	H5
	G10N (3)	Poor medium	235	159	217	125	157	116	123	148	187	H5
	G10P (3)	Pyrrolnitrin	235	159	217	125	157	116	123	148	187	H5
	G20P (3)	Pyrrolnitrin	235	159	217	125	157	116	123	148	187	H5
	G20I (3)	Iprodione	235	159	217	125	157	116	123	148	187	H5
BC25	G0		**215**	**151**	**213**	**125**	**155**	**132**	**117**	**144**	**175**	**H437**
	G20C (3)	Rich medium	215	151	213	125	155	132	117	144	175	H437
	G10N (3)	Poor medium	215	151	213	125	155	132	117	144	175	H437
	G10P (3)	Pyrrolnitrin	215	151	213	125	155	132	117	144	175	H437
	G20P (3)	Pyrrolnitrin	215	151	213	125	155	132	117	144	175	H437
	G20I (3)	Iprodione	215	151	213	125	155	132	117	144	175	H437
BC26	G0		**219**	**173**	**219**	**125**	**159**	**120**	**119**	**148**	**181**	**H829**
	G20C (2)	Rich medium	219	173	219	125	159	120	119	148	181	H829
	G10N (2)	Poor medium	219	173	219	125	159	120	119	148	181	H829
	G10P (3)	Pyrrolnitrin	219	173	219	125	159	120	119	148	181	H829
	G20P (2)	Pyrrolnitrin	219	173	219	125	159	120	119	148	181	H829
	G20P (3)	Iprodione	219	173	219	125	159	120	119	148	181	H829
H6	G0		**235**	**159**	**217**	**125**	**157**	**124**	**121**	**146**	**173**	**H830**
	G20C (2)	Rich medium	235	159	217	125	157	124	121	146	173	H830
	G10N (2)	Poor medium	235	159	217	125	157	124	121	146	173	H830
	G20P (3)	Pyrrolnitrin	235	159	217	125	157	124	121	146	173	H830

a Between brackets are the number of lineages tested from each generation produced.

b Rich medium = PDA, poor medium = agar–glucose (1 g L^{-1}), pyrrolnitrin = PDA + pyrrolnitrin, Iprodione = PDA + iprodione.

c Name and repeat motif of each microsatellite locus used in the study (Fournier *et al.* 2002). In bold, the allele sizes of microsatellite loci for the wild type isolates G0.

d Haplotype code is based on the allele size of the nine microsatellite loci (Decognet *et al.* 2009).

amplicons obtained were all of similar sizes at the nine microsatellite loci whatever the motif and the size of the microsatellites core repeat are (Table 4). The haplotypes remained identical whatever the selective pressure endured by the fungus, revealing a good stability of the microsatellites genetic markers in *Botrytis cinerea* isolates (Table 4).

Discussion

The main purpose of the present study was to assess if an exposure of the filamentous fungus *Botrytis cinerea* to different environmental stresses during successive conidial generations could have an effect on the stability of the microsatellite markers. Four isolates of *B. cinerea* with three independent replicates per isolate were exposed to four different selective pressures. Whatever the number of generations realised on Petri plates (G10 or G20), the duration of the consecutive transfers (280–300 d of successive cultures) and the nature of stress endured by the fungus, no mutations were observed in alleles at the nine microsatellite loci studied. After 20 generations, approximately 2×10^9 asexual spores were produced for each lineage. As conidia of *B. cinerea* are multinucleate with numbers usually in the range of 3–6 (Beever & Weeds 2004), the number of nuclei produced for each of the four conditions tested probably approximates 10^{10}, without a single genotypic change observed in the microsatellite loci while phenotypic changes have been observed. Moreover, stability was not affected by the microsatellite loci tested. In yeasts, microsatellite instability increased as repetitive tracts became longer (Wierdl *et al.* 1997). This is the first study that reveals the stability of microsatellite markers after successive generations of a spore-producing fungus exposed to different growth conditions. This knowledge is essential in interpreting data from population genetics studies, for instance to differentiate mutation from genetic exchange or recombination. The stability in alleles at seven microsatellite loci was also observed in the non-sporulating plant pathogenic ascomycete *Sclerotinia sclerotiorum* after 400 d of continuous *in vitro* hyphal growth (Kohn *et al.* 2008). The short-term stability of polymorphic microsatellite marker profiles was also demonstrated in *Penicillium marneffei* isolates after 7–8 weeks of serial culture in liquid media (Lasker & Ran 2004).

Moreover, even if *in vitro* serial generations of conidia of *B. cinerea* is probably different from what really happen in the nature, the number of generations realised in this study is realistic compared to the theoretical number of infection cycle produced by the fungus on its plant host during a growing season. Actually, *B. cinerea* is able to produce massive amount of spores in only 7 d of incubation under optimal conditions on different plant tissues (Nicot *et al.* 1996). Therefore, we can assume that microsatellite markers may be used with confidence as population genetics markers for this spore-producing fungus.

Meanwhile, some of the selective pressures tested in this study induced phenotypic changes in *B. cinerea* isolates, such as a resistance to pyrrolnitrin and to some fungicides, and a reduction in aggressiveness to tomato plants and apple fruits (G20P and G20I). The stability of the microsatellite markers combined with the phenotypic modifications illustrates the fact that different phenotypes could possess the same genotype based on the use of these microsatellite markers. Moreover, the generations produced on PDA medium show that periodical transfer of isolates of *B. cinerea* to fresh medium, as commonly done in many laboratories, does not modify their microsatellite pattern. This stability is particularly important in population diversity studies when researchers test strains from collections that need to be regularly propagated on medium in the laboratory. To complement this study, the stability of microsatellites after successive cycles of sexual reproduction should be evaluated. Moreover, it would be interesting to understand better at the genome level what is happening during this selection process.

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REFERENCES

Ajouz S, Nicot PC, Bardin M, 2010. Adaptation to pyrrolnitrin in *Botrytis cinerea* and cost of resistance. *Plant Pathology* **59**: 556–566.

Beever RE, Weeds PL, 2004. Taxonomy and genetic variation of *Botrytis* and *Botryotinia*. In: Elad Y, Williamson B, Tudzynski P, Delen N (eds), Botrytis*: biology, pathology and control*. Kluwer Academic Press, Dordrecht, The Netherlands, pp. 29–52.

Decognet V, Bardin M, Trottin-Caudal Y, Nicot PC, 2009. Rapid change in the genetic diversity of *Botrytis cinerea* populations after the introduction of strains in a tomato glasshouse. *Phytopathology* **99**: 185–193.

Drake JW, Charlesworth B, Charlesworth D, Crow JF, 1998. Rates of spontaneous mutations. *Genetics* **148**: 1667–1686.

Fournier E, Giraud T, Loiseau A, Vautrin D, Estoup A, Solignac M, Cornuet JM, Brygoo Y, 2002. Characterization of nine polymorphic microsatellite loci in the fungus *Botrytis cinerea* (Ascomycota). *Molecular Ecology Notes* **2**: 253–255.

Ikeda K, Nakayashiki H, Takagi M, Tosa Y, Mayama S, 2001. Heat shock, copper sulfate and oxidative stress activate the retrotransposon MAGGY resident in the plant pathogenic fungus *Magnaporthe grisea*. *Molecular Genetics and Genomics* **266**: 318–325.

Jarne P, Lagoda PJL, 1996. Microsatellites, from molecules to populations and back. *Trends in Ecology & Evolution* **11**: 424–429.

Jarvis WR, 1980. Epidemiology. In: Coley-Smith JR, Verhoeff K, Jarvis WR (eds), *The Biology of Botrytis*. Academic Press, London, UK, pp. 219–250.

Karchani-Balma S, Gautier A, Raies A, Fournier E, 2008. Geography, plants, and growing systems shape the genetic structure of Tunisian *Botrytis cinerea* populations. *Phytopathology* **98**: 1271–1279.

Kohn LM, Schaffer MR, Anderson JB, Grunwald NJ, 2008. Marker stability throughout 400 days of in vitro hyphal growth in the filamentous ascomycete, *Sclerotinia sclerotiorum*. *Fungal Genetics and Biology* **45**: 613–617.

Lasker BA, Ran Y, 2004. Analysis of polymorphic microsatellite markers for typing *Penicillium marneffei* isolates. *Journal of Clinical Microbiology* **42**: 1483–1490.

Leroux P, Chapeland F, Desbrosses D, Gredt M, 1999. Patterns of cross-resistance to fungicides in *Botryotinia fuckeliana* (*Botrytis cinerea*) isolates from French vineyards. *Crop Protection* **18**: 687–697.

Mes JJ, Haring MA, Cornelissen BJC, 2000. Foxy: an active family of short interspersed nuclear elements from *Fusarium oxysporum*. *Molecular and General Genetics* **263**: 271–280.

Nicot PC, Baille A, 1996. Integrated control of *Botrytis cinerea* on greenhouse tomatoes. In: Morris CE, Nicot PC, Nguyen-The C (eds), *Aerial Plant Surface Microbiology*. Plenum Press, New-York, pp. 169–189.

Nicot PC, Mermier M, Vaissiere BE, Lagier J, 1996. Differential spore production by *Botrytis cinerea* on agar medium and plant tissue under near-ultraviolet light-absorbing polyethylene film. *Plant Disease* **80**: 555–558.

Schug MD, Mackay TFC, Aquadro CF, 1997. Low mutation rates of microsatellite loci in *Drosophila melanogaster*. *Nature Genetics* **15**: 99–102.

Vaczy KZ, Sandor E, Karaffa L, Fekete E, Arnyasi M, Czegledi L, Kovics GJ, Druzhinina IS, Kubicek CP, 2008. Sexual recombination in the *Botrytis cinerea* populations in Hungarian vineyards. *Phytopathology* **98**: 1312–1319.

Weber JL, Wong C, 1993. Mutation of human short tandem repeats. *Human Molecular Genetics* **2**: 1123–1128.

Wierdl M, Dominska M, Petes TD, 1997. Microsatellite instability in yeast: dependence on the length of the microsatellite. *Genetics* **146**: 769–779.