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Two new primers highly specific for the detection of *Botrytis cinerea* Pers.: Fr.

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Summary. Of thirty-nine *Botrytis cinerea* isolates originating in different host-plants and grown in pure cultures, twenty-six produced abundant grey aerial mycelium and sporulated intensely, whilst thirteen produced a thin mycelial layer, abundant sclerotia and secreted an unidentified yellow pigment in PDA culture media. The commonly used $C_{729}^{+/-}$ primers (5'-AGCTCGAGAGAGATCTCTGA-3'; 5'-CTGCAATGTTCTGCGTGGAA-3') designed to detect *B. cinerea* did not amplify the DNA fragment of 0.73 kb in this smaller group of strains under standard conditions, whereas a shorter DNA fragment (0.60 kb) was amplified at a lower annealing temperature (50°C). This fragment was sequenced and two new internal primers were designed, BC_{108}^{+} (5'-ACCCGCACCTAATTCGTCAAC-3') and BC_{563}^{-} (5'-GGGTCTTCGATACGGGAGAA-3'). These new primers were used to amplify a DNA fragment of 0.48 kb for the main group of 26 *B. cinerea* strains and a shorter fragment (0.36 kb) for the smaller group of 13 strains due to a deletion of 0.12 kb, which was not detected with the primers $C_{729}^{+/-}$. All the strains were amplified to detect the presence or absence of *Boty* and *Flipper* transposable elements. No correlation was found between strains possessing the deletion and those belonging to either the *vacuma* or the *transposa* sibling species. Other closely related *Botrytis* species such as *B. allii* and *B. fabae* were not amplified with these primers, confirming their specificity for *B. cinerea* and enhancing the sensitivity of the molecular tools available to detect this fungus in host-plants.

Key words: molecular marker, specific primers, PCR.

Introduction

Botrytis cinerea Pers.: Fr. is a necrogenous saprophyte (Whetzel, 1945) that causes grey mould on many economically important crops, including grapes, berries, vegetables and ornamental crops (Jarvis, 1977). It has been identified in more than 200 plant species, with no apparent host specificity (Jarvis, 1980).

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Variation in the phenotypic features of this fungus has led mycologists to consider *B. cinerea* a collective anamorph (Whetzel, 1945). Genetic and phenotypic variability within and among isolates of *B. cinerea* have long been reported (Hansen and Smith, 1932; Grindle, 1979; Van Kan *et al.*, 1993; Van der Vlugt-Bergmans *et al.*, 1993; Vallejo *et al.*, 1996; Kerssies *et al.*, 1997; Levis *et al.*, 1997; Vallejo *et al.*, 2002). The genetic diversity was attributed to the multinucleated heterokaryotic state of the fungus, which was mainly due to mycelial anastomosis (Hansen and Smith, 1932; Faretra and Antonacci, 1987). Phenotypic diversity could be due to various mechanisms that silence

some nuclei, leading to their inactivation (Hamada *et al.*, 1997). Some authors (Giraud *et al.*, 1997; Giraud *et al.*, 1999) identified two sibling species in the *B. cinerea* complex, named *transposa* and *vacuma*. These species are genetically distinct since both transposable elements *Boty* and *Flipper* occur in *transposa,* while they are lacking in *vacuma*. Muñoz *et al*. (2002) also detected molecular differences between the Chilean isolates of *B. cinerea,* which allowed both sibling species to be recovered. Like Giraud *et al*. (1997), they attributed this genetic diversity between the sibling species to sexual reproduction, even if apothecia of the fungus are rarely observed under natural conditions (Faretra and Antonacci, 1987; Pezet *et al.*, 2004). Parasexuality and aneuploidy may also be involved (Buxton, 1956; Pontecorvo, 1956; Leach and Rich, 1969; Genovesi and Magill, 1976; Büttner *et al.*, 1994).

The isolation of this fungus from many host-plants, presented in this paper, showed that about a third of pure *B. cinerea* strains produce fewer conidia, more sclerotia and a yellow pigmentation in the culture medium. The primers designed for the specific DNA marker previously described by Rigotti *et al*. (2002) did not amplify the DNA fragments of this smaller group of strains under standard conditions, so that these particular *B. cinerea* strains were not detected by those primers. In this paper, we report on the design of improved new specific primers allowing amplification of distinct bands for both the standard and the special *B. cinerea* strains isolated.

Materials and methods

Fungal isolates and PCR amplification

The origins of the *B. cinerea* isolates used in this study are shown in Table 1. The strains were grown in Petri dishes on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA) at 21°C and a 12-h day.

PCRs were performed directly on each strain without DNA extraction, according to Gindro *et al*. (Gindro *et al.*, 2005), except that on non-sporulating strains mycelium was used as starting material. The collected fungal cells were briefly swirled in 18 μl sterile water. The tubes were transferred to a thermocycler (PC-Personal Cycler, Biometra, Gottingen, Germany) pre-heated to 97°C for 15 min to break the cells. The thermocycler was then held at 4°C and 7 μl of the PCR mix was added, namely 2 units of Taq (Taq DNA Polymerase, Qiagen, Hilden, Germany); 0.4 μM of each primer (C729+/C729–) according to Rigotti *et al*. (2002) which amplify a 0.73 kb band specific to *B. cinerea*; 0.2 mM of each dNTP; 3 mM $MgCl_2$ and 1× PCR buffer. Amplification was performed with one cycle at 95°C for 3 min, followed by 34 cycles at 94°C for 20 s, 54°C for 20 s and 72°C for 30 s. Conserved primer sequences for PCR amplification of the nuclear ribosomal RNA (partial small subunit rDNA (SSU) - internal transcribed spacer 1 (ITS1) - 5.8s - internal transcribed spacer II (ITSII) - partial large subunit rDNA (LSU), namely primers LR1 (5'- GGTTGGTTTCTTTTCCT -3') and SR6R (5'- AAGWAAAAGTCGTAACAAGG -3') were used in the same conditions as described above, except that the annealing temperature was 54°C. The transposable elements *Boty* and *Flipper* were amplified in the same reaction conditions as above except that the annealing temperature was 55°C. A fragment of 604 bp for *Boty* was amplified using primers Boti442+ (5'-ACGGGCTATACGAGTACCAAG-3') and Boti1006 (5'-GGTTGGCTCAGAACTGCTCC-3'); in the same way, a fragment of 1.25 kb for *Flipper* was amplified using primers F300 (5'-GCACAAAACCTACAGAAGA-3') and F1500 (5'-ATTCGTTTCTTGGACT-3'). Gel electrophoresis of PCR products was performed on 1% agarose gel.

Cloning and sequencing

A final extension at 72°C for 3 min was added to clone the PCR product into pCR4Blunt-TOPO® (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Plasmids were purified on spin columns (Qiagen) and sent to Microsynth for sequencing. The sequences were aligned and analyzed using Blast search (http://www.ncbi.nlm.nih.gov/BLAST/).

Conidial size

The length and width of 100 conidia per isolate were measured with a light microscope (Laborlux S, Leica, Wetzlar, Germany), using a digital image analysis device (IM 500 - Leica). Classification analysis was carried out on the conidial length and width using discriminant analysis. Statistical anal-

Table 1. Code and origin of fungal isolates.

Isolate code	Species	Host plant	Origin
B16	*Botrytis cinerea*	*Vitis vinifera*	Switzerland[1] (Nyon)
B23	*B. cinerea*	*Fragaria* × *ananassa* (cv. Elsanta)	Switzerland (Conthey)
B36	*B. cinerea*	*Lactuca sativa*	Switzerland (Nyon)
B48	*B. cinerea*	*Lycopersicon esculentum*	Switzerland (Cadenazzo)
B54	*B. cinerea*	*Daucus carota* ssp. *sativus*	Switzerland (Granges)
B99	*B. cinerea*	*Allium cepa*	Switzerland (inconnu)
B105	*B. cinerea*	*Beta vulgaris* (var. conditiva)	Switzerland (inconnu)
B118	*B. cinerea*	*F.* × *ananassa* (cv. inconnu)	Switzerland (inconnu)
B125	*B. cinerea*	*Pelargonium zonale*	Switzerland (Trélex)
B157	*B. cinerea*	*Malus domestica* (cv. Gala)	Switzerland (Vétroz)
B158	*B. cinerea*	*Rubus fructicosus*	Switzerland (Begnins)
B161	*B. cinerea*	*Ocimum basilicum*	Switzerland (Orney)
B186	*B. cinerea*	*Heliantus annuus*	Switzerland (Denges)
B188	*B. cinerea*	*Rubus idaeus*	Switzerland (Begnins)
B190	*B. cinerea*	*Juglans* sp.	Switzerland (Gland)
B191	*B. cinerea*	*Valerianella locusta*	Switzerland (Riddes)
B192	*B. cinerea*	*Lactuca sativa* (var. Emerold)	Switzerland (Saillon)
B194	*B. cinerea*	*Cynara scolymus*	Italy
B197	*B. cinerea*	*Eriobotrya japonica*	France (Comps)
B199	*B. cinerea*	*Solanum melongena*	Switzerland (Tessin)
B201	*B. cinerea*	*Helianthus annuus* (var. Cadsol)	Switzerland (Neuchâtel)
B203	*B. cinerea*	*Capsicum annuum*	Switzerland (Prangins)
B205	*B. cinerea*	*Cydonia oblonga*	Switzerland (Begnins)
B207	*B. cinerea*	*Chrysanthemum* sp.	Switzerland (Trélex)
B208	*B. cinerea*	*F.* × *ananassa* (cv. Marmolada)	Switzerland (Cadenazzo)
B214	*B. cinerea*	*F.* × *ananassa* (cv. Kimberly)	Switzerland (Conthey)
B51*	*B. cinerea*	*Phaseolus vulgaris*	Switzerland (Marchissy)
B111*	*B. cinerea*	*Valerianella locusta*	Unknown
B142*	*B. cinerea*	*Brassica napus*	Unknown
B162*	*B. cinerea*	*Dahlia* sp.	Switzerland (Begnins)
B166*	*B. cinerea*	*Brassica oleracea*	Switzerland (Valeyres–sous–Rances)
B185*	*B. cinerea*	*Taraxacum officinale*	Switzerland (La Sarraz)
B187*	*B. cinerea*	*Ribes rubrum*	Switzerland (Sullens)
B189*	*B. cinerea*	*Lycopersicon esculentum*	Switzerland (Begnins)
B193*	*B. cinerea*	*Cucurbita maxima* (var. little Jack)	Switzerland (Nyon)
B198*	*B. cinerea*	*Prunus avium*	Switzerland (Nyon)
B200*	*B. cinerea*	*Helianthus annuus* (var. Sunluca)	Switzerland (Aubonne)
B204*	*B. cinerea*	*Origanum vulgare*	Switzerland (Nyon)
B206*	*B. cinerea*	*Rosa* sp.	Switzerland (Trélex)
B195	*B. allii* (= *B. aclada*)	*Allium cepa*	Switzerland (Vully)
B202	*B. allii*	*Vitis vinifera* (var. Gamay)	Switzerland (Nyon)
Bac1	*B. allii*	*Allium cepa*	CBS[2] (260.71)
Bfa1	*B. fabae*	*Vicia faba*	CBS (120.29)
Bfa2	*B. fabae*	*Vicia faba*	Switzerland (Lugnez)
F8	*Fusarium acuminatum*	*F.* × *ananassa* (cv. Kimberly)	Switzerland (Conthey)

[1] Swiss, Italian and French strains belong to the ACW mycology collection, Nyon, Switzerland.
[2] Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
*, Isolates producing yellow pigment.

ysis was carried out with the Systat 10 program (SPSS Inc., Chicago, IL, USA).

Monoconidial cultures

Single-spore cultures were obtained from isolates of *B. cinerea* grown on oatmeal agar (OMA, Difco) at 21°C for 7 days, with a 16-h day. Conidia were collected by vacuum aspiration and stored at -80°C until use, as described by Gindro and Pezet (2001).

A 200 μl aqueous conidial suspension (7×10^3 conidia ml^{-1}) was plated on water agar (WA, Oxoid, Unipath Ltd, Basingstoke, England). After overnight incubation at 21°C, germinated conidia were picked out, transferred individually to PDA and incubated at 21°C for 10 days, with a 16-h day. From single-spore cultures, a second generation was produced as described above. PCR amplification was performed directly on the aqueous conidial suspensions. PCR conditions were the same as above.

Pathogenicity assays

The aggressiveness of some *B. cinerea* isolates was determined by pathogenicity tests on apples according to the method of Shüepp and Küng (1978) with minor modifications. Five fruits were surface-sterilized with 70% ethanol and infected with 5-mm PDA discs of mycelial culture. Similar-sized PDA discs without the fungus served as the negative control. The infected apples were kept in a humid chamber at 21°C, with a 16-h day. After 5 days of incubation the size of the necrotic tissues was measured. The fungus from the necrotic zones was isolated on PDA in order to confirm by molecular techniques the identity of the *B. cinerea* strain used in the experiment.

Results

Thirty-nine isolates of *B. cinerea* were isolated each from a different host-plant. Of these, twenty-six showed the common characteristics of *B. cinerea* cultures, producing abundant grey mycelium and aerial sporulation. In further experiments, strain B23 was used as a type or reference strain for this more common group of *B. cinerea* strains. Another, smaller group of thirteen isolates produced less aerial mycelium in pure culture and fewer conidia but more sclerotia, and also produced a chemically unidentified yellow pigment in the PDA culture medium (*yellow strains)*. This yellow pigmentation was however not detected in isolates grown on other common media, such as OMA, corn meal agar, malt extract agar, Czapek dox broth, bean pod agar, Lima bean agar and Cooke rose bengal agar. In further experiments, isolate B204 was used as a reference strain for this group of strains. Cloning and sequencing of the entire ITS1-5.8s-ITS2 ribosomal DNA fragment confirmed the sequence gene of these strains and that they were species of *B. cinerea* (Holst-Jensen *et al*., 1997; EMBL accession number Z73765). Statistical analysis detected no significant differences in conidial size between the B23 type strains and the B204 type strains (12.24±1.12×6.9±0.66 μm for the B23-type strains vs. 11.75±1.08×6.83±0.53 μm for the B204 type strains) (Table 2). The average F-value of these isolates (4.63) was slightly smaller than the theoretical F-value (4.66). The conidial size of *B. fabae* (16.45±1.41×11.04±1.32 μm) and *B. allii* (7.75±0.98×4.20±0.51 μm) on the other hand was significantly different from that of *B. cinerea*. Dried specimens of isolates B23 (BPI 842199) and B204 (BPI 842200) were deposited in the U.S. National Fungus Collections in Beltsville (USA).

Table 2. Discriminant analysis of the conidial length and width of *Botrytis cinerea* isolates B23 and B204, *B. allii* and *B. fabae* ($F_{0.01\ (1)\ 2,395} = 4.66$).

F-matrix	*B. cinerea* (B23)	*B. cinerea* (B204)	*B. allii*	*B. fabae*
B. cinerea (B23)	0.00			
B. cinerea (B204)	4.63[a]	0.00		
B. allii	468.27	395.93	0.00	
B. fabae	706.49	777.41	2253.41	0.00

[a] No significant difference at the 0.01% level.

The $C_{729}^{+/-}$ primers described above (Rigotti *et al.*, 2002), amplified a DNA fragment of 0.73 kb in the B23-type strains and a fragment of 0.60 kb band for the two isolates of *B. fabae* used in this experiment (Table 1). However, these primers did not amplify the B204-type strains, nor the *B. allii* and *Fusarium* sp. strains tested (Table 1). In order to confirm the presence or absence of a similar sequence in the B204-type strains, the PCR was performed at lower annealing temperatures ranging from 50°C to 60°C (Biometra T Gradient) to decrease the specificity of the $C_{729}^{+/-}$ primers. A DNA fragment of 0.6 kb was amplified between 50°C and 55°C for all the B204-type strains; this fragment was not amplified at temperatures above 55°C. The PCR product was cloned and sequenced (EMBL accession No. AJ539088). The 0.76 kb sequence amplified from strain B23 (EMBL accession No. AJ422103), was aligned with the 0.61 kb sequence of strain B204, and with the 0.60 kb sequence of *B. fabae* isolate Bfa2 (EMBL accession No. AJ422104) (Fig. 1). Various mutations were observed in the sequence alignment, including a 0.12 kb internal deletion for *B. cinerea* isolate B204 and *B. fabae* isolate Bfa2. Two new primers (Bc_{108}^{+}, 5'-ACCCGCACCTAATTCGTCAAC-3'; Bc_{563}^{-}, 5'-GGGTCTTCGATACGGGAGAA-3') were designed within the 0.76 kb sequence of *B. cinerea* isolate B23, so that mismatches occurred between the sequence of the primers and the sequence of *B. fabae* (Fig. 1). The new primers were tested on 1 ng total genomic DNA extracted from the fungi listed in Table 1. As expected, they amplified fragments of the predicted size for all B23-type strains (0.48 kb) and B204-type strains (0.36 kb). Fig. 2 shows some examples of the PCR products obtained after PCR amplification with the primers Bc_{108}^{+} and Bc_{563}^{-}. No bands were amplified for *B. allii*, *B. fabae*, or the *Fusarium* sp. tested.

Experiments were carried out to see if the genetic diversity between the B23 and B204-type strains was linked to the presence or absence of the transposable elements *Boty* and *Flipper*. These two elements were amplified on all isolated strains. The presence or absence of the transposable elements did not differentiate the B23 and B204-type strains of the two subgroups (Table 1).

Thirty-six single-spore cultures were obtained from each of the B23 and B204-type strains. From these cultures, fifty second-generation single-spore cultures were produced. The stability of the deletion of the B204-type strains was tested by PCR using the amplification of the Bc_{108}^{+} and Bc_{563}^{-} DNA fragments, which remained characteristic for the B23 and B204-type strains (respectively 0.48 and 0.36 kb) when conidia from second-generation single-spore cultures were used in the PCR amplifications.

No difference in aggressiveness was observed between the B23 and the B204-type strains when grown on apple. The size of the necrotic lesions was the same for both type strains. After re-isolation from contaminated apple tissue, the amplification of the 0.48 kb and 0.36 kb DNA fragments characteristic of the B23 and B204-type strains respectively was maintained.

Discussion

When *B. cinerea* is isolated from different host-plants, it often produces a grey aerial mycelium, dark sclerotia in a circle, and abundant conidia in pure culture, as did the B23 type-strain in this study. However, about a third of the isolates, irrespective of the host-plant, produce only a thin mycelium sticking to the medium, abundant light-brown sclerotia, and form a yellow pigment, not yet chemically identified, in PDA medium but not on any other media tested. This could be due to the chemical conversion of some compound found in PDA, which is lacking in the other media. Although no morphological differences were found between the B23 and the B204 type-strains, a stable deletion in the fragment amplified by the new described primer C_{108}^{+} / C_{563}^{-} differentiated both types and thus enhanced the sensitivity of the molecular tools available to detect *B. cinerea* in infected host-plants.

At a standard annealing temperature of 60°C, primer $Bc_{729}^{+/-}$ (Rigotti *et al.*, 2002) amplified a DNA fragment of 0.73 kb only in the B23-type strain. A suspicion that there was a mutation point between the $Bc_{729}^{+/-}$ primers and the annealing site of the B204-type strain was confirmed when the PCR was performed at a lower stringency by decreasing the annealing temperature to 50°C, which led to the amplification of a 0.60 kb DNA fragment. A comparison of the nucleotide sequences of the two type-strains re-

Fig. 1. Nucleotide sequence of the 0.76-kb fragment from *Botrytis cinerea* (EMBL accession No. AJ422103). Primers C_{729}^{+}, C_{729}^{-}, Bc_{108}^{+}, and Bc_{563}^{-} are shadowed. Mismatches between *B. cinerea* isolates B23 and B204, and *B. fabae* isolate Bfa2 are indicated by nucleotides below the sequence, and continuous lines indicate the deletion for B204 and Bfa2.

vealed 95% homology and a single 0.12 kb deletion in the B204 type-strain. A deletion of a similar size but at a different position was also identified in the sequence of *B. fabae*. Two new PCR primers were designed within the aligned sequences, incorporating mismatches between the sequence of *B. cinerea* and the annealing site of *B. fabae*.

The new primers enabled both type-strains to be detected and discriminated by amplifying a DNA fragment of 0.48 kb in the B23 isolates, and of 0.36 kb in the B204 isolates. The new primers Bc_{108}^{+} and Bc_{563}^{-} detected all type-strains of *B. cinerea* occuring in many different host-plants, particularly during the quiescent stage that this fungus has in many crops (Pezet *et al.*, 2004). The multinucleate status of *B. cinerea* suggests that its phenotypic features in pure culture can be very

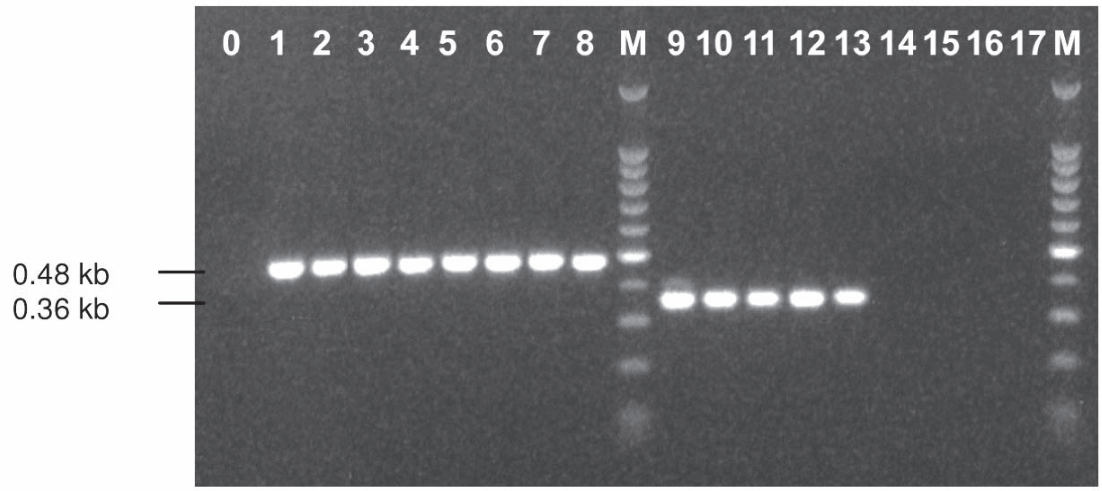

Fig. 2. PCR amplification using primers Bc_{108}^{+} and Bc_{563}^{-} on 1 ng *Botrytis cinerea* genomic DNA of *grey strains* B23 (1), B190 (2), B192 (3), B194 (4), B197 (5), B199 (6), B201 (7), B203 (8), and of *yellow strains* B189 (9), B193 (10), B198 (11), B200 (12) and B204 (13). *Botrytis allii* isolate B195 (14); B202 (15); *B. fabae* Bfa2 (16), *Fusarium* sp. F8 (17). Lane 0, negative control (no DNA); Lane M, molecular weight marker (100 bp DNA ladder, Promega).

variable, but it also confers on this fungus a high adaptability to many different hosts and environmental conditions.

Some years ago, two sub-populations of *B. cinerea*, *transposa* and *vacuma,* were described (Giraud *et al.*, 1997; Giraud *et al.*, 1999). These sibling species, which seem to belong to two different ecological niches, are not morphologically distinguishable except by a faster mycelial growth rate for the *vacuma* type (Martinez *et al.*, 2003). The transposable elements *Boty* and *Flipper* were also tested by PCR on all *B. cinerea* isolates described in this paper according to Muñoz *et al.* (2002) and Levis *et al.* (1997). All the B204-type strains lacked both the transposable elements, and consequently formed part of the *vacuma* type.

The DNA marker identified by Rigotti *et al.* (2002) has been exploited by researchers to detect *B. cinerea* (Pezet *et al.*, 2004; Holguin-Pena and Arcos, 2005), and its specificity seems to be greater in real-time PCR than other markers reported for *B. cinerea* (Suarez *et al.*, 2005). The new nucleotide sequence data for this DNA marker seem ideally suited to specifically detect and quantify all *B. cinerea* isolates and to investigate the epidemiology of both the subgroups identified.

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Literature cited

Büttner P., F. Koch, K. Voigt, T. Quidde, S. Risch, R. Blaich, B. Brückner and P. Tudzynski, 1994. Variations in ploidy among isolates of *Botrytis cinerea*: implications for genetic and molecular analyses. *Current Genetics* 25, 445–450.

Buxton E.W., 1956. Heterokaryosis and parasexual recombination in pathogenic strains of *Fusarium oxysporum*. *Journal of General Microbiology* 15, 133–137.

Faretra F. and E. Antonacci, 1987. Production of apothecia of *Botryotinia fuckeliana* (de Bary) Whetz. under controlled environmental conditions. *Phytopathologia Mediterranea* 26, 29–35.

Genovesi A.D. and C.W. Magill, 1976. Heterokaryosis and parasexuality in *Pyricularia oryzae* Cavara. *Canadian Journal of Microbiology* 22(4), 531–536.

Gindro K. and R. Pezet, 2001. Effects of long–term storage at different temperatures on conidia of *Botrytis cinerea* Pers.: Fr. FEMS Microbiology Letters 204, 101–104.

Gindro K., R. Pezet, O. Viret and H. Richter, 2005. Development of a rapid and highly sensitive direct-PCR assay to detect a single conidium of *Botrytis cinerea* Pers.: Fr. *in vitro* and quiescent forms *in planta*. *Vitis* 44(3), 139–142.

Giraud T., D. Fortini, C. Levis, P. Leroux and Y. Brygoo, 1997. RFLP markers show genetic recombination in *Botryotinia fuckeliana* (*Botrytis cinerea*) and transposable elements reveal two sympatric species. *Molecular Biology Evolution* 14(11), 1177–1185.

Giraud T., D. Fortini, C. Levis, C. Lamarque, P. Leroux, K. LoBuglio and Y. Brygoo, 1999. Two sibling species of the *Botrytis cinerea* complex, *transposa* and *vacuma*, are found in sympatry on numerous host plants. *Phythopathology* 89(10), 967–973.

Grindle M., 1979. Phenotypic differences between natural and induced variants of *Botrytis cinerea*. *Journal of General Microbiology* 111, 109–120.

Hamada W., M.C. Soulié, P. Malfatti, G. Bompeix and M. Boccara, 1997. Stability and modulated expression of a hygromycin resistance gene integrated in *Botrytis cinerea* transformants. *FEMS Microbiology Letters* 154, 187–193.

Hansen H.N. and R.E. Smith, 1932. The mechanism of variation in imperfect fungi: *Botrytis cinerea*. *Phytopathology* 22, 953–964.

Holguín-Peña R.J. and F.G. Arcos, 2005. First report of gray mold in tomato caused by *Botrytis cinerea* in Baja California, Mexico. *Plant Disease* 89(5), 528.

Holst-Jensen A., L.M. Kohn and T. Schumacher, 1997. Nuclear rDNA phylogeny of the Sclerotiniaceae. *Mycologia* 89, 885–899.

Jarvis W.R., 1977. Botryotinia *and* Botrytis *Species*: *Taxonomy, Physiology, and Pathogenicity*. Monograph No. 15, Research Branch, Canada Department of Agriculture, Ontario, Canada, 195 pp.

Jarvis W.R., 1980. Taxonomy. In: *The Biology of* Botrytis (J.R. Coley-Smith, K. Verhoeff, W.R. Jarvis, ed.), Academic Press Inc., London, UK, New York, NY, USA, 1–18.

Kerssies A., A.I. Bosker-van Zessen, C.A.M. Wagemakers and J.A.L. van Kan, 1997. Variation in pathogenicity and DNA polymorphism among *Botrytis cinerea* isolates sampled inside and outside a glasshouse. *Plant Disease* 81(7), 781–786.

Leach S.S. and A.E. Rich, 1969. The possible role of parasexuality and cytoplasmic variation in race differentiation in *Phytophthora infestans*. *Phytopathology* 59(10), 1360–1365.

Levis C., D. Fortini and Y. Brygoo, 1997. Flipper, a mobile Fot1-like transposable element in *Botrytis cinerea*. *Molecular Genomics and Genetics* 254, 674–680.

Levis C., T. Giraud, M. Dutertre, D. Fortini and Y. Brygoo, 1997. Telomeric DNA of *Botrytis cinerea*: a useful tool for strain identification. *FEMS Microbiology Letters* 157, 267–272.

Martinez F., D. Blancard, P. Lecomte, C. Levis, B. Dubos and M. Fermaud, 2003. Phenotypic differences between *vacuma* and *transposa* subpopulations of *Botrytis cinerea*. *European Journal of Plant Pathology* 109, 479–488.

Muñoz G., P. Hinrichsen, Y. Brygoo and T. Giraud, 2002. Genetic characterization of *Botrytis cinerea* populations in Chile. *Mycological Research* 106(5), 594–601.

Pezet R., O. Viret and K. Gindro, 2004. Plant microbe interaction: the *Botrytis* grey mould of grapes. Biology, biochemistry, epidemiology and control management. In: *Advances in Plant Physiology* Vol. 7. (A. Hemantaranjan, ed.), Varanasi, India, 75–120.

Pontecorvo G., 1956. The parasexual cycle in fungi. *Annual Review in Microbiology* 10, 393–400.

Rigotti S., K. Gindro, H. Richter and O. Viret, 2002. Characterization of molecular markers for specific and sensitive detection of *Botrytis cinerea* Pers.: Fr. in strawberry (*Fragaria* × *ananassa* Duch.) using PCR. *FEMS Microbiology Letters* 209, 169–174.

Schüepp H. and M. Küng, 1978. Gegenüber Dicarboximid-Fungizide tolerante Stämme von *Botrytis cinerea*. *Berichte der Schweizerischen Botanischen Gesellschaft* 88, 63–71.

Suarez B., K. Walsh, N. Boonham, T. O'Neill, S. Pearson and I. Barker, 2005. Development of real-time PCR (TaqMan,) assays for the detection and quantification of *Botrytis cinerea* in planta. *Plant Physiology and Biochemistry* 43(9), 890–899.

Vallejo I., M. Carbù, F. Muñoz, L. Rebordinos and J.M. Cantoral, 2002. Inheritance of chromosome-length polymorphisms in the phytopathogenic ascomycete *Botryotinia fuckeliana* (anam. *Botrytis cinerea*). *Mycological Research* 106(9), 1075–1085.

Vallejo I., M. Santos, J.M. Cantoral, I.G. Callado and L. Rebordinos, 1996. Choromosomal polymorphism in *Botrytis cinerea* strains. *Hereditas* 124, 31–38.

Van Kan J.A.L., A. Goverse and C.J.B. Van der Vlugt–Bergmans, 1993. Electrophoretic karyotype analysis of *Botrytis cinerea*. *Netherland Journal of Plant Pathology* 99(3), 119–128.

Van der Vlugt–Bergmans C.J.B., B.F. Brandwagt, J.W. Van't Klooster, C.A.M Wagemakers, and J.A.L. Van Kan, 1993. Genetic variation and segregation of DNA polymorphism in *Botrytis cinerea*. *Mycological Research* 97(10),1193–1200.

Whetzel H.H., 1945. A synopsis of the genera and species of the Sclerotiniaceae, a family of stromatic inoperculate Discomycetes. *Mycologia* 37, 648–714.

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