Characterization of *Botrytis cinerea* from Table Grapes in Chile Using RAPD-PCR

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ABSTRACT

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Random amplified polymorphic DNA (RAPD) analysis was performed on 29 isolates of *Botrytis cinerea* Pers. ex Fr. isolated from table grapes (*Vitis vinifera* L.) and other crops in Chile with 29 decaprimers. No single primer was found to differentiate either the host or the geographical origin of each of the *B. cinerea* isolates tested. The DNA profiles obtained, particularly with primers OPA4 and OPA11, distinguished isolates of *B. cinerea* from other epiphyte fungi found on table grapes, including *Alternaria alternata*, *Aspergillus niger*, *Cladosporium herbarum*, *Epiccoccum nigrum*, *Rhizopus stolonifer*, a *Penicillium* sp., and yeasts (*Cryptococcus laurentii*, *Rhodotorula glutinis*, and *Saccharomyces cerevisiae*). Regardless of host origin, primers OPA4 and OPA11 amplified a strong fragment of 1.2 kilobases (kb) and two fragments of 1.10 and 0.7 kb, respectively. These DNA fragments were obtained even when only one conidium of *B. cinerea* was in the test sample. Three main groups were clearly defined based on the genetic similarities found in additional RAPD analysis with 19 arbitrary decaprimers and 15 selected isolates of *B. cinerea*. The overall similarity coefficients (SC) between the groups obtained ranged from 0.326 to 0.891. Interestingly, all isolates from table grapes were included in group I (SC: 0.761 to 0.826), isolates from apple and tomato were in group II (SC: 0.739 to 0.848), while isolates from blueberry were either in group I (SC: 0.804) or III (SC: 0.673). Consequently, the genetic variability determined by RAPD analysis among these *B. cinerea* isolates suggested a possible host: pathogen relationship. However, further research is needed to clarify its pathological significance.

Additional keywords: apple, blueberry, *Botryotinia fuckeliana*, Botrytis bunch rot, gray mold, tomato

Botrytis cinerea Pers.:Fr. (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel) is the cause of Botrytis bunch rot of table grapes (*Vitis vinifera* L.). Significant losses occur when long periods of high relative humidity (>95%) or wetness and cool temperatures (15 to 22°C) coincide with blossoming and ripening (soluble solids > 8%; 1,2,9,11). Besides grapes, *B. cinerea* infects a wide range of important vegetable, ornamental, bulb, and fruit crops in temperate regions, colonizing and surviving on senescing and dead plant tissues (2,9,12). Host specialization has not been reported among populations of *B. cinerea*, but differences in virulence toward one given host plant has been observed (4,5,19).

Molecular techniques based on the polymerase chain reaction (PCR; 17,18) have been used as a tool in genetic mapping, molecular taxonomy, evolutionary studies, and diagnosis of several fungal species. The analysis of DNA products generated through random amplified polymorphic DNA (RAPD) has provided information on variation (10) and segregation (16) of genetic traits among strains of *B. cinerea*. Restriction fragment length polymorphism (RFLP) analysis has also revealed important genetic variation in *B. cinerea*, suggesting the presence of two species (6,8). The objectives of this work were to characterize both intra- and interspecies isolates of *B. cinerea* from table grapes in Chile, using RAPD, and to identify specific markers to distinguish *B. cinerea* from epiphyte fungi commonly associated with table grapes.

MATERIALS AND METHODS

Fungal isolates. A total of 19 single-spore isolates of *B. cinerea* were established from samples of grape berries collected at harvest from commercial 'Thompson Seedless' vineyards in the Aconcagua Valley and Central Valley of

Chile. *B. cinerea* was also isolated from lettuce (*Lactuca sativa* L.) and from fruits of blueberry (*Vaccinium corymbosum* L.), apple (*Malus communis* DC.), kiwifruit (*Actinidia chinensis* Planchon), tomato (*Lycopersicon esculentum* Mill.), and pear (*Pyrus communis* L.). With the exception of isolates from blueberry, all *B. cinerea* isolates were obtained during the 1997 to 1998 growing season from samples collected in the same geographical region, across an area of about 200 km from north to south where most of the fruit and vegetable crops are cultivated. Those from blueberry were obtained in southern Chile (Gorbea, Temuco) in 1998. Isolates of several fungi found as epiphytes on table grapes in Chile were included to establish the specificity of the RAPD primers. The fungi included: *Alternaria alternata* (Fr.:Fr) Keissl., *Aspergillus niger* Teigh., *Cryptococcus laurentii* (Kuff.) C. E. Skinner, *Cladosporium herbarum* (Pers.:Fr.) Link., *Epiccoccum nigrum* Link ex. Fr., a *Penicillium* sp., *Rhizopus stolonifer* (Ehrenb.:Fr) Vuill., *Rhodotorula glutinis* (Fresen.) F. C. Harrison, and *Saccharomyces cerevisiae* Meyen ex Hansen. All isolates were cultivated on potato dextrose agar (PDA) at 23°C in complete darkness and were stored on PDA at 5°C.

DNA extraction. Conidia from 5- to 8-day-old cultures were removed aseptically by flooding each plate with 20 ml of BG-broth (0.5% wt/vol Bacto-Broth, 0.5% wt/vol glucose) and scraping the surface. The conidial suspension was filtered through fiberglass into a conical flask and placed in an orbital shaker for 24 h at 20°C. The germinated conidia were separated from the surrounding medium either by decanting or by filtrating the broth through muslin cloth and then drying the mycelium with paper towels. Samples of yeasts (*Cryptococcus laurentii*, *R. glutinis*, and *S. cerevisiae*) for DNA extraction were taken directly from PDA plates. Nucleic acids were extracted according to Möller et al. (13), except the volumes of all reagents were reduced by 60% and isopropanol replaced ethanol for DNA precipitation. All DNA extracts were quantified according to their optical density at 260 nm, using a Spectronic Genesys 5 spectrophotometer (Milton Roy, Rochester, NY). DNA extraction was repeated at least twice for each isolate.

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RAPD analysis. Primers (10-mer) were selected at random from Operon primer kits A, B, and G (Operon Technology, Inc., Alameda, CA). Amplification reactions were done in a 20- μ l reaction mixture containing 10 to 20 ng of template DNA in 2.0 μ l of sterile distilled water, 2.0 μ l of 10 $\times$ reaction buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0], and 1% Triton; Promega Corp., Madison, WI), 2.0 μ l of dNTPs (each at 2.5 mM), 0.4 μ l of *Taq* DNA polymerase (5 U/ μ l; Promega Corp.), 2.0 μ l of MgCl (25 mM), 5.0 μ l (30 μ M) of primer, and 6.6 μ l of sterile distilled water. Samples were overlaid with mineral oil. Control reactions contained water instead of template DNA. Reactions were amplified in a programmable thermocycler (M. J. Research, Inc., Watertown, MA) for 45 cycles of 1 min at 94°C (melting), 30 s at 48°C (annealing), and 1.5 min at 72°C (extension), with a final extension at 72°C for 4 min.

DNA was separated by electrophoresis in a 1.2% (wt/vol) agarose gel in 1 $\times$ TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0; 15). A 100-base pair (bp) ladder (Life Technologies, Gaithersburg, MD) was used as a size standard. To visualize DNA, gels were stained with ethidium bromide (0.1 mg/liter) and then photographed by transillumination using a Polaroid DS-34 with 667 film (Polaroid Corp., Cambridge, MA). All RAPD analyses were repeated at least three times for each isolate.

DNA polymorphism among isolates of *B. cinerea* and of epiphyte fungi. In a preliminary study to determine suitable primers, a total of 29 decaprimers (Table 1) were used to characterize two isolates of *B. cinerea* obtained from table grapes (G.4.1 and G.4.4), and single *B. cinerea* isolates obtained from apples (A1.1) and blueberries (B2.1) were included. Additionally, single isolates of each of the following epiphytes were included for comparison: *Alternaria alternata*, *Aspergillus niger*, *Cladosporium herbarum*, *Cryptococcus laurentii*, *E. nigrum*, a *Penicillium* sp., *Rhizopus stolonifer*, and *Rhodotorula glutinis*.

Primers selected as useful in discriminating *B. cinerea* from other epiphyte fungi were used to study the uniformity and reliability of the amplification products from 25 additional isolates of *B. cinerea* from grapes and other hosts. As further controls, we examined one isolate of *S. cerevisiae* and two isolates each of *Alternaria alternata*, *Aspergillus niger*, and a *Penicillium* sp.

Genetic characterization of *B. cinerea* from table grape. Genetic variability among isolates of *B. cinerea* from table grapes and from other host plants was assessed by RAPD analysis using 19 decaprimers selected from Operon Technologies kit A (OPAs: 1, 2, 9, 12, 13, and 18), kit B (OPBs: 1, 7, 8, 10, 12, and 18), and

kit G (OPGs: 2, 3, 7, 8, 9, 15, and 18) (Table 1), following the same protocol described above. Data were analyzed and scored as 0 or 1 based on the absence or presence, respectively, of DNA fragments that consistently appeared in standard positions. Data were analyzed statistically and a phenogram was constructed by the unweighted pair-group method with averages linkage (UPGMA) based on Jaccard similarity coefficients using the cluster analysis program NTSYS, PC version 2.01.

Sensitivity of RAPD analysis. To study the relative sensitivity of the RAPD analysis in detecting conidia of *B. cinerea*, samples of *B. cinerea* (G.1.1), *Alternaria alternata* (A16.2), *Aspergillus niger* (As4.2), and a *Penicillium* sp. (Pe1.2) were cultured for 48 h in BG-broth as described above. Germinated conidia (approximately 0.6 mg) were then individually separated under a dissecting microscope and test samples composed of 1, 3, or 10 conidia of *B. cinerea* alone or in combination with three germinated conidia of the *Penicillium* sp. or with three germinated conidia each of *Alternaria alternata*, *Aspergillus niger*, and the *Penicillium* sp. were prepared. Control samples contained three germinated conidia of the *Penicillium* sp. alone, or samples containing three germinating conidia

of one of the fungi (*Alternaria alternata*, *Aspergillus niger*, and the *Penicillium* sp.). DNA extracted from each sample was then tested with primers OPA4 and OPA11.

RESULTS

DNA polymorphism among isolates of *B. cinerea* and of epiphyte fungi. Strong non-polymorphic fragments were PCR amplified consistently from the four isolates of *B. cinerea* with primers OPA4 (Opa4_{1.30}) and OPA11 (Opa11_{1.10} and Opa11_{0.70}) (Fig. 1). The other 27 primers produced variable results. Some primers amplified several strong fragments (e.g., OPAs 2 and 18 and OPBs 7 and 10) and others (e.g., OPBs 1, 8, 9, 15, and 18) produced common strong fragments of the same size as two or more of the fragments amplified from the epiphyte fungi. In a second test, fragments Opa4_{1.30}, Opa11_{1.10}, and Opa11_{0.70} were also amplified from each of the 25 additional *B. cinerea* isolates obtained from table grapes and other hosts in different localities in Chile (Figs. 1 and 2).

With the exception of primer OPA11 with *R. glutinis* DNA, primers OPA4 and OPA11 generated banding patterns from each of the other fungal species that were easily distinguished from those of *B. cinerea* (Fig. 1).

Table 1. Sequences of primers used for random amplified polymorphic DNA analysis of *Botrytis cinerea* isolated from table grapes and other host plants in Chile

Primers ^b	Nucleotide sequences	DNA fragments ^a	
	5' to 3'	Amplified	Polymorphic
OPA1*	CAGGCCCTTC	1	0
OPA2*	TGCCGAGCTG	9	4
OPA4	AATCGGGCTG	1	0
OPA7	GAAACGGGTG	4	1
OPA8	GTGACGTAGG	2	0
OPA9*	GGGTAACGCC	7	1
OPA11	CAATCGCCGT	2	0
OPA12*	TCGGCGATAG	6	2
OPA13*	CAGCACCCAC	2	0
OPA16	AGCCAGCGAA	2	0
OPA18*	AGGTGACCGT	6	5
OPA20	GTTGCGATCC	2	0
OPB1*	GTTTCGCTCC	5	2
OPB5	TGCGCCCTTC	1	0
OPB7*	GGTGACGCAG	11	7
OPB8*	GTCCACACGG	2	0
OPB10*	CTGCTGGGAC	8	5
OPB11	GTAGACCCGT	1	0
OPB12*	CCTTGACGCA	5	3
OPB14	TCCGCTCTGG	3	2
OPB15	GGAGGGTGTT	2	1
OPB18*	CCACAGCAGT	2	1
OPG2*	GGCACTGAGG	6	1
OPG3*	GAGCCCTCCA	5	0
OPG7*	GAACCTGCGG	5	3
OPG8*	TCACGTCCAC	8	3
OPG9*	CTGACGTCAC	2	1
OPG15*	ACTGGGACTC	3	2
OPG18*	GGCTCATGTG	4	2
Total		117	46

^a Total number of strongly stained and reproducible fragments and number of polymorphic fragments obtained among 15 isolates of *B. cinerea*.

^b Primers from Operon Technology (Alameda, CA) kits A, B, and G. Those primers followed by an asterisk were selected for genetic variability studies of isolates from table grapes and other host plants.

Fig. 1. Characterization of *Botrytis cinerea* and epiphyte fungi obtained from table grapes by random amplified polymorphic DNA analysis with primers (A) OPA4 and (B) OPA11. Isolates of *B. cinerea* from table grapes are in lanes 4 (G4.1) and 7 (G4.4), and those from apple and blueberry are in lanes 10 (A.1.1) and 13 (B.2.1), respectively. Lane 3, *Alternaria alternata*; lane 5, *Aspergillus niger*; lane 6, *Cladosporium herbarum*; lane 8, *Cryptococcus laurentii*; lane 9, *Epicoccum nigrum*; lane 11, a *Penicillium* sp.; lane 12, *Rhizopus solonifer*; and lane 14, *Rhodotorula glutinis*. Lane 1, distilled water and lane 2, 100-base pair molecular size marker in kilobases. The positions of fragments Opa4_{1.30}, Opa11_{1.10}, and Opa11_{0.70} are marked with an arrow.

Fig. 2. Random amplified polymorphic DNA patterns obtained with primers (A) OPA4 and (B) OPA11 for isolates of *Botrytis cinerea* isolated from hosts other than table grapes and for isolates of epiphyte fungi commonly associates with table grape fruit clusters. *Alternaria alternata* isolates: lane 2, Al.6.2, and lane 3, Al.8.2; *Aspergillus niger* isolates: lane 4, As.4.2, and lane 5, As.9.2; *Penicillium* sp. isolates: lane 6, Pe.1.2, and lane 7, Pe.4.1; and *B. cinerea*: lanes 8 to 12, isolates from apple, lettuce, kiwifruit, pear, and tomato, respectively. Lane 1, 100-base pair molecular marker in kilobases. The positions of fragments Opa4_{1.30}, Opa11_{1.10}, and Opa11_{0.70} are marked with an arrow.

Genetic characterization of *B. cinerea* from table grape. A total of 97 strongly stained and reproducible fragments, 42 of which revealed polymorphism, were amplified from the 15 isolates of *B. cinerea* with the 19 primers (Table 1). These primers amplified 1 to 11 fragments per primer, ranging in size from 0.4 to 2.5 kilobases (kb), although the majority were below 1.5 kb. One to seven polymorphic fragments per primer were obtained with 15 of these primers. Similarity coefficients (SC) between pairs of isolates ranged from 0.326 to 0.891 (Table 2). The genetic distances among all 15 isolates of *B. cinerea* showed significant genetic variability and three main groups of isolates (I, II, and III) were identified (Fig. 3). Interestingly, each group consisted of isolates from different host plants. Group I contained all the isolates from table grapes, isolate P1 from pear, and isolates B1 and B4 from blueberry. Isolates from table grapes had SC values ranging from 0.761 to 0.891 and were distributed in subgroups Ia, Ib, or Ic, while the two isolates from blueberry were in subgroup Id and the pear isolate was in subgroup Ia. The SC values between isolates from table grapes and isolates B1 and B4 from blueberry varied from 0.326 to 0.826 (Table 2, Fig. 3). Group II contained the apple and tomato isolates and SC ranged from 0.717 to 0.848. Group III contained isolates B2.2 and B2.3 (B2 and B3) from blueberry (SC: 0.673; Table 2).

Sensitivity of RAPD analysis. Using primer OPA11, fragment Opa11_{1.10} was amplified from each test sample whether or not it contained DNA from *B. cinerea*. However, fragment Opa11_{0.70} was amplified only from samples containing 1, 3, or 10 conidia of *B. cinerea* and from those containing 10 conidia of *B. cinerea* mixed with 3 conidia of either *Alternaria alternata*, *Aspergillus niger*, or the *Penicillium* sp. (Fig. 4). The results obtained with primer OPA4 were not specific, because this primer amplified a fragment the size of Opa4_{1.30} from *Penicillium* sp. isolate Pe1.2.

DISCUSSION

RAPD analysis was a useful tool for characterizing genetic variability among isolates of *B. cinerea* from table grapes and other hosts. Significant genetic variability was observed among 15 different isolates of *B. cinerea*, using 19 arbitrary oligonucleotide primers with SC values ranging from 0.326 to 0.891 (Table 2). Similar variability and SC values were previously reported for *B. cinerea* populations obtained on hosts other than table grapes, suggesting a great genetic diversity among isolates of *B. cinerea* (10,14,16). No relationship was found between geographical origin and the DNA profiles obtained. However, based on the genetic similarities, the cluster analysis divided the 15 isolates of *B. cinerea* into three main groups. Interestingly, all isolates from table grapes were

included in group I, isolates from apple and tomato were in group II, while isolates from blueberry were either in group I (subgroup Id) or in group III (Fig. 3). It is generally accepted that no host specialization occurs among *B. cinerea* populations because isolates of *B. cinerea* can infect a wide range of host plants in the laboratory (9). Nevertheless, it is still possible that host specificity may occur in the field, and our results support this hypothesis. Similar results were reported previously in Chile using RAPD analysis (14), and results obtained with other molecular techniques showed great genetic diversity among *B. cinerea* isolates, also suggesting host specialization in *B. cinerea* populations (3,4,6,8). Consequently, isolates from table grapes may be genetically distinct from isolates of *B. cinerea* obtained on other hosts. Nevertheless, additional studies

using a rather large number of *B. cinerea* isolates would be advisable in order to clarify the pathological significance of these RAPD groups.

The experiments to test the sensitivity of the RAPD analysis using primers OPA4 and OPA11 were designed to include the range of fungi likely to be encountered when grape clusters are sampled. The choice of using *Alternaria alternata*, *Aspergillus niger*, and a *Penicillium* sp. in the extraction with *B. cinerea* was based on the high frequency of occurrence of these fungi on grape clusters as previously reported in Chile and elsewhere (7,12). For OPA4 and OPA11, even one germinated conidium could be detected by this method, although competition presumably from excess DNA from other sources during amplification results in no fragment being detected. In the case of primer OPA4, the results showed that the fragment

Opa4_{1.30} was not specific, because it appeared with varying degrees of intensity in all extracts, including those without *B. cinerea*, most likely because of a minor band of the same size produced by the *Penicillium* sp. sample. With primer OPA11, fragment Opa11_{1.10} or a fragment of the same size was present in all extracts. Fragment Opa11_{0.70} could only be detected in those samples containing *B. cinerea*, and it was absent in fungal mixes with only one germinated conidia of *B. cinerea*. Taking into account the amount of inter- and intraspecific variation detected in this study, it can be assumed that the chance of there being a fungal source that is not *B. cinerea*, producing a fragment of similar or identical size to that of one found in all *B. cinerea* isolates, is relatively low. Nevertheless, this fragment should be cloned, sequenced, and specific primers designed before it is used to detect *B. cinerea*.

Fig. 3. Phenogram based on random amplified polymorphic DNA profiles (RAPD) showing the genetic relationships among 15 isolates of *Botrytis cinerea* isolated from different host plants in Chile that were generated with 19 arbitrary decaprimers. Isolates G1 to G6 were from table grapes, A1 and A2 from apple, B1 to B4 from blueberry, P1 from pear, and T1 and T2 from tomato.

Fig. 4. Sensitivity of the random amplified polymorphic DNA analysis using primer OPA11 to detect *Botrytis cinerea*. Lanes 2, 3, and 4, test samples containing DNA from 1, 3, or 10 germinated conidia of *B. cinerea* (G.9.1) alone, respectively; lanes 5, 6, and 7, in mixed samples containing DNA from 1, 3, or 10 germinated conidia of *B. cinerea* and from three germinated conidia of *Penicillium* sp. (P1.2), respectively; or lanes 9, 10, and 11, from germinated conidia of each *Alternaria alternata* (A16.2), *Aspergillus niger* (As4.2), and *Penicillium* sp., respectively. Lane 1, 100-base pair molecular size marker indicated in kilobases. Lane 8, DNA from three germinated conidia of the *Penicillium* sp. alone. Lane 12, DNA from three germinated conidia each of *Alternaria alternata* (A16.2), *Aspergillus niger* (As4.2), and the *Penicillium* sp. (Pe1.2). The positions of fragments Opa11_{1.10} and Opa11_{0.70} are marked with an arrow.

Table 2. Similarity coefficients for isolates of *Botrytis cinerea* isolated from table grape and other host plants in Chile^a

Isolates	Host origin of isolates														
	Table grapes						Blueberries				Apples		Pear	Tomatoes	
	G1	G2	G3	G4	G5	G6	B1	B2	B3	B4	A1	A2	P2	T1	T2
G1	1	...	...	...	...	...	...	...	...	...	...	...	...	...	...
G2	0.826	1	...	...	...	...	...	...	...	...	...	...	...	...	...
G3	0.826	0.870	1	...	...	...	...	...	...	...	...	...	...	...	...
G4	0.804	0.848	0.848	1	...	...	...	...	...	...	...	...	...	...	...
G5	0.761	0.891	0.804	0.783	1	...	...	...	...	...	...	...	...	...	...
G6	0.761	0.761	0.848	0.870	0.783	1	...	...	...	...	...	...	...	...	...
B1	0.783	0.739	0.826	0.804	0.717	0.761	1	...	...	...	...	...	...	...	...
B2	0.391	0.348	0.348	0.370	0.326	0.413	0.435	1	...	...	...	...	...	...	...
B3	0.500	0.457	0.500	0.435	0.478	0.435	0.500	0.673	1	...	...	...	...	...	...
B4	0.717	0.761	0.804	0.826	0.696	0.783	0.804	0.457	0.565	1	...	...	...	...	...
A1	0.652	0.652	0.696	0.630	0.630	0.587	0.652	0.435	0.587	0.630	1	...	...	...	...
A2	0.674	0.587	0.587	0.609	0.609	0.565	0.630	0.543	0.609	0.609	0.761	1	...	...	...
P2	0.826	0.783	0.783	0.717	0.761	0.717	0.696	0.435	0.543	0.717	0.739	0.717	1	...	...
T1	0.587	0.544	0.544	0.522	0.522	0.565	0.500	0.587	0.478	0.565	0.717	0.739	0.674	1	...
T2	0.630	0.674	0.674	0.696	0.652	0.652	0.630	0.543	0.652	0.696	0.848	0.826	0.717	0.826	1

^a Similarity coefficients were calculated based on Jaccard similarity coefficients from DNA profiles obtained with decaprimers OPAs 1, 2, 9, 12, 13, and 18; OPBs 1, 7, 8, 10, 12, and 18; and OPGs 2, 3, 7, 8, 9, 15, and 18 (Operon Technologies, Alameda, CA).

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