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Chromosomal polymorphism in *Botrytis cinerea* strains

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The electrophoretic karyotypes of five strains of *Botrytis cinerea* from different locations were determined. A unique karyotype was observed for each strain; although general features of the banding pattern of chromosome sized DNA were conserved between strains not two strains had identical karyotypes, indicating that considerable length polymorphism exists in this species. The size of the bands ranged from 1.88 Mb to 3.72 Mb and the genome sizes from 11.22 to 22.49 Mb using chromosomal standards from *Hansenula wingeii*. Three of the strains were quite similar both in number and size of bands and genome total size and would be the diploid form of the species. The other two presented lower band number and, according to the total genome size, one of them could be haploid and the other an aneuploid form. The mitochondrial DNA band was also shown on gels and its origin confirmed by the cytochrome c oxidase specific reaction. Infection assays were carried out both in *Nicotiana tabacum* and *Vitis vinifera* and differences in pathogenicity were shown by the strains, although parasitic degree could not be related to any ploidy state or specific band.

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Botrytis cinerea is a phytopathogenic fungus producing the "grey mould" on many economically important crops including grapes. The biology of the fungus shows considerable phenotypic diversity and a life cycle including both sexual and asexual forms, sclerotia and microconidia being the female and male gametes, respectively, which by fusion produce apothecia containing asci. However, the asexual cycle is the most common form of reproduction and propagation of the fungus, and it occurs by mitotic division of the asci giving macroconidia, which germinate and produce mycelium and eventually develop sclerotia and microconidia. Also high levels of somatic variability appear when the fungus is grown "in vitro", depending on the medium, temperature, light, and other factors, which even determine differences in agressiveness of spores (PHILLIPS et al. 1987).

Trying to diminish the economic damage that *B. cinerea* causes, special attention has been paid to the study of its chemical control (COLEY-SMITH et al. 1980). However, high levels of resistance to fungicides have appeared in the fungus. In addition, contamination problems due to the persistence of these chemicals both in soil and water and decrease of wine quality from contaminated grapes, result in important economic damages (PÉREZ et al. 1991; VAN DER VLUGT-BERGMANS et al. 1993).

Cytogenetic studies on lower fungi have been limited by their small size and/or the difficulty to condense sufficiently the chromosomes to make them visible by microscope. Instead, the chromosomal complement has been inferred from the determination of linkage groups. Despite this, their analysis is only reliable when a high number of genetic markers are studied. Genetic data about *Botrytis* are scarce due to the absence of induced stable mutants because of the difficulty to reproduce the fungus sexually in the laboratory, the multinucleate and heterokaryotic composition of macroconidia, and the non-haploid state of the fungus (GRINDLE 1979; FARETRA et al. 1988).

The development of pulsed-field gel electrophoresis allows the separation of large DNA molecules which would all co-migrate in conventional agarose gels, and has proved to be a very useful technique to study aspects of genome organization in several yeasts and fungi. One version of pulsed-field gel electrophoresis is the so called contour-clamped homogeneous electric field (CHEF) gel system, which provides the advantage of extending the upper limit of resolution to about 10 Mb so that each band could correspond to a

complete DNA molecule, like mitochondrial DNA, or intact chromosomes. Species studied by this method have been *Histoplasma capsulatum* (STEELE et al. 1989) *Phytophthora megasperma* (HOWLETT 1989), *Ustilago maydis* (BUDDE and LEONG 1990), *Podospora anserina* (OSIEWACZ et al. 1990), *Aspergillus nidulans* (MONTENEGRO et al. 1992), and *Penicillium chrysogenum* (FIERRO et al. 1993).

An electrophoretic study in *Botrytis cinerea* would allow to determine both the number of chromosomes and the ploidy level as well as to contribute to the knowledge of the genomic organization of the fungus. Thus, the aim of the present work is to study the karyotype of *Botrytis cinerea* by pulsed-field gel electrophoresis and to compare the patterns between several strains in order to gain insight into the genetics of the fungus. Our results indicate a high degree of chromosomal polymorphism and ploidy differences among *Botrytis* strains.

Material and methods

Fungal strains. — Fungal strains used are listed in Table 1.

Preparation of DNA in agarose plugs. — Young mycelium grown on Czapek Dox liquid medium was collected for filtration, washed with 0.9 % NaCl, squeezed between filter papers, and suspended (1 g of wet weight in 20 ml) in TPP isotonic buffer (0.7 M KCl, 50 mM potassium phosphate buffer, pH 5.8). The suspension was treated with Lysing Enzymes (Sigma Chemical Co.) to a concentration of 0.5 mg/ml. The mixture was incubated with gentle agitation (50 rpm) at 25°C for 3 h to obtain protoplasts and then filtered through a 30 μm nylon filter (Nytal) to remove the undigested mycelia. The filtrate was centrifuged and the pellet washed three times with isotonic solution (0.7 M KCl, 50 mM EDTA, pH 8.0). The protoplasts were suspended in the same solution and embedded in low-melting-point agarose (10 g/l in 0.7 M KCl, 50 mM EDTA, pH 8.0) at about 45°C. The final concentration of agarose in the plugs was 0.5 % with a protoplast content of 1×10^8/ml of agarose. The plugs were cooled at 4°C and treated with ESP (0.5 M EDTA, pH 9.0, 1 % lauroylsarcosine, 1 mg/ml proteinase K) at 50°C for 24 h to release the DNA. After lysis of the cells, the plugs were washed three times with 50 mM EDTA, pH 8.0 and kept at 4°C in the same buffer. Under these conditions the released DNA was stable for several months.

Pulsed-field gel electrophoresis. — Gels (14 × 12.5 cm) were cast using 0.7 % agarose in 1 × TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0). The buffer was maintained at a constant temperature of 11°C. Electrophoresis was conducted in a CHEF-DR-II (BioRad) and carried out using a pulse time gradient of 250 to 900 s for a total time of 52 h at 3 V/cm. Gels were stained with 0.5 μg/ml ethidium bromide for 30 to 45 min and destained with the electrophoresis buffer (1 × TAE) for several hours when required.

Chromosomal size estimation. — *Hansenula wingeii* chromosomes were used as markers for calculation of band sizes smaller than 3.30 Mb. Size of bands

Table 1. Characteristics of the fungal strains used in the work

Strain	Characteristics	References
Botrytis cinerea		
UCA 992	Isolated from grapewines on 1992	Domecq (Jerez de la Frontera, Spain)
UCA 993	Isolated from grapewines on 1993	Osborne (Puerto de Santa María, Spain)
UCA 994	Isolated from grapewines on 1994	This work (collected in Alicante, Spain)
2100	Isolated from *Vicia faba* on 1979	CECT[a]
2850	Isolated from saffron bulb on 1987	CECT[a]
Penicillium chrysogenum	Chromosome sizes: 10.4, 9.6, 7.3, and 6.8 Mb	BARREDO et al. 1989
Hansenula wingeii	Chromosome sizes: 3.13, 2.70, 2.35, 1.81, 1.66, 1.37, and 1.05 Mb	BIO-RAD
Saccharomyces cerevisiae	12 chromosomes ranging from 225 to 2,200 kb	BIO-RAD

[a] Colección Española de Cultivos Tipo (Valencia, Spain)

higher than 3.30 Mb were calculated by a regression analysis. Sizes of the seven chromosomes present in this yeast are shown on Table 1.

Chromosomal bands from *B. cinerea* strains were numbered from faster to slower mobility by roman numbers.

Isolation of mitochondria. — Mitochondria were obtained from lyophilized mycelia and purified by a sucrose step-gradient, and the cytochrome c oxidase activity was measured (DARLEY-USMAR et al. 1994). Preparation of mtDNA in agarose plugs was carried out as described above for total DNA.

Hybridization conditions. — Procedures for DNA transfer to membranes (Hybond N^+, Amersham), hybridization and probe labelled using ^{32}P-dCTP by nick translation were according to SAMBROOK et al. (1989). Probes used were a fragment of 800 pb from the *pyr G* gene (coding for oritidine-5'-phosphate decarboxylase, involved in the biosynthesis of uridine 5'-monophosphate) from *Penicillium chrysogenum* (CANTORAL et al. 1987) and 2 kb of the *AMA1* region (genomic sequence that produces plasmids replicating autonomously) from *Aspergillus nidulans* (GEMS et al. 1991). Washes were carried out at low stringency conditions.

Bioassays. — Tobacco leaves from *Nicotiana tabacum* var. Xanthi NC and from *Vitis vinifera* var. Aledo (susceptible to *B. cinerea*) were used. Leaves were sterilized with 10 % sodium hypochlorite for 3 min, washed four times with sterile water, dried between filter papers, and placed on Petri dishes containing Whatman paper wet with sterile water. Defined mycelial plugs were placed on the leaves and the plates kept at 25–28°C. Bioassays were read five days after infection.

Results

To resolve the karyotype of *B. cinerea*, we first used CHEF gel conditions suitable for separation of chromosomes ranging in size between 5 and 7 Mb, typical of most filamentous fungi, using *Penicillium chrysogenum* as control of size. Samples were electrophoresed at 40 V for 170 h with ramped pulse times of 3300 to 5400 s. However, electrophoretic patterns indicated that chromosome sizes in *Botrytis cinerea* were smaller than in *Penicillium*, indicating that a higher voltage and shorter times were required. In a second assay the

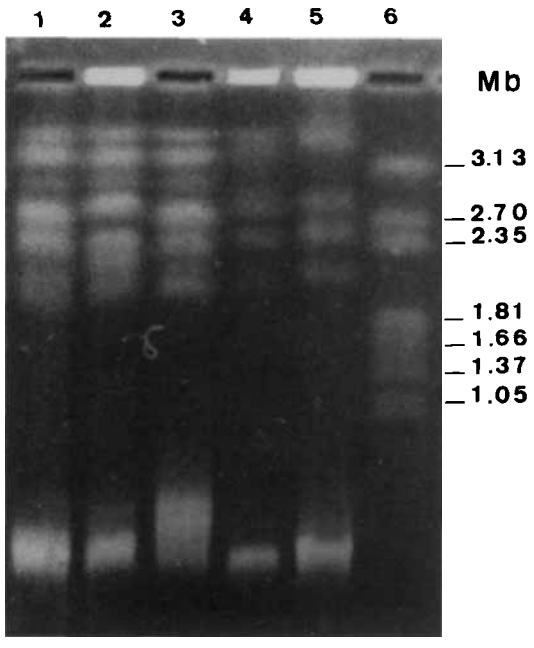

Fig. 1. CHEF analysis of chromosomal DNAs from *Botrytis cinerea* strains. Chromosomal DNAs were prepared from *Botrytis cinerea* UCA 994 (1), *Botrytis cinerea* 2100 (2), *Botrytis cinerea* UCA 992 (3), *Botrytis cinerea* 2850 (4), *Botrytis cinerea* UCA 993 (5) and electrophoresed at 100 V with a switch time of 250 to 900 s and a run time of 52 h. The size of the chromosomal bands from *Hansenula wingeii* (6), which was used as size marker, is shown on the right.

electrophoresis was carried out at 45 V with a pulse time gradient of 2100 to 2700 s, and seven bands were then seen. Band I appeared on an intermediate position between the two highest ones of *Saccharomyces cerevisiae* (1.6 and 2.2 Mb), and it would be the minimal size of the chromosomes. If we consider the previous results, compared with *Penicillium*, the range of size for *Botrytis* chromosomes would be 1.6 and 6.8 Mb. After trying 66 V and pulse times from 1500 to 2000 s, the best conditions resulted with 100 V and a switch time of 250 to 900 s and a run time of 52 h. Under these conditions, differences in size of most of the chromosomes were observed among the five *B. cinerea* strains (Fig. 1).

The smallest band size was 1.88 Mb, present in UCA 994 strain. An equivalent band of 1.91 Mb was present both in 2100 and UCA 992, but was missing in 2850 and UCA 993. The second, third, and fourth bands appeared as the most conserved

among the strains and were present in all of them, so that bands II and III showed the same size in 3 strains (band II in UCA 992, 2850, and UCA 993 strains and Band III in 2100, UCA 992, and 2850), and band number IV presented 2.81 Mb in strains 2850 and UCA 992 and was rather similar in the other three ones.

Band V was missing in UCA 993, showed 3.01 Mb in UCA 994 and 2850, and was slightly different in 2100 (3.04 Mb) and UCA 992 (3.02 Mb). Bands VI and VII were absent from 2850 and UCA 993, but the differences between the other three strains were minimal. Band VIII was lacking in UCA 993, presented the same size in UCA 994 and 2100 (3.51 Mb), and was very similar in UCA 992 (3.53 Mb) and 2850 (3.54 Mb). And, finally, band IX with the higher molecular band size (3.72 Mb) was only present in strains UCA 993 (3.68 Mb) and 2850. The relative intensities of ultraviolet fluorescence of bands I, III, and IV after ethidium bromide staining suggested that they could represent doublets. Also when pulse times were changed to 250–900 s and the running time extended until 70 h, a new band migrating faster than III (IIIa) appeared on UCA 992.

Strains UCA 994, 2100, and UCA 992 showed the lowest variability level and the variation was due mainly to the smallest chromosomes. Zymograms of these strains were clearly different to the ones showed by the strains 2850 and UCA 993, which were also different among themselves. Fixed electrophoretic conditions never produced variation on any of the karyotypes studied, and at least 5 electrophoreses carried out in the same conditions always gave the same pattern of bands.

From these results, the minimal size of genome for each of these strains was obtained (Table 2). The highest size was 22.49 Mb (2100) and UCA 994 and UCA 992 presented similar sizes of 22.04 and 22.36, respectively, whereas 2850 strain showed 17.48, and UCA 993 presented only 11.22.

There was one band that showed a higher mobility than any of the bands described before, and that corresponded to mitochondrial DNA. Mobility of this band was slightly different among the five strains and also varied for the same strain on different electrophoreses, with indications to be a circular molecule. The position of this band on the gel corresponded to the mtDNA band of *Penicillium chrysogenum*, with a size of 25.8 kb. To determine with certainty the origin of this DNA band we purified mitochondria with two purposes, firstly to conduct the cytochrome c oxidase specific reaction of mitochondria, which is shown on Fig. 2, and secondly to load them on the gel and to compare with the bands of the complete zymograms.

Some more bands were visible between the chromosomal bands and the mtDNA band, but their presence varying with the extraction, gels, etc., they were not considered on the strain karyotypes.

In relation to hybridization results, no bybridization bands were detected when heterologous probes *pyr G* and *AMA1* were used, under low strigency conditions.

Results on phytopathogenicity indicated that strain UCA 992 was the most phytotoxic one, both in *Nicotiana* and *Vitis*. However, differences in wounded and unwounded leaves were obtained for the rest of the strains. In this way, results were

Table 2. Chromosome-sized DNA and total genome size of five strains of *Botrytis cinerea*

Band number	Size (Mb)				
	UCA 994	2100	UCA 992	2850	UCA 993
I[a]	1.88	1.91	1.91	–[b]	–
II	1.97	2.15	2.05	2.05	2.05
III[a]	2.27	2.35	2.35	2.35	2.57
IV[a]	2.74	2.87	2.81	2.81	2.92
V	3.01	3.04	3.02	3.01	–
VI	3.30	3.30	3.31	–	–
VII	3.36	3.36	3.38	–	–
VIII	3.51	3.51	3.53	3.54	–
IX	–	–	–	3.72	3.68
Minimal genome size	22.04	22.49	22.36	17.48	11.22

[a] Indicates possible doublet bands
[b] Indicates absent band

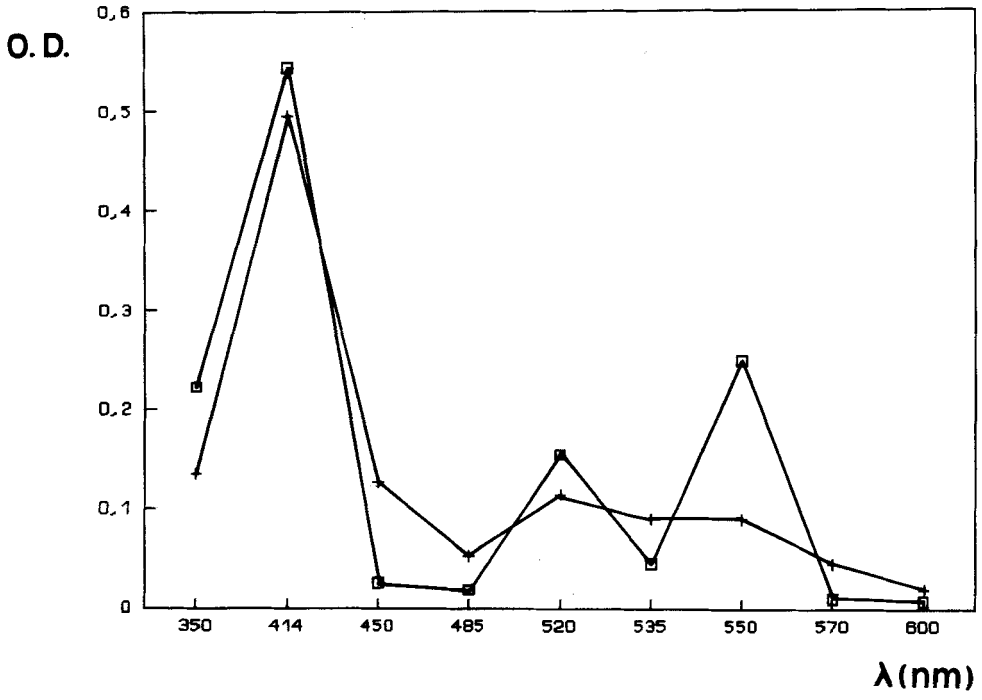

Fig. 2. Variation of the cytochrome c spectrum in the presence of purified mitochondria extracts.

similar for tobacco and for unwounded *Vitis* leaves, 2100 and 2850 appearing as non-infective, and UCA 993 and UCA 994 showing similar virulence, but on a lower degree than for UCA 992. Interestingly, when wounded *Vitis* leaves were used, not only did all strains produce the grey mould symptoms on leaves but also did 2100 appear as a very phytotoxic strain, just below UCA 992. On the parasitic level after 2100 UCA 994 appeared and then, with the same activity, UCA 993 and 2850 (Fig. 3).

Discussion

Pulsed-field gel electrophoresis rendered a unique karyotype for each of the five *Botrytis* strains analyzed. Bands listed in Table 2 were sensitive to changes in electrophoretic conditions, indicating that they were linear molecules and non-circular forms, which often produce irregular migrations. Some additional bands that appeared sporadically in some of the karyotypes, could be the result of breaks of the DNA because of manipulation or the existence of "fragile sites" on the DNA as described by SUTHERLAND and HECHT (1985). A different origin for these extra-bands could be supernumerary or B-chromosomes as described by ORBACH (1989) in *Magnaporthe grisea*. Moreover, in B-chromosomes of *Schizosaccharomyces pombe*, functional genes have been reported (NIWA et al. 1986) and even genes involved in pathogenicity in *Nectria haematococca* (MIAO et al. 1991). But because they appeared sporadically on our gels, they were not considered in karyotyping although the knowledge of their existence is interesting for further analysis.

The number of chromosome DNA bands varied from 6 (if we consider doublet bands III and IV), showed by strain UCA 993, to 12 considering the doublet bands I, III, and IV and one extra-band resolved after varying the electrophoretic conditions, in UCA 992. The difference between the cytological data published by SHIRANE et al. (1989) and the reported 16 chromosomes in *B. cinerea* and the total chromosomal number found

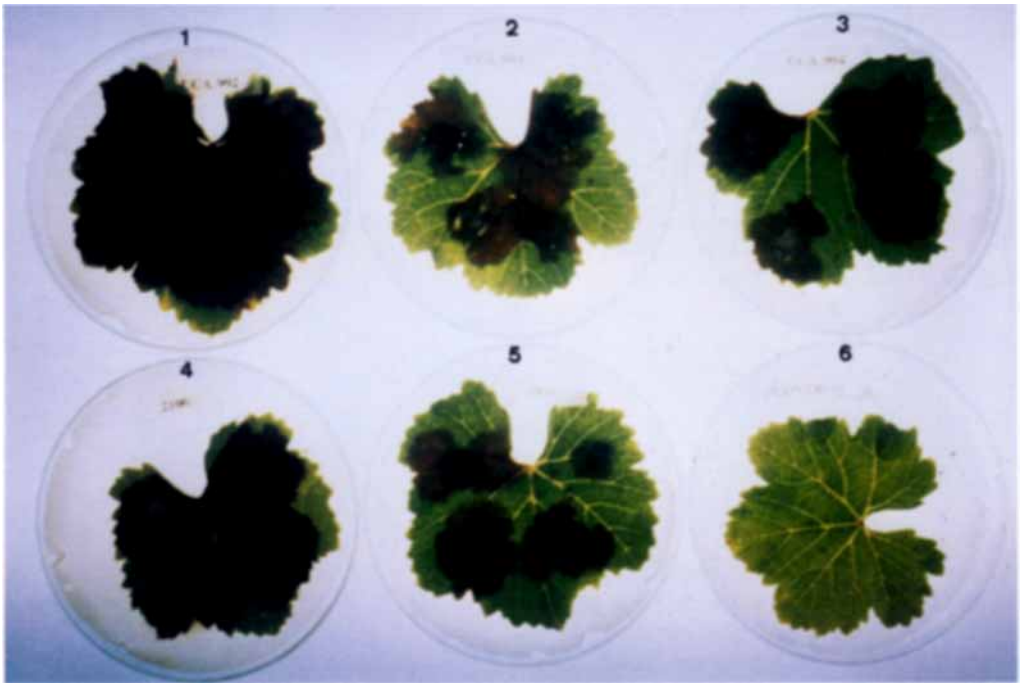

Fig. 3. Wounded *Vitis vinifera* leaves infected with mycelial plugs corresponding to *Botrytis cinerea* UCA 992 (1), *Botrytis cinerea* UCA 993 (2), *Botrytis cinerea* UCA 994 (3), *Botrytis cinerea* 2100 (4), and *Botrytis cinerea* 2850 (5). A wounded leaf with plugs of non-infected medium was used as control (6).

by electrophoresis, could be explained by the high degree of aneuploidy detected in *B. cinerea* (BÜTTNER et al. 1994). On the other hand, chromosomes VII, VIII, and IX migrating slower than the largest of *H. wingeii* and no size markers of higher molecular weight being available in the electrophoretic conditions studied, the sizes of these chromosomes could be determined only approximately through a regression analysis. Anyway, two considerations have to be made: (1) a doublet band could be composed of two compounds of a couple of homo-logous chromosomes or of two heterologous chromosomes of similar size, and (2) two homologous chromosomes can differ in size and appear like two heterologous ones. Thus, hybridization studies are necessary in order to determine the linkage groups for each of the bands, However, a high number of probes would be necessary, which can be difficult to obtain. Therefore, the most accurate size determination of the slowest chromosomes and/or those ones migrating as doublets may be possible only after the isolation of single (or double) bands from pulsed field gels, digestion of the corresponding chromosomes with rare restriction endonucleases that produce large fragments, and electrophoresis of the corresponding fragments.

Our results evidenced polymorphism between all kinds of band sizes, indicating that the electrophoretic conditions chosen were able to discriminate at all DNA size levels. However, an extra-band appeared when conditions were changed, at least in UCA 992, indicating that two or more different electrophoretic conditions could be necessary in order to improve the resolution of a localized zone on the gel, the same as in other genotypes (FIERRO et al. 1993; BRODY and CARBON 1989; DEBETS et al. 1990).

The presence of this high level of polymorphism reveals the facility to support chromosomal rearrangements in this species and could be the basis of the high degree of adaptability to new fungicides. Moreover, it is unlikely that the chromosomal rearrangements appear as a result of meiotic recombination, if we consider that the teleomorphic state is unusual in *Botrytis cinerea*. Instead, the mechanism producing variable karyotype must be activated during the somatic growth or by parasex-

ual recombination of the fungus. A high degree of DNA polymorphism among strains of *B. cinerea* has also been described using RAPD analysis (VAN DER VLUGT-BERGMANS et al. 1993).

Chromosome size polymorphisms had been reported in several fungal strains, in *S. cerevisiae*, where a 1:1 proportion indicated a stable mechanism of transmission (ONO and ISHINO-ARAO 1988), in *Aspergillus* ssp. (KELLER et al. 1992), and the phytopathogenic species *Ustilago maydis* (KINSCHERFF and LEONG 1988), *Ustilago hordei* (MCCLUSKEY and MILLS 1990), and *Colletotrichium gloeosporioides* (MASEL et al. 1990).

The minimal genome size indicated that strain UCA 993 is exactly half the size of strains UCA 992, UCA 994, and 2850 and could represent the haploid content of the DNA, the above strains being the diploid forms and 2100 being an aneuploid. In this regard, BÜTTNER et al. (1994) concluded that the *Botrytis cinerea* strains are not haploids and that aneuploidy is a widespread phenomenon in this species, after determination of the DNA content per nucleus. Segregation results of VAN DER VLUGT-BERGMANS et al. (1993) of DNA polymorphisms in the progeny showed segregation ratios compatible with a diploid level but inexplicable for a haploid.

Nevertheless, if doublet bands and IIIa are considered, the minimal total size of the diploid state would be around 32 Mb, and the haploid one ranging 16–17 would be similar to the diploid form of most fungal genome sizes described (FIERRO et al. 1993), indicating that the filamentous fungi have a larger amount of genetic information than have yeasts with sizes of 12–14 Mb.

Instead, hybridization results showed non-homology to filamentous fungi with two different kind of genes, structural and regulatory (*pyr* and *AMA1*, respectively).

In relation to phytopathogenicity, all strains were parasitic, and no relationships could be established between any karyotype or chromosome and the degree of virulence. 2850 and 2100 were the lowest phytotoxic strains and UCA 992 the highest one. This relation was obtained both in *Nicotiana* and *Vitis*. However, the infective capacity of 2850 and 2100 was increased when wounded *Vitis* leaves were used. This could be explained by enzymes taking part in the first step of the infection for softening the tissue and before the grey mould symptoms are produced by other compounds. Thus, a first approach for further work would be to analyse enzymes such as cutinases, proteases or polygalacturonases involved in leaf tissue softening, looking for a reduced production of these enzymes in 2100 and 2850 strains. We are already working to identify metabolites responsible for phytotoxicity produced by *B. cinerea* (COLLADO et al. 1996). The electrophoretic karyotypes presented will aid the knowledge of genome organization of *Botrytis* and help in the long-term identification and isolation of genes involved in the interaction between fungus and plant and pathogenicity.

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