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STORAGE OF *BOTRYTIS CINEREA* USING DIFFERENT METHODS

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ABSTRACT

Different methods were used to store a collection of field isolates of *B. cinerea.* Five isolates each of the fungus were stored: (*i*) in silica gel at 4ºC, (*ii*) in sand at 4ºC, (*iii*) in potato dextrose agar slants at 4ºC, (*iv*) as dry spores in tubes at –20ºC, or (*v*) as spores in glycerol (20%) at –20ºC. Different characteristics were tested after 1, 2 and 4 years of storage. Viability was poor when spores were stored dried, and contamination was frequent when stored in potato dextrose agar. Linear growth rates of cultures stored for 4 years were similar to initial values, except for one isolate in sand (9% reduction) and for another in glycerol (14% reduction). Sclerotia size and sporulation were reduced after 4 years of storage, independently of the method used. Fungicide resistance to procymidone changed to sensitive in all the isolates, except for one isolate, after four years of storage, also regardless of the storage method used. Storage in sand at 4ºC or in glycerol at –20ºC were the best methods for preserving *B. cinerea* isolates.

Key words: *Botrytis cinerea*, gray mould, storage methods.

INTRODUCTION

Several techniques are available for long-term preservation of fungi such as lyophilization (Fisher *et al.,* 1982), cryopreservation (Holden and Smith, 1992), mineral oil (Lima, 1991), sterile soil (Smith, 1988), and silica gel (Trollope, 1975). These methods are effective in preserving fungal species, but each has limitations. Lyophilization and cryopreservation ensure long-term viability with minimal culture variation, but the equipment is expensive and cultures cannot be returned to storage for reuse. Mineral oil is difficult to use and contamination is common. Storage in soil or silica gel carries a risk of mutation that can result in loss of morphological characters and, sometimes, virulence (Windels *et al.,* 1993).

Botrytis cinerea is a haploid, filamentous, heterothallic fungus that attacks a wide range of plant species in temperate zones and causes grey mould on many economically important crops. Due to its widespread distribution and often destructive effects *B. cinerea* has been studied in a wide range of areas (Coley-Smith *et al.,* 1980; Salinas, 1992; Verhoeff *et al.,* 1992; Kerssies *et al.,* 1997), and interest in this fungus continues. Studies on population genetics or fungicide resistance of this fungus are important to establish better control strategies. These studies are based on experiments using large numbers of field isolates, originating from laboratory fungal collections, which should be properly conserved. Since the variability of the fungus is well documented (Grindle, 1979; Leone, 1990; Van der Vlugt-Bergmans, 1996; Alfonso *et al.*, 2000) a storage method which preserves the characteristics of the isolates should be used. Methods currently used to preserve *B. cinerea* use cryopreservation, sterile soil, and silica gel (Prof. F. Faretra, personal communication). This paper reports the viability and fitness components of some isolates of *B. cinerea* after being stored for several years using different methods.

MATERIALS AND METHODS

Fungal isolates. Five *B. cinerea* isolates were used. Isolations were made from single lesions on tomato (isolate 12.7), cucumber (isolates 30.1 and 43.2), and pepper (isolates 45.3 and 45.4) from crops grown under plastic in commercial greenhouses in Almería (Spain) in November 1992. To isolate *B. cinerea* small pieces of tissue taken from the edge of a lesion were incubated in Petri dishes on fresh potato dextrose agar (PDA) medium amended with 0.5 g l^{-1} streptomycin sulphate at 20-25ºC. Growing mycelium was transferred to PDA slants and stored at 4ºC until needed (approx. 12 months).

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Methods of storage. Mycelia and conidia of *B. cinerea* isolates were produced on eggplant-agar, a medium that enhances conidia production of the fungus. Each isolate was placed in Petri dishes containing eggplant-agar (300 g eggplant were ground in 300 ml of water to which 700 ml of water was then added. The mixture was maintained in a water bath at 50ºC for 2 h and then autoclaved at 120ºC for 30 min after adding 20 g of agar) with a cellophane layer on the surface. The plates were then incubated in a growth chamber at 20-25ºC under fluorescent lights (100 µEm^{-2} s^{-1}, 16 h photoperiod) for two weeks for mycelium and spore production. Isolates were stored in January 1994 in five different ways: (*i*) as dry conidia and conidiophores at –20ºC. Conidia and conidiophores of each isolate were taken from eggplant-agar cultures with a sterile needle and placed into sterile 1.5 ml eppendorf tubes. Tubes were then stored in a freezer at –20ºC. (*ii*) In glycerol at –20ºC. Conidia and conidiophores of each isolate were taken from eggplant-agar cultures with a sterile needle and placed into sterile 1.5 ml Eppendorf tubes containing 1 ml of 20% glycerol. Tubes were then stored in a freezer at –20ºC. (*iii*) In silica gel at 4ºC. A concentrated spore suspension of each isolate was prepared in sterile, reconstituted skim-milk (10% w/v) previously cooled to 4ºC. Aliquots of 0.5 ml were slowly and evenly added to Pyrex glass test tubes (160 x 20 mm) with tightly sealing cotton wool plugs containing sterile silica gel without indicator dye (6-20 mesh, Merck). The tubes were stored at 4ºC. (*iv*) In sand at 4ºC. Pyrex glass test tubes (160 x 20 mm), containing fine sand (garden sand was passed through a sieve 1 mm diam) with 3% oatmeal to give 1/3 of the volume of the tubes, were sealed with cotton wool plugs and autoclaved at 120ºC for 60 min for three consecutive days. Then 0.5 ml aliquots of an aqueous concentrated spore suspension of each isolate were added to the test tubes. The test tubes were incubated at room temperature under continuous fluorescent light in one week. They were then sealed with parafilm and stored in a refrigerator at 4ºC. (*v*) In tubes containing slanting PDA at 4ºC. Slanting PDA (20 ml) in Pyrex glass test tubes (160 x 20 mm) was inoculated with each isolate, incubated at 20-25ºC for one week and stored in a refrigerator at 4ºC.

Viability, resistance to fungicides and fitness assessment. Viability, fitness components (growth rate, sclerotia production and conidia production) and qualitative tests for fungicide resistance of each isolate were assessed initially and after 1, 2 and 4 years of storage.

Viability was determined by inoculating each culture onto 9 cm culture plates containing PDA followed by incubation at 20-25ºC in the dark for three days. Growth indicated that the culture was alive.

The linear growth rate of each isolate was determined *in vitro* by transferring an actively-growing mycelium plug (6 mm diameter) onto PDA plates. After two days of incubation at 20ºC in the dark, the mean colony diameter was determined by two measurements at right angles after subtraction of the 6 mm agar inoculum plug. Radial growth of the colonies was measured daily at the same hour until the colonies covered the entire plate. The growth rate was expressed in mm h^{-1}. Each isolate was cultured on three plates.

To determine the *in vitro* sclerotia production, plates prepared as described above were incubated for three weeks. The average area of sclerotia in the colony (mm^2 cm^{-2}) on each of the three plates was determined by image analysis (CUE-2 version 4.5 software; Galsi Production Ltd.).

To quantify the *in vitro* conidia production, each isolate was grown on three plates containing PDA for 10 days at 20ºC in the dark. The viable colony, covering the whole plate, was cut into small pieces, placed in 150 ml of a 1% Tween-80 solution and sonicated for 5 min. The number of conidia was determined with a haematocytometer from four subsamples per plate and the number of conidia per cm^2 was calculated.

Fitness components were also estimated *in vivo* by measuring lesion growth rate and sporulation on 2 cm leaf disks cut with a sterile cork borer from the second leaf of cucumber plants. Cucumber seeds (cv. 'Hyclos' Mix F1 RS; Royal Sluis) were sowed in compost (Brill 1, GmbH&Co.KG, Germany) and maintained in a greenhouse (day/night temperature 30-15 ± 5ºC, day/night HR 30-96 and no artificial illumination) until leaves developed. Five leaf disks were placed in one Petri dishes on two filter papers soaked with 5 ml of 100 mg l^{-1} gibberellic acid (Hsiang and Chastagner, 1992). Disks were inoculated with actively-growing mycelial plugs (4 mm diameter) and incubated at 22ºC in the dark. Each isolate/storage method was tested three times. The mean colony diameter of the lesion was obtained by two measurements at right angles after 2 days of inoculation. The growth rate was expressed in mm h^{-1}. Spore production was quantified after 7 days of incubation as follows. The leaf disks of each plate were immersed in individual flasks containing 150 ml of 50% ethanol and sonicated for 5 min. The number of conidia was counted with a haematocytometer in four subsamples per flask, and the number of conidia per cm^2 of lesion was determined.

Qualitative tests for fungicide resistance were carried out using discriminatory doses as described in Raposo *et al.* (1996). An actively growing mycelial plug (6 mm diameter) was transferred to each sector of a three sec-

tor plate. Sectors contained PDA (Oxoid) amended with either acetone (0.5%) (control) or one of the following fungicides (dissolved in acetone): 1 mg ml^{-1} a.i. of carbendazim, 5 mg ml^{-1} a.i. of diethofencarb, 6 mg ml^{-1} a.i. of the mixture of carbendazim and diethofencarb (1:5, vol.), and 5 mg ml^{-1} a.i. of procymidone. The final content of acetone in all the media was 0.5%. Plates were incubated at 20ºC in the dark for 3 days and then rated. The fungal colony was considered resistant if it grew on fungicide-amended media and was sensitive if it did not.

All the experiments were repeated at least twice. The second experiment confirmed the results obtained in the first one; thus only results from the first experiment are reported. All data were subjected to analysis of variance as a completely factorial design, with different replicates in each experiment, and 'isolates' and 'storage methods' as main factors. When factors were significant, means were compared by Student-Newman-Keuls range tests ($P = 0.05$).

RESULTS

Fitness components (linear growth rate, sclerotia and conidia production) and fungicide resistance of isolates after six months of isolation are shown in Table 1.

Cultures of all isolates were viable after four years of storage in sand at 4ºC, silica gel at 4ºC or glycerol at –20ºC. However spores stored dried at –20ºC were not viable after 1 or 2 years of storage except for isolate 45.3 (Table 2). Contamination usually occurred in PDA, occasionally in silica gel (Table 2).

Linear growth rates of *B. cinerea*, measured *in vitro* and *in vivo,* were largely unaffected by storage. Linear growth rates of cultures stored 4 years were similar or even greater than initial values (data not shown), except for isolates 45.3 stored in sand (9% reduction in growth) and 45.4 in glycerol (14% reduction) when measured in *in vitro* tests and pathogenicity was not reduced. All isolates continued to infect cucumber leaves, even after 4 years of storage.

Other fitness components of *B. cinerea* isolates, such as sclerotia production, were affected by storage, and were isolate dependent. The area of the sclerotia of isolates 12.7 and 30.1 was reduced after one year of storage, regardless of the storage method. This level then remained constant for isolate 30.1, but increased from 40 to 70% over 4 years for isolate 12.7 (Table 3). A decrease in sclerotia production began after 2 years of storage in isolates 43.2 and 45.3 and then stopped regardless of storage method. In the case of isolate 45.4, a reduction of the sclerotia production was observed only after storage in sand for 2 or 4 years.

In vitro, the conidia production of the isolates was always negatively affected by storage, except for isolate 45.3, particularly in silica gel (Table 4).

In vivo, the number of conidia in cucumber leaf discs was reduced after four years of storage, independent of the method used. In some cases, the reduction of conidia production in *in vivo* tests was observed after one year of storage (Table 5).

Problems arose as well with fungicide resistance phenotypes. Initial fungicide resistance phenotypes of isolates 43.2, 45.3 and 45.4 were $Car^RProc^RDiet^R$ (resistant to carbendazim, procymidone and diethofencarb) whereas 12.7 and 30.1 isolates were resistant to $Car^RProc^RDiet^S$ and $Car^SProc^RDiet^R$, respectively (Table 6).

Table 1. Fitness components and fungicide resistance of *B. cinerea* isolates after one year of isolation[a].

Isolate	Linear growth rate (µm h^{-1})		Sclerotia production (mm^2 cm^{-2})	Conidia production (10^5 conidia cm^{-2})		Fungicide resistance		
	In vitro	*In vivo*	*In vitro*	*In vitro*	*In vivo*	Car	Proc	Diet
12.7	112 ± 1	224 ± 20	5.70 ± 0.30	128.00 ± 43.00	21 ± 4	R	R	S
30.1	99 ± 2	188 ± 20	9.50 ± 0.30	210.00 ± 29.00	10 ± 3	S	R	R
43.2	111 ± 2	244 ± 20	4.70 ± 0.30	1.50 ± 1.100	14 ± 2	R	R	R
45.3	118 ± 2	271 ± 30	4.30 ± 0.30	0.16 ± 0.160	12 ± 3	R	R	R
45.4	118 ± 2	269 ± 20	4.20 ± 0.40	4.20 ± 2.500	16 ± 4	R	R	R

[a] Fitness components: linear growth rate and spore production both measured *in vitro* and *in vivo;* sclerotia production measured *in vitro*. Fungicides: carbendazime (Car), procymidone (Proc) and diethofencarb (Diet). Mean of three and five replicates respectively in *in vitro* and *in vivo* assays. R: resistant; S: sensitive.

Table 2. Viability of *B. cinerea* isolates stored using five different methods for one, two or four years[a].

Storage method[b]	Isolate														
	12.7			30.1			43.2			45.3			45.4		
	Year			Year			Year			Year			Year		
	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
Dry	–[b]	–	–	–	–	–	+	–	–	+	+	+	–	–	–
Silica gel	+	+	+	+	+	+	+	+	+	+	+	+	+	+	C
Sand	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PDA	–	–	–	+	+	C	+	+	C	+	+	C	+	+	C

[a] – : no mycelial growth; + : mycelial growth; C : contaminated culture.
[b] Methods of storage are described in Materials and Methods.

Table 3. Percent reduction in sclerotia production of *B. cinerea* isolates stored for 4 years using different methods[a].

Storage method[b]	Isolate														
	12.7			30.1			43.2			45.3			45.4		
	Year			Year			Year			Year			Year		
	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
Silica gel	**38**	**43**	**40**	**60**	**62**	**65**	0	**38**	**30**	0	**36**	**49**	0	**32**	**42**
Sand	**40**	**54**	**70**	**67**	**78**	**63**	0	**23**	**20**	0	**12**	**10**	0	0	0
Glycerol	**36**	**54**	**50**	**45**	**66**	**61**	0	**37**	**30**	0	**38**	**25**	0	0	0

[a] Data are the percent reduction over the initial values shown in Table 1. ANOVA was carried out and statistical significance was tested according to Student-Newman-Keuls range test at $P = 0.05$. Data whose percentage reduction is significant compared to data shown in Table 1 are in bold.
[b] Methods of storage are described in Materials and Methods.

Table 4. Percent reduction in conidia production *in vitro* of *B. cinerea* isolates stored for 1, 2 or 4 years using different methods[a].

Storage method[b]	Isolate														
	12.7			30.1			43.2			45.3			45.4		
	Year			Year			Year			Year			Year		
	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
Silica gel	**98**	**99**	**100**	**97**	**96**	**98**	**87**	**93**	**87**	0	0	0	**76**	**98**	C
Sand	**97**	**99**	**100**	**96**	**98**	**99**	**98**	**100**	**80**	0	**100**	**100**	**83**	**98**	**90**
Glycerol	**98**	**98**	**99**	**97**	**96**	**99**	**93**	**80**	**80**	0	**100**	**100**	**90**	**98**	**88**

[a] Data are the percent reduction over the initial values shown in Table 1. ANOVA was carried out and statistical significance was tested according to Student-Newman-Keuls range test at $P = 0.05$. Data whose percentage reduction is significant compared to data shown in Table 1 are in bold. C: contaminated culture.
[b] Methods of storage are described in Materials and Methods.

Table 5. Percent reduction in conidia production *in vivo* of *B. cinerea* isolates, stored for 1 or 4 years using different methods[a].

Storage method[b]	Isolate									
	12.7		30.1		43.2		45.3		45.4	
	Year		Year		Year		Year		Year	
	1	4	1	4	1	4	1	4	1	4
Silica gel	**81**	**81**	0	**60**	8	**78**	38	**75**	12	C
Sand	25	**95**	0	**60**	22	**93**	**67**	**67**	0	**44**
Glycerol	**48**	**66**	0	**60**	**57**	**86**	33	**67**	**75**	**87**

[a] Data are the percent reduction over the initial values shown in Table 1. ANOVA was carried out and statistical significance was tested according to Student-Newman-Keuls range test at $P = 0.05$. Data whose percentage reduction is significant compared to data shown in Table 1 are in bold. C: contaminated culture.
[b] Methods of storage are described in Materials and Methods.

Table 6. Changes in sensitivity of *B. cinerea* isolates to fungicides after 4 years of storage using different methods.

Storage method	Isolate														
	12.7			30.1			43.2			45.3			45.4		
	Fungicide[a]			Fungicide			Fungicide			Fungicide			Fungicide		
	Car	Proc	Diet	Car	Proc	Diet	Car	Proc	Diet	Car	Proc	Diet	Car	Proc	Diet
Initial sensitivity	R	R	S	S	R	R	R	R	R	R	R	R	R	R	R
Silica gel	R	R	S	S	R	R	R	**S**	R	R	R	R	R	**S**	R
Sand	R	R	S	S	R	R	R	R	R	R	**S**	R	R	**S**	R
Glycerol	R	R	S	S	**S**	R	R	**S**	R	R	**S**	R	R	**S**	R

[a] Car: carbendazim; Proc: procimidone; Diet: diethofencarb; R: resistant; S: sensitive. Changes in sensitivity of fungicides compared to data shown in Table 1 are in bold.

The phenotypes of all isolates, except isolate 12.7, changed with time in storage. Procymidone resistance was affected by storage after four years. Isolate 30.1 stored in glycerol, isolate 43.2 stored in silica gel or glycerol; isolate 45.3 stored in sand or glycerol and isolate 45.3 stored in silica gel, sand or glycerol became sensitive (Table 6).

DISCUSSION

Although cryopreservation has been used to successfully store isolates of some fungal species, the survival of *B. cinerea* after freezing was very low (Table 1). However, when a cryoprotectant (such as glycerol) was added to the conidia before freezing, survival was enhanced. Cryoprotectants are usually added to spore suspensions of fungi to avoid cell injury during the freezing process (Morris *et al.*, 1988, Smith and Onions, 1994). However, in some cases, the addition of cryoprotectants results in deleterious effects. In cryopreservation of urediniospores of *Puccinia abrupta* var. *partheniicola,* the addition of the cryoprotectants glycerol, dimethyl sulphoxide, trehalose or polyvinyl pyrrolidine prior to freezing reduced both viability and virulence (Holden and Smith, 1992).

Contamination is a general problem in fungal collections. Several authors described problems of contamination in collections of fungi stored in PDA due to mites or other causes (Holden and Smith, 1992). Storage of *B. cinerea* in PDA resulted in contamination problems as well.

Virulence is a characteristic easily lost by fungal isolates in axenic culture (Morris *et al.,* 1988; Holden and Smith, 1992). However, *B. cinerea* isolates continued to be pathogenic after being stored for 4 years in glycerol, sand or silica gel.

Reduction of sclerotia and/or conidia production caused by storage has been also reported in other fungi (Holden and Smith, 1992). Since the normal cytological condition of hyphal cells in *B. cinerea* is multinucleate and frequently heterokaryotic (Lorbeer, 1980), any change in nutrition or environment would allow for different patterns of intermingling of the nuclei and hence would alter the morphological expression. Perhaps the reduction of conidia or sclerotia production of *B. cinerea* could be a transient phenomenom induced by the methods of preservation employed. Like other fungi, *B. cinerea* easily adapts to dryness and abnormal conditions of osmosis and consequently it may temporarily change its behaviour temporarily.

Loss of resistance to procymidone (a dicarboximide) occurred after 4 years of storage in several isolates of *B. cinerea*. Resistance to procymidone (a dicarboximide) in *B. cinerea* implies resistance to other dicarboximide fungicides, since cross-resistance was previously demonstrated (Raposo *et al.,* 1996). Recently, phenotype instability in *B. cinerea* in the absence of benzimidazole and dicarboximide fungicides has been reported (Yourman *et al*., 2001). Yourman *et al*. (2001) state that, in the absence of fungicides, sensitive populations of *B. cinerea* can develop resistance to thiophanate-methyl (a benzimidazole) and vinclozolin (a dicarboximide), and this resistance can be maintained in populations through multiple generations. Results also indicate that populations resistant only to vinclozolin were the most unstable and had the highest incidence of change to a different phenotype. As previously discussed, *B. cinerea* is an anamorph species that has exceptional potential for genetic variation; cells in the hyphae and conidia can be either multinucleate or heterokaryotic, and nuclei can vary in ploidy (Hansen and Smith, 1932; Lorbeer, 1980; Büttner *et al.*, 1994). These inherent characteristics could affect the stability of fungicide sensitive phenotypes in *B. cinerea*. Resistance to procymidone and other dicarboximide fungicides in *B. cinerea* is controlled by the single locus *Daf1* with three alleles: those responsible for normal sensitivity to the fungicides (*Daf1S*), those that cause low resistance (*Daf1LR*), and those that cause high resistance (*Daf1HR*) (Faretra and Pollastro, 1993). A multinucleate hypha or conidiospore is in reality a colony which cannot produce a genetically pure culture unless its nuclei are all identical. Differences in the allele, that codifies for fungicide resistance, when carried in different nuclei, could account for the changes in dicarboximide resistance observed here. Faretra and Pollastro (1993) suggested that the instability of mutants of *Botryotinia fuckeliana* (the teleomorph of *B. cinerea*) grown in fungicide and salt-amended-media was the result of heterokaryosis containing both sensitive and resistant nuclei and that nuclear ratios for sensitive or resistant phenotypes may have been modified under selection pressure.

Changes in fungicide sensitivity after storage could also be due to mutation. The natural mutation frequencies of *B. cinerea* to some dicarboximide fungicides ranged from 4 mutants in 10^6 total conidia to 1 mutant in 10^8 total conidia (Hisada *et al.,* 1979; Davis, 1981; Delcán, 1997). The risk of mutation has been reported for storage methods such as silica gel or sand (Holden and Smith, 1992). Cryopreservation is reported to have less risk of mutation (Moline *et al.,* 1962), but perhaps in *B. cinerea* lower temperatures than –20ºC should be used to avoid this problem.

Problems arise when trying to preserve *B. cinerea* fitness components and fungicide resistance in storage. Among the storage methods tested, the best results were obtained with storage in sand at 4ºC or in glycerol at –20ºC. In addition, viability and virulence of *B. cinerea* were maintained for 4 years using these methods of storage. Whatever method of storage is used, the viability and virulence must be sustained, not only during storage, but also for a period thereafter.

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