An approach for long-term preservation of *Blumeria graminis* f. sp. *tritici*

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Abstract *Blumeria graminis* f. sp. *tritici* (*Bgt*) is an obligate parasite that only infects living tissues of wheat (*Triticum aestivum* L.). Long term preservation of *Bgt* is useful for genetic studies but it is challenging due to difficulty of artificial cultivation. In this study a simple protocol was developed in which desiccating the conidia using crystals (3–6 mm in diameter) of silica gel allowed viable storage for 12 months. The conidia were mixed with silica gel, dried for 5 h at 23 °C, and then stored at −80 °C. The preserved *Bgt* isolates still maintained their viability after 12 months and successfully infected detached leaves of wheat seedlings. Analysis of 20 *Bgt* isolates revealed that the cryopreservation process had no effect on two key phenotypic variants: virulence against 12 near-isogenic wheat cultivars and their sensitivity to the fungicide triadimefon. DNA sequencing analysis confirmed that the nucleotide sequences of the 1.4 α-demethylase inhibitor (*cyp51*), chitin synthase 1 (*chs1*), and β-tubulin (*tub2*) genes had not changed and still contained the corresponding single nucleotide mutations. These results indicate that the silica gel-based desiccation protocol is suitable for the long term preservation of large numbers of *Bgt* isolates.

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Introduction

Blumeria graminis (DC.) Speer f. sp. *tritici* (*Bgt*) is an obligate parasite that only infects living tissues of wheat (*Triticum aestivum* L.). Understanding the genetic background and fungicide resistance response of *Bgt* populations is essential for the development of effective control strategies. However, such epidemiological studies require large numbers of field isolates that need to be properly maintained. As an obligate biotroph, *Bgt* has conventionally been maintained by subculturing on infected seedlings of susceptible hosts. This approach is not practical when storing large numbers of isolates as it is time consuming, prone to contamination, and to genetic and physiological changes.

Cryopreservation, one of the most widespread preservation techniques used for the maintenance of microbial culture collections (Challen and Elliott 1986; Smith 1998; Ryan and Smith 2004) has been successfully used to store a number of obligate biotrophs (Hughes and Macer 1964; Hermansen 1972; Satou 2000; Pérez-Garcia et al. 2006). A key step of the published methods is the partial dehydration of spores followed by storing at low temperature. Several specialized dehydration treatments have been developed for obligate biotrophic pathogens. For example, the conidia of *Erysiphe necator* Schwein. were air-dried for approximately 18 h in a laminar flow cabinet before being snap-frozen in liquid nitrogen (Stummel et al. 1999), while the conidia of *Podosphaera fusca* (Fr.) U. Braun & Shishkoff were desiccated with $CaCl_2$ before treatment with liquid nitrogen (Bardin et al. 2007). It has also been found that the conidia of *Podosphaera fusca* were viable for up to 5 years after silica gel treatment at 22 °C for 8 h and storage at −80 °C (Pérez-Garcia et al. 2006). Although being effective in some species, these techniques are not generally applicable to all obligate pathogens, which can differ in their physiological characteristics.

The aim of this study was to develop a simple method for long term preservation of large collections of *Bgt* isolates. Having reviewed the literature regarding the cryopreservation of obligate biotrophs (Bardin et al. 2007; Stummel et al. 1999; Pérez-Garcia et al. 2006; Wei and Wang 2011), we selected desiccation using silica gel as the most promising approach. After establishing the efficacy of this method, the recovered isolates were assessed to confirm the stability of their genetic and physiological characteristics.

Materials and methods

Source and production of conidia

The *Bgt* isolates E01, E02, E05, E06, E09 and E21 were kindly donated by Dr. Yi-Lin Zhou (Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China), while the others were collected from the major wheat growing regions of China for the present study (Table 1). Conidia were produced by inoculating detached, surface-sterilized leaves of wheat cv. Chancellor as described previously (Yang et al. 2007). The response of the isolates to triadimefon and their virulence against 12 near-isogenic wheat

cultivars (Table 2) were tested prior to preservation. Silica gel was obtained from Sigma (catalog no. 85332).

Optimization of desiccation time

Viability of conidia from *Bgt* isolate HN-12 was tested to optimize the duration of desiccation. Approximately 20 mg of fungal biomass (mainly conidia) was collected from infected leaves and deposited into 1.5 mL cryovials containing 4–5 crystals of the anhydrous silica gel that were oven-sterilized at 160 °C for 3 h. The closed cryovials were then kept in a dark incubator at 23 °C for 1, 5, 10, 15, 24, 36 and 48 h before being placed directly into a DW-86 L628 ultra low temperature refrigerator (Haier Co.) and stored at −80 °C for 1 day. The vials were then thawed to room temperature, and the silica gel removed from the conidia samples using a sterile forceps. The viability of 100 conidia, assessed as the percentage of germinated conidia, was compared with the percent germination of 100 non-desiccated, fresh conidia using the protocol of Yang et al. (2008). Similar samples of conidia were also desiccated for various durations but kept at room temperature for 1 day instead of −80 °C, then assessed for viability as a comparison. The entire experiment was repeated three times.

Effect of storage time and quantity of conidia

The viability of 20 isolates was evaluated after desiccation with 4–5 oven-sterilized crystals of anhydrous silica gel for 5 h at 23 °C and stored at −80 °C for 1, 3, 6 and 12 months. The effect of three quantities of conidia (10, 20 and 30 mg fresh weight) was also assessed for each storage duration. The recovered conidia were then thawed, and their viability were assessed as described. Each treatment was replicated three times.

Table 1 Isolates of *Blumeria graminis* f. sp. *tritici* used in the present study

Isolates[a]	Year	Sampling	Colony color	Sensitivity to triadimefon
E01	1993	Beijing	Whitish-brown	S
E02	1993	Beijing	Whitish-brown	S
E05	1993	Yunnan	Whitish-brown	S
E06	1991	Sichuan	Whitish-brown	S
E09	1993	–	Whitish-brown	S
E21	1992	–	Whitish-brown	S
JZ15	2007	Hubei	whitish-brown	S
HN-12	2009	Henan	Whitish-brown	S
HN-15	2009	Henan	Whitish-brown	M
SX-5	2009	Shanxi	Whitish-brown	M
AH-12	2009	Anhui	Whitish-brown	S
AH-13	2009	Anhui	Whitish-brown	S
SX −112	2011	Shanxi	Pale Yellow	M
HN −17	2011	Henan	Whitish-brown	S
JS-1	2011	Jiangsu	Whitish-brown	S
GS-1	2011	Gansu	Whitish-brown	S
HB-36	2011	Hebei	Albino	M
GZ-82	2011	Guizhou	Albino	M
SD-10	2011	Shandong	Whitish-brown	M
SC-65	2011	Sichuan	Whitish-brown	R

[a] All strains were maintained using the methods developed in this study

[b] *S* sensitive, *M* moderately resistant, *R* resistant to triadimefon

Table 2 Differentials wheat lines used to characterize the virulence of *Blumeria graminis* f. sp. *tritici* isolates

Cultivar name	Pm gene
Chancellor	–
Axminster	1
Ulka	2
Assosan/8 cc	3a
Chul/8 cc	3b
Sonora/8 cc	3c
Michigan	3f
Hope/8 cc	5
Timgalen	6
CII4189	7
Kavkaz	8
Amigo	17
Bounty	19

Pathogenicity test

The virulence profiles of 20 *Bgt* isolates stored by cryopreservation were assessed relative to the same isolates maintained by conventional subculturing to determine the stability of their virulence phenotype after preservation. Each isolate was used to inoculate 12 near-isogenic cultivars as described previously (Niewoehner and Leath 1998). Leaf segments (2.5 cm long) were cut from each cultivar and placed on sterile water agar amended with benzimidazole (0.5 % w/v) in 10×10 cm plastic boxes. A miniature settling tower, composed of a 2 L cup with a 1.5 cm opening at the top, was used to aerially distribute the test conidia onto the leaf in the plate below. After incubating for 14 days at 17 °C in a growth chamber with a 12 h photoperiod and 70 % relative humidity, leaf sections were assessed for disease severity using the scale of Niewoehner and Leath (1998).

Fungicide sensitivity

The sensitivity of the cryopreserved *Bgt* isolates to the fungicide triadimefon was assessed using detached leaf sections as described previously (Brown and Wolfe 1990) in conjunction with the resistance scale of Wang et al. (2011). Each isolate was exposed to triadimefon concentrations of 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 12.8, 25.6 and 51.2 mg/L, and the number of colonies was counted 7 days after inoculation using a 2× magnifying lens. All the sporulation data was collected in 1 day by a single researcher to minimize the possibility of subjective differences when determining whether or not particular colonies had sporulated. The EC_{50} value (the concentration that resulted in 50 % mycelial growth inhibition) was determined using the Data Program System computer program (Hangzhou Reifeng Information Technology Ltd.). Four replicates were used for each treatment, and the entire experiment was performed twice. The average EC_{50} values from the two replicates were used in the data analysis. Each experiment included samples of the cryopreserved isolates and the same isolates conventionally subcultured.

DNA isolation and sequence analysis

Total genomic DNA was isolated from 20 *Bgt* isolates (Table 1) according to the protocol of Gong et al. (2011) before and after cryopreservation and used as template for a polymerase chain reaction (PCR) analysis using the following primer sets: CHS1f (5′-CCT ACT CTG CTC GGC ACT CT-3′) and CHS1r (5′-CTC ATG GAG ATT TCG CGT TT-3′); TUB2f (5′-CCG CGT TCT GTT CGT AAA AT-3′ and TUB2r (5′-AAC CGA GAA GGT TGC CAT C-3′); CYP51f (5′-TAG CAT TGC GTT GGC TAG TG-3′) and CYP51r (5′-GTC CGA CGG CTG TTG ATA AT-3′), which amplified the chitin synthase 1 (*chs1*), β-tubulin (*tub2*) and 1.4 α-demethylase

inhibitor (*cyp51*) genes, respectively. PCR was performed using a PTC-225 Peltier Thermal Cycler (MJ Research) with the following settings: 5 min at 95 °C, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with a final extension of 72 °C for 5 min. The resulting PCR products were 657, 613 and 844 bp in length, respectively, which were then purified, ligated into the PMD18-T Vector (TaKaRa Biotech), and sequenced by Invitrogen Biotech (Shanghai, China). The PCR analysis was performed independently three times for each isolate to avoid mismatch sequences during the PCR amplification and sequencing. Sequence alignment and manual editing of the sequence data were performed using the Bioedit software package (http://www.mbio.ncsu.Edu/BioEdit/bioedit.html).

Data analysis

The data expressed as percentages were transformed to arcsine square root equivalents prior to an ANOVA and comparison of means by Duncan's multiple range tests ($P<0.05$). The statistical analyses were performed using the SAS software package (SAS Institute).

Results

The effect of desiccation time on conidia viability

The effect of desiccation time and freezing on the viability of *Bgt* conidia is shown in Fig. 1. The viability of desiccated conidia stored at room temperature decreased steadily with increasing periods of desiccation, resulting in a complete loss of viability at 48 h. In general, freezing at −80 °C caused a more rapid decline in conidia viability. However, although the samples desiccated for 1 h lost almost 80 % of their viability,
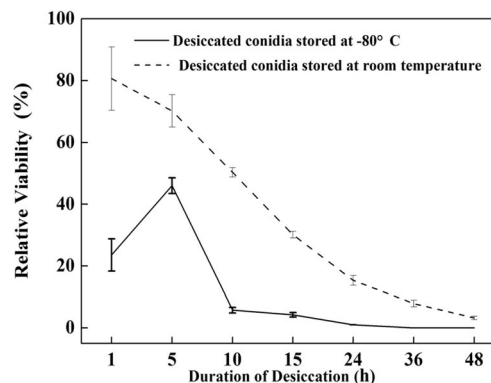

Fig. 1 Effect of desiccation time at 23 °C. Room temperature and −80 °C treatments are represented by dashed and solid lines, respectively. Relative viability was calculated as the percent germination of the treated conidia relative to fresh conidia (usually 45–50 %). Each point represents the mean of three independent experiments. Vertical bars represent standard deviations (SD) of the means

samples desiccated for 5 h retained 40–50 % of their viability compared with fresh conidia. Longer periods of desiccation dramatically reduced the viability to 10 % after 10 h and resulted in a complete loss of viability at 48 h. The optimal time period for silica gel desiccation was thus determined to be 5 h, with the conidia retaining on average 46 % viability (Fig. 1).

Effect of storage time and Quantity of conidia on viability

For the 20 *Bgt* isolates assessed, none of the isolates, regardless of mass of the sample, had lost viability after 6 months of storage (Table 3). However, by 12 months only the 20 mg samples still had 100 % of the isolates remaining viable, whereas 90 % and 80 % of the isolates saved as 10 and 30 mg samples, respectively, maintained viability.

Phenotypic analyses

No differences were detected for any of the 20 cryopreserved *Bgt* isolates compared with the same isolates that had been maintained by the conventional subculture method (Fig. 2). Nor did the color or density of the resulting colonies differ. In particular, isolates SX-112 and HB-36 remained pale yellow, and GZ-82 retained its albino characteristics (Table 1).

Genetic stability

The EC_{50} values calculated for both the cryopreserved and conventionally maintained isolates showed that desiccation and freezing did not significantly ($P>0.05$) affect the sensitivity of any of the isolates tested (Table 4). In addition, DNA sequencing confirmed that the nucleotide sequences of the *cyp51*, *chs1* and *tub2* genes had not

Table 3 Recovery frequency over time of isolates of *Blumeria graminis* f. sp. *tritici* stored in various sample sizes at −80 °C (n=20)

	Months in storage														
	0			1			3			6			12		
	Sample size (mg)			Sample size (mg)			Sample size (mg)			Sample size (mg)			Sample size (mg)		
Isolate	10	20	30	10	20	30	10	20	30	10	20	30	10	20	30
E01	3/3[a]	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
E02	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
E05	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3
E06	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
E09	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	1/3	3/3	3/3
E21	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
JZ15	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3
HN-12	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
HN-15	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
SX-5	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	3/3	3/3
AH-12	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3	3/3	3/3
AH-13	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3
SX-112	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
HN-17	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	3/3	2/3
JS-1	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3
GS-1	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3	3/3	3/3
HB-36	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
GZ-82	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
SD-10	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	1/3	3/3	3/3
SC-65	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3
Total no. of recovered isolates	20	20	20	20	20	20	20	20	20	20	20	20	18	20	16
Recovery frequency of isolates (%)	100	100	100	100	100	100	100	100	100	100	100	100	90	100	80
Recovery for each test[b] (%)	60/60	60/60	60/60	60/60	60/60	60/60	60/60	60/60	60/60	60/60	60/60	60/60	12/60	60/60	14/60

[a] No. of recovered vials/no. tested (repeated)
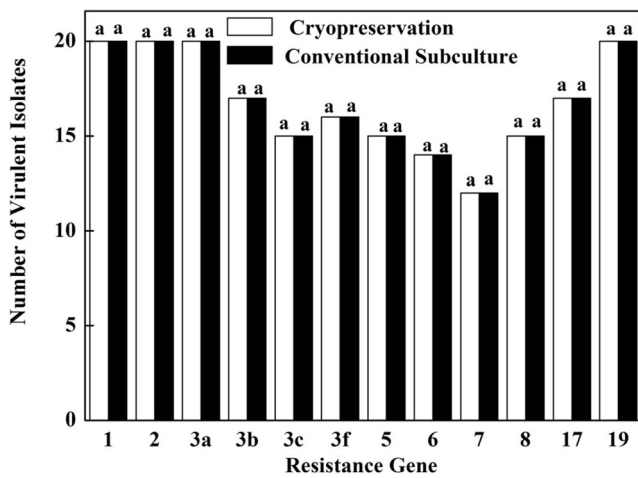
[b] No. of survived tube/frozen tube each test

Fig. 2 Virulence of 20 isolates of *Blumeria graminis* f. sp. *tritici* on near-isogenic wheat lines having one of 12 resistance genes, after either desiccation with cryopreservation at −80 °C (unfilled squares) or conventional subculture method (filled squares). Values followed by the same letter in each column were not significantly different (*P*>0.05)

changed and still contained single nucleotide mutations from the original isolate (data not shown). These results provide evidence that desiccation and freezing did not result in any mutations or genetic rearrangements.

Discussion

A successful method for the long term storage of *B. graminis* f. sp. *tritici* was developed. The new method, which does not require a living host, combined the use of silica gel as a desiccating agent and cryopreservation at −80 °C. The simple three-step protocol can be summarized as follows: (1) place 20 mg conidia suspension into 1.5 mL cryovials containing 4–5 crystals of sterilized anhydrous silica gel; (2) dehydrate the conidia by keeping them in the closed cryovials at 23 °C for 5 h in the dark; (3) place the samples directly in an ultrafreezer at −80 °C. The stored conidia rapidly thawed in the cryovials at room temperature to use as inoculum after the silica gel crystals were removed with a sterile forceps.

Silica gel has been used as a desiccating agent in several methods for long term storage of microorganisms. For example, Perkins (1962) suspended the spores or mycelia of the fungus *Neurospora crassa* in skim milk, which was adsorbed to anhydrous silica gel particles, while Trollope (1975) utilized silica gel for the storage of both Gram-positive and Gram-negative bacilli. However, there have also been many failed attempts (Hopwood and Ferguson 1969; Kutzner 1972) to extend these methods to other microbial groups, which are

Table 4 Comparison of sensitivity of 20 isolates of *Blumeria graminis* f. sp. *tritici* to the 1.4 α-demethylase inhibitor (DMI) fungicide triadimefon after conventional subculturing or storage at −80 °C after desiccation

Isolates	EC$_{50}$ (mg/L) [a]		Resistance to tradimefon [b]
	Routine preservation	Preserved isolates	
E01	0.07±0.012[*]	0.06±0.006	S
E02	0.08±0.005[*]	0.09±0.003	S
E05	0.08±0.001[*]	0.09±0.001	S
E06	0.09±0.002[*]	0.08±0.003	S
E09	0.05±0.005[*]	0.05±0.002	S
E21	0.06±0.016[*]	0.04±0.014	S
JZ15	0.07±0.017[*]	0.07±0.002	S
HN-12	1.25±0.252[*]	1.46±0.462	S
HN-15	20.13±0.13[*]	18.92±8.92	M
SX-5	22.34±2.34[*]	25.37±5.37	M
AH-12	1.23±0.237[*]	1.12±0.127	S
AH-13	1.26±0.267[*]	1.15±0.157	S
SX-112	17.52±7.52[*]	18.23±8.23	M
HN-17	0.04±0.014[*]	0.03±0.015	S
JS-1	0.03±0.017[*]	0.02±0.017	S
GS-1	0.04±0.015[*]	0.04±0.001	S
HB-36	23.25±3.25[*]	25.42±5.42	M
GZ-82	19.28±9.28[*]	17.49±7.49	M
SD-10	21.16±1.16[*]	23.25±3.25	M
SC-65	137.25±37.2[*]	132.23±32.2	R

[a] Data are means of three replicates±standard error. Means followed by [*] in each column were not significantly different (*P*>0.05)

[b] S sensitive, M moderately resistant, R resistance to tradimefon

known to respond differently to established preservation methods. The duration of desiccation (usually 3–10 h) seems to be a critical factor. A shorter time is insufficient to prevent the formation of damaging ice crystals upon freezing, but an extended long time is damaging from the desiccation process itself. In the present study, 5 h was optimal for 20 mg samples mixed with 4–5 crystals of anhydrous silica gel. For the closely related species *P. fusca*, a longer period of desiccation of 8 h was optimum, which enabled the spores to survive for at least 5 years at −80 °C (Pérez-Garcia et al. 2006). Although it is possible that the two species respond differently to desiccation, it is also possible that the type of silica gel may influence the duration of desiccation. We therefore recommend the use of Sigma silica gel (catalog no. 85332) to preserve *Bgt* conidia using our method.

The desiccated *Bgt* conidia were stored for up to 12 months at −80 °C without a significant loss of viability. Indeed, the 20 mg samples had a 100 % recovery rate. However, it was noted that the ratio of silica gel was an important factor, with the lower (10 mg) and higher (30 mg) quantities of spores resulting in a 10-20 % decline in viability respectively. It is likely that this loss of viability resulted from sub-optimal desiccation, since it has long been known that the successful preservation of powdery mildew conidia is highly influenced by their moisture content (Hermansen 1972). There have also been several reports (Nicot et al. 2002; Singh et al. 2004; Koitabashi et al. 2011) that cryopreservation at −80 °C is beneficial in preventing the formation of ice crystals, which can cause physical damage during storage. We found here that freezing at −80 °C was essential for the long-term storage of desiccated *Bgt* conidia, as the viability of conidia at room temperature steeply declined over time with a complete loss of viability within 48 h.

Another key requirement of any preservation process is that it does not induce any genetic or physiological changes (Homolka et al. 2010). The morphological characteristics of the *Bgt* isolates in the present study seemed to be mostly unaffected by the cryopreservation process. For example, the characteristic color and colony density of isolates SX-112, HB-36 and GZ-82 were unaffected. However it was noted that the time required for conidiation (about 14 days) of the cryopreserved fungi was slightly longer than for colonies from freshly harvested conidia (about 10 days). Neither the triadimefon sensitivity nor the virulence phenotype of any of the *Bgt* isolates differed significantly from isolates maintained by conventional subculture. Thus, our cryopreservation method had a negligible effect on the phenotype of *Bgt* isolates.

The two studies that have so far evaluated the genetic stability of obligate biotrophic pathogens after cryopreservation seem to indicate that cryopreservation does not cause any genetic changes (Stummel et al. 1999; Pérez-Garcia et al. 2006). Pérez-Garcia et al. (2006) used random amplified polymorphic DNA (RAPD) to confirm the genetic stability of

P. fusca isolates, while Stummerel et al. (1999) used restriction fragment length polymorphism (RFLP) analysis with *E. necator*. While these fingerprinting methods are sensitive enough to detect certain genetic rearrangements, they are not suitable to detect minute yet important changes in the genome, such as point mutations or short indel mutations. Our genetic analysis here confirmed that the known point mutations in the 1.4 α-demethylase inhibitor (*cyp51*) gene associated with triadimefon resistance, and the sequences of the chitin synthase 1 (*chs1*) and β-tubulin (*tub2*) housekeeping genes in the 20 *Bgt* isolates were unaffected by cryopreservation.

Taken together, our findings provide further evidence that cryopreservation does not induce morphological and genetic changes and confirm the reliability of this method of storage. To date, we have stored more than 800 isolates of *Bgt* using this method, and we hope that future analysis of these isolates will validate the long term viability of conidia stored using this cryopreservation method.

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