

Short Communication

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Long-term Preservation of *Podosphaera fusca* Using Silica Gel

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Abstract

Podosphaera fusca is the main causal agent of cucurbit powdery mildew in Spain and one of the most important limiting factors for cucurbit production worldwide. As an obligate biotrophic parasite, this fungus has been traditionally cultured and conserved by periodical transfers of conidia to fresh plant material. Here we describe a simple protocol for preservation of *P. fusca* isolates in absence of living tissue based on the dry spore, slow-freezing technique, and demonstrate that storage of silica gel desiccated conidia at -80°C is an efficient method for the long-term preservation of the pathogen.

Introduction

Powdery mildew is a devastating disease of cucurbits worldwide. The disease on cucurbits can be caused by either *Podosphaera fusca* (syn. *Sphaerotheca fuliginea*, syn. *Podosphaera xanthii*) or *Golovinomyces cichoracearum* (syn. *Erysiphe cichoracearum*), species that induce identical symptoms but can be distinguished easily under light microscopy (Braun et al., 2002). In southern Spain, however, *P. fusca* has been identified as the sole cause of powdery mildew in cucurbits (Del Pino et al., 2002). Studies on population genetics or fungicide resistance of this fungus are essential to establish better control measures. Such epidemiological studies are based on experiments using large numbers of field isolates, which should be properly conserved. Due to its nature of obligate biotrophic parasite, *P. fusca*, like other powdery mildew fungi, has been traditionally conserved by its propagation on cotyledons of susceptible hosts and periodical transfers to fresh plant material. This method is efficient but not very practical for maintaining large numbers of isolates. Moreover, it is time-consuming, prone to contamination and does in some cases not prevent genetic or physiological changes during long-term and frequent subculturing (Nicot et al., 2002).

Cryopreservation in liquid nitrogen has been considered the best and the most widely applicable preservation technique available for filamentous fungi (Smith, 1998), and it has been also reported for long-term storage of *P. fusca* (O'Brien and Weinert, 1994; Bardin and Nicot, 1999). Briefly, conidia are dried in air or using a desiccating agent such as CaCl_2 , and stored at -196°C in liquid nitrogen. However, freezing in ultra-low freezers is the most commonly used method to preserve microbial culture collections and the method that adjusts better to the requirements of any standard laboratory. For powdery mildews, a method involving the storage at -80°C of infected leaves preserved in paper bags is of routine use for *Blumeria graminis* (Hermansen, 1972). Air-dried conidia of *Erysiphe necator* have been successfully preserved at -70°C after fast freezing in liquid nitrogen (Stummer et al., 1999). For *P. fusca*, freezing at -80°C using cryoprotectants such as dimethylsulphoxide, glycerol, skimmed milk or powdered agar has been also described (Ozaki et al., 1995). In preliminary tests in our laboratory the use of such agents failed. The aim of this study was, therefore, to develop a simple freezing method for the long-term preservation of large collections of isolates of *P. fusca*.

Results and Discussion

Successful preservation of powdery mildew conidia appears to be influenced by their moisture content (Hermansen, 1972). Keeping in mind this observation, we started initially evaluating the suitability of silica gel as desiccating agent to preserve *P. fusca* conidia at -80°C , as silica gel is considered one of the methods for preservation of spore forming microorganisms (Trollope, 1975). Three isolates of *P. fusca* were used in this first approach. The isolates were grown *in planta* on cotyledons of zucchini (*Cucurbita pepo*, cv. Negro Belleza), maintained *in vitro* as described by Álvarez and Torés (1997). Prior to storage at -80°C ,

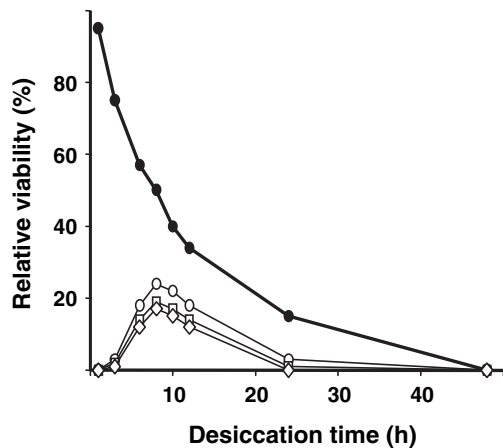


Fig. 1 Relationship between silica gel desiccation time at 22°C and viability of *Podosphaera fusca* conidia in storage at -80°C. Relative viability is indicated as percentage of germinated conidia referred to germination rate of fresh conidia (usually 45–50% of germination). Data points are mean values of three independent experiments using three isolates of *P. fusca*. Symbols are desiccation without storage (●), desiccation and storage at -80°C for 1 week (○), for 1 month (□) or for 1 year (◇)

abundant fungal biomass (mainly conidia) were collected from infected cotyledons with the help of an ethanol-disinfected eyelash, deposited into 1.5 ml cryovials containing 4–5 oven-sterilized crystals of anhydrous silica gel, and desiccated at 22°C in the dark for different time periods varying from 1 to 48 h. Relative viability of isolates after different storage periods was assessed as percentage of germinated conidia on zucchini cotyledons referred to the germination rate of non-desiccated fresh conidia. Conidia germination was evaluated by light microscopy 24–48 h after inoculation and for that, cotyledon discs were taken, cleared and stained as previously described (Romero et al., 2004).

Relationship between viability of *P. fusca* conidia after different storage periods (1 week, 1 month and 1 year) at -80°C and desiccation time at 22°C is shown in Fig. 1. On non-stored material, silica gel desiccation had a negative impact on viability and a typical inactivation curve could be observed, viability being completely abolished after 48 h of desiccation. Storage at -80°C of non-desiccated conidia also suppressed viability (data not shown); however, when conidia were storage at -80°C after desiccation at 22°C, the profiles of the inactivation curves changed substantially, and either after a week, a month or a year in storage, a maximum of viability of around 20–25% of viability of fresh conidia was observed. In all cases, this maximum corresponded to desiccation periods of 6–12 h, which seemed to protect conidia from freezing damage to levels high enough to maintain viability of isolates of *P. fusca* at least for up to 1 year. This result improved twofold the viability previously reported for cryopreservation in liquid nitrogen of air-dried conidia of *P. fusca* (O'Brien and Weinert, 1994). Hermansen (1972) described that conidia of *B. graminis* produced

Table 1

Recovery frequency of isolates of *Podosphaera fusca* after several years of storage at -80°C of silica gel desiccated conidia ($n = 20$)

Years of storage	Isolates recovered	Recovery frequency (%)
1	20	100
2	20	100
3	20	100
4	16	80
5	15	75

at high relative humidity lost their infectiveness after freezing, whereas conidia produced at low relative humidity retained their viability during storage at below-freezing temperature. Similarly, our results show that only a limited desiccation of *P. fusca* conidia to reduce spore water content to a certain extent seems to be beneficial against freezing damage.

To test the hypothesis that long-time preservation at -80°C of *P. fusca* conidia could be achieved after silica gel desiccation, 20 isolates were subjected to desiccation for 8 h at 22°C and the recovery frequencies of isolates after periods of 1–5 years in storage at -80°C was estimated by their ability to infect zucchini cotyledons. As it can be observed in Table 1, recovery frequencies were 100% for storage periods up to 3 years, which is similar to that reported for cryopreservation in liquid nitrogen of *P. fusca* conidia after desiccation with CaCl₂ (Bardin and Nicot, 1999). For longer storage periods, a 20–25% decline in the recovery frequency was observed after 4–5 years. Nevertheless, as a few spores viable would be enough to get a couple of powdery mildew colonies, it is likely that survival periods longer than 5 years could be achieved with this method, like the one described for *B. graminis*, which has been tried up to 10 years (L. Munk, personal communication). Furthermore, phenotypic stability of the isolates was analysed by testing specific traits, such as race-specific avirulence and host range (Del Pino et al., 2002). No differences were observed between data recorded before and after storage (data not shown). In addition, genetic stability of isolates was tested by random amplification of polymorphic DNA (RAPD) analysis. In accordance with phenotypic stability, no differences were found between the RAPD profiles observed before and after storage for any of the isolates tested (data not shown), which suggests that this preservation method does not appear to cause

Table 2

Freezing/thawing method for long-term preservation of *Podosphaera fusca* in ultra-low freezers proposed in this study

1. For freezing, collect abundant conidia and place them into 1.5 ml cryovials containing 4–5 anhydrous silica gel crystals.
2. Incubate at 22°C in the dark for 8 h and store at -80°C.
3. For thawing, wait until tube reaches room temperature.
4. Remove silica gel crystals and deposit conidia onto fresh cotyledons.

any mutations or genomic rearrangements detectable by this technique.

On the basis of these results we have designed a protocol for the long-term preservation of *P. fusca* in absence of living host tissue, which is shown in Table 2. This method complies with the three main objectives that should be achieved for any microbial preservation method: (i) pureness, (ii) survival, and (iii) genetic stability of the isolates during storage. Currently, more than 1100 isolates of *P. fusca* have been stored at -80°C using this method in our laboratory.

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