EVALUATION OF DIFFERENT STORAGE METHODS FOR RAPID AND COST-EFFECTIVE PRESERVATION OF *BOTRYOSPHAERIA* SPECIES

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ABSTRACT

Four *Botryosphaeria* species isolated from grapevine, namely *Diplodia seriata* (teleomorph *B. obtusa*), *Diplodia mutila* (teleomorph *B. stevensii*), *Neofusicoccum parvum* (syn. *B. parva*) and *Neofusicoccum luteum* (syn. *B. lutea*), were stored using 11 different methods. Duplicate isolates were stored for 1, 3 and 6 months as mycelium-colonised agar plugs and pycnidia, in sterile water, mineral oil, 20% glycerol or potato dextrose agar (PDA) at room temperature (RT), 4°C or -80°C. The viability of the four *Botryosphaeria* species was similar (81-100%) for all storage methods apart from pycnidia on pine needles at RT (68%), but their overall growth rates differed between the storage methods ($P < 0.001$) and storage times ($P < 0.001$). Of the methods tested, mycelium plugs stored in sterile water at 4°C or in glycerol at -80°C retained highest growth rates.

Keywords: *Botryosphaeria* species, culture preservation, mycelium plugs, pycnidia.

INTRODUCTION

The preservation of fungal strains as reference stocks for ongoing research requires that the stored cultures remain viable for long time periods without any morphological or physiological alterations. Of the various methods used for extended storage of fungal cultures, researchers have reported that certain methods may be more or less efficacious for a particular species or genus. Common methods used to date include storage in sterile water, saline, grain or soil at room temperature, storage under mineral or paraffin oil, freezing at -20°C or -80°C, freeze-drying and cryopreservation in liquid nitrogen. The methods reported for storing *Botryosphaeria* species include storage of agar plugs under mineral oil, which retained the viability of two unidentified *Botryosphaeria* species for 31 years, of *B. obtusa* for 20 years and *B. ribis* for 6 years (Smith & Onions 1994), as well as freeze-drying and liquid nitrogen storage (M. Fletcher, ICMP Landcare Research, Auckland, pers. comm.). Culture viability is a common criterion of success for fungal preservation methods. Radial growth rate may also be used (Burdall & Dorworth 1994), but has not been reported for *Botryosphaeria* spp. The pathogenicity of some fungi to host plants has also been assessed, especially after long-term storage (Hajek et al. 1995).

This study evaluated different storage methods for simplicity, speed and cost-effectiveness, in preserving two isolates each of four *Botryosphaeria* spp. from grapevine, on which they cause trunk decline and plant death (van Niekerk et al. 2006). A storage method that preserves culture viability was considered essential for current research into the modes of action of these pathogens.

MATERIALS AND METHODS

Fungal isolates

Two isolates from each of four *Botryosphaeria* species, namely *Diplodia seriata* (teleomorph *B. obtusa*), *Diplodia mutila* (teleomorph *B. stevensii*), *Neofusicoccum parvum* (syn. *B. parva*) and *Neofusicoccum luteum* (syn. *B. lutea*), were used in this study. These isolates were freshly isolated from grapevine samples collected in Auckland, Gisborne, Nelson and Blenheim regions in New Zealand during 2007/2008 and had been identified using molecular (Alves et al. 2005) and morphological criteria (http://www.crem.fct.unl.pt/botryosphaeria_site/).

Storage methods

The 11 storage methods chosen included those commonly used by plant pathologists (Smith & Onions 1994; Waller et al. 2002), but excluded storage by freeze-drying and within liquid nitrogen, as these methods were deemed impractical for rapid storage of large numbers of isolates or costly to maintain.

The isolates were grown on potato-dextrose agar (PDA; Oxoid) for 7 days at 23.5°C with 12:12 h dark:light to provide mycelium colonised agar plugs (5 mm diameter) for the different storage methods. Slant cultures on PDA were inoculated with mycelial agar plugs, incubated for 7 days and stored at 4°C. Pycnidia, produced on water agar (2%; Oxoid) embedded with autoclaved mature pine needles (*Pinus* sp.) at room temperature under near UV light for 2 weeks (Slippers et al. 2005), were harvested using a sterile needle. For *D. seriata* and *D. mutila*, mycelium-colonised agar plugs and pycnidia were stored in all the combinations of substrate and temperature (Table 1). No pycnidia were produced by *N. parvum* and *N. luteum*, so only treatments with mycelium-colonised agar plugs (M1-4 and M7; Table 1) were used for these species.

In this study, the sterile plastic screw cap vials (2 ml) were filled with sterile distilled water (Invitrogen), sterile mineral oil (Sigma, Molecular biology grade) or 20% glycerol (BioLab) and glass screw cap vials (20 ml) used for 10 ml PDA slants. Storage was at room temperature, 4°C and -80°C. Water storage of mycelium plugs was initially set up at room temperature (RT), as described by Smith & Onions (1994), but these cultures continued to grow in the storage vials, becoming dark coloured and full of mycelia, so this treatment was discontinued. Each vial contained six colonised agar plugs or ten pycnidia, with five replicates for each treatment and sampling time point.

Assessment of storage methods

Initial growth rate ($T=0$) of all isolates on PDA at 23.5°C was measured between 24 h and 48 h, with four measurements per plate (one in each quarter) to give a mean growth rate (mm) per day. This was calculated for the four replicates per treatment and isolate. PDA brand, plate size (90 × 15 mm) and agar depth (5-6 mm) was consistent throughout the experiment.

Radial growth of fungi from stored mycelium plugs was measured after 1, 3 and 6 months of storage. Mycelium plugs retrieved from 20% glycerol, sterile water and mineral oil were blotted dry on sterile filter paper prior to plating. For each pycnidium assessment a 2-day-old, 5 mm mycelium plug derived from plating a single pycnidium was transferred to a fresh PDA plate and the radial growth measured as above. A subculture was used because the stored pycnidia grew slowly and unevenly.

The growth rate data for the *Botryosphaeria* species, storage methods and storage periods were analysed using univariate analysis of variance. The non-viable replicates were treated as missing data for the statistical analysis.

RESULTS

Viable cultures of the four species were recovered from all of the storage methods at each time point, with treatments M1-4 and M8 having 100% viability at all times. Over all treatments, RT storage and pycnidia had the lowest viability, with pycnidia stored at RT on pine needles being least viable (68%; Table 1).

Radial growth rates were similar for the four species ($P=0.755$) so the data from the species were combined. Storage period affected radial growth rate ($P<0.001$), which

decreased over the length of the storage period from means of 18.6 mm/day ($T=0$) to 17.9, 17.6 and 16.5 mm/day at 1, 3 and 6 months, reductions of 3.5%, 5% and 10.9%, respectively. Significant effects were observed for storage method ($P<0.001$), with radial growth generally being greater for cultures stored at cold temperatures (4°C and -80°C) than at RT, with pycnidia at RT (M9 and M10) being lowest. After 6 months storage, mycelium plugs stored in sterile water at 4°C (M1) and in glycerol at -80°C (M2) had greatest ($P<0.001$) radial growth rates of 17.9 and 17.2 mm/day, respectively, compared with other treatments, and pycnidia stored on pine needles at RT (M9) had the least (15.7 mm/day).

TABLE 1: Viability (%) of isolates at 1, 3 and 6 months of storage with different storage methods (M1-M11), and their mean radial growth rates (mm/day) after 6 months storage, with 95% confidence intervals in parentheses.

Code	Storage method	Viability (months)			Mean growth rate after 6 months
		1	3	6	
M1	Mycelium in sterile water at 4°C	100	100	100	17.9 (17.5-18.2)
M2	Mycelium in 20% glycerol at -80°C	100	100	100	17.2 (16.9-17.6)
M3	Potato dextrose agar slant at 4°C	100	100	100	16.1 (15.7-16.4)
M4	Mycelium in mineral oil at 4°C	100	100	100	16.5 (16.1-16.8)
M5	Pycnidia in 20% glycerol at -80°C	94	100	81	16.5 (16.1-17.0)
M6	Pycnidia in sterile water at 4°C	100	100	94	16.5 (16.0-16.9)
M7	Mycelium in mineral oil at RT	100	97	100	16.1 (15.7-16.4)
M8	Pycnidia in mineral oil at 4°C	100	100	100	16.1 (15.6-16.5)
M9	Pycnidia on pine needles at RT	81	81	68	15.7 (15.2-16.3)
M10	Pycnidia in sterile water at RT	94	94	88	15.9 (15.4-16.3)
M11	Pycnidia in mineral oil at RT	88	81	88	16.5 (16.0-16.9)

DISCUSSION

This study was initiated to develop storage methods that could preserve culture viability and characteristics essential for research into the modes of action of these pathogens. Results showed that culture storage methods that incorporated mycelium had 100% viability after 6 months for all four *Botryosphaeria* species tested, whereas pycnidia had 68-100% viability. This concurs with Smith & Onions (1994) who also reported excellent survival (up to 31 years) of *Botryosphaeria* species following storage as agar slants covered by mineral oil. Radial growth rates were also greater for stored mycelium than for pycnidia, being greatest for mycelium plugs held in sterile water at 4°C and glycerol at -80°C . The failure of the method for storing mycelium plugs in water at RT due to continuous growth could have been resolved by storing only mycelium with no attached agar, a method that was recommended by Smith & Onions (1994). However, this was deemed too labour intensive for the purpose of rapidly storing large numbers of isolates.

Storage of fungi under sterile water has been used to successfully store a range of different fungal species (Smith & Onions 1994), being simple, rapid and inexpensive. However, like many other studies they investigated only viability. The present study has demonstrated that although storage under mineral oil retained 100% viability there was a decrease in growth rate of recovered isolates. Other studies have also shown that although colony characteristics can be retained after long term storage, frequent serial transfer following recovery can cause the loss of physiological attributes. For example, *B. obtusa*

retained ascospore producing capacity when stored under mineral oil for 20 years but this characteristic was lost during frequent sub-culturing (Smith & Onions 1994).

Higher radial growth rates for recovered isolates were evident in treatments at the lower temperatures of 4°C and -80°C when compared to room temperature (approximately 20°C). It is possible that either the higher temperature, which maintains a greater respiratory activity, or the regular fluctuations of ambient temperature (day/night effect) are detrimental to longevity of stored cultures of *Botryosphaeria* species.

These results indicate that storage for 6 months can alter the growth rate of recovered isolates of *Botryosphaeria* species. It is not known whether this reduced growth rate would persist following serial sub-culturing or after passage through plant material. Longer storage periods are required before the methods recommended can be finally confirmed as best able to maintain characteristics of the isolates. It would also be useful to extend this study by investigating the effect of storage method on pathogenicity of *Botryosphaeria* species. In summary, this work has shown that storage of *Botryosphaeria* species as mycelium plugs in sterile water at 4°C was the best method for maintaining viability and preservation of growth rates and is suitable for storage of the >400 isolates that form the basis of the current research.

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