Viability and pathogenicity of *Phytophthora clandestina* after storage in water and liquid nitrogen

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Abstract

Survival, pathogenicity, and in vitro growth rate of three isolates of *Phytophthora clandestina* were tested at monthly intervals for up to 10 months following storage in liquid nitrogen and sterile deionised water on millet seeds at 4°C. Storage of cultures in sterile deionised water on millet seeds maintained high recovery rates (75-80%) of all three isolates after 10 months without loss of pathogenicity. The success of liquid nitrogen storage of *P. clandestina* was variable with only one isolate surviving more than 3 months storage. There was also an effect of the protective solutions used on long term storage and maintenance of pathogenicity. Glycerol (10%) allowed a high recovery rate (89%) of one isolate without loss of pathogenicity for 10 months whilst DMSO (5%) provided a maximum storage period of 3 months with a significant reduction in pathogenicity in the three isolates tested. This study demonstrates that sterile water storage is superior to liquid nitrogen for the medium to long term storage of *P. clandestina*.

Introduction

*Phytophthora clandestina* Taylor, Pascoe and Greenhalgh was first isolated from rotted roots of subterranean clover (*Trifolium subterraneum* L.) in Victoria (Taylor 1984) and later in Western Australia (Wong et al. 1985). This fungus is now recognised as the major cause of severe tap root rot of subterranean clover throughout Australia (Murray and Davis 1996).

When maintained in pure culture through the laborious technique of periodic transfer, phytopathogenic fungi may lose pathogenicity and the ability to sporulate (Dahmen et al. 1983). Further, genetic degeneration or attenuation of strains has been recorded (Shaw 1988). The continued handling and maintenance of such plant pathogens can also increase the chance of contamination. Commonly used methods for preserving fungal cultures include freezing (Bromfield and Schmitt 1967), freeze-drying (Heckly 1961; Staffeldt 1961), oil-cover slants, soil or sand cultures, and drying (Fennell 1960). Although freeze-drying is useful for preserving many fungi, alternative techniques are needed for many *Phytophthora* species whose sporangia do not survive this process (Fennell 1960; Hwang 1966).

The storage of *Phytophthora* spp. in liquid nitrogen is not widely used, even though it is generally regarded as the most effective method of storage (Smith 1982). The reason for this is the high cost of programmable slow-freezing units required for liquid nitrogen storage of *Phytophthora* spp. Such units need a controlled temperature decrease of about 1°C per minute for the fungi to survive the freezing process (Alexander et al. 1980; Dahmen et al. 1983; Hwang 1966; Hwang and Howells 1968). However, the need for a programmable freezer can be avoided by using an uncontrolled freezing protocol that achieves gradual freezing using a −80°C freezer (Tooley 1988). Tooley (1988) found that isolates of several species of *Phytophthora* (not including *P. clandestina*) could be stored for up to 9 months in liquid nitrogen. He suspended agar plugs with mycelium in 1 mL of cryoprotectant (10% glycerol or 5% dimethylsulfoxide) in polypropylene vials. The vials were placed in a mechanical freezer at −80°C for 60 min before storage in liquid nitrogen.

As an alternative to expensive storage in liquid nitrogen, McGinnis et al. (1974) have recorded
survival rates of 93% with filamentous fungi maintained in sterile distilled water over periods ranging from 12 to 60 months. Boesewinkel (1976) has reported the storage of many phytopathogenic fungi in sterile water including *Fusarium*, *Phytophthora* spp., *Rhizoctonia solani* and *Sclerotium cepivorum* for 14 to 18 months. Water storage is now used routinely for a wide range of fungi (Dhingra and Sinclair 1985).

This study aimed to find a suitable technique for the routine storage of *P. clandestina*. The technique of Tooley (1988) for liquid nitrogen storage of *P. clandestina*, using the two cryoprotectants dimethylsulfoxide and glycerol was therefore compared with storage of the fungus on sterile millet seed in sterile deionised water at 4°C. The suitability of these two storage techniques was assessed at monthly intervals in terms of recovery, growth rate and maintenance of pathogenicity of the stored material.

**Methods**

**Isolates of Phytophthora clandestina** Three isolates of *P. clandestina*, NSW1, NSW4 and NSW5, obtained from Department of Agriculture Herbarium, Rydalmere (Herb DAR) were used in these studies. They were isolated by baiting from soil from the Holbrook area of southern New South Wales, Australia in 1991 by Ms J. Eskdale (Eskdale and Murray 1991). The cultures were maintained for around one month by periodic transfer on lima bean agar (LBA) and passaged back through susceptible subterranean clover seedlings (cv. Woogenellup) to restore pathogenicity before commencing the experiment.

**Liquid nitrogen storage and recovery of P. clandestina** Fungal material for storage was prepared by cutting six 7-mm–diameter plugs of fungal mycelium from within the colony margins of 12–day–old cultures of each isolate of *P. clandestina* grown on LBA. These plugs were placed into 1 mL of either sterile 10% (v/v) glycerol (in deionised water) or sterile 5% (v/v) aqueous solution of dimethylsulfoxide (DMSO) in 2 mL screw-cap polypropylene vials. For each cryoprotectant (glycerol or DMSO) x fungal isolate x storage time combination, three vials each containing six plugs were prepared (i.e. a total of 18 plugs per isolate). The vials were clipped into aluminium vial holders (six vials per holder) and covered with cardboard cryosleeves before placing inside stainless steel pipette cans and transferred to a −75°C freezer for 65 min. The vials were then removed from the pipette cans and cardboard cryosleeves and plunged into liquid nitrogen (−196°C). A further 18 agar plugs (three vials of six plugs) of each isolate of *P. clandestina* suspended in sterile deionised water and stored at room temperature served as controls to compare with the plugs suspended in liquid nitrogen at the start of the experiment.

Immediately after reaching the storage temperature (−196°C), three frozen vials of each cryoprotectant x isolate combination were thawed by placing in a 37°C water bath for 4 min. Because DMSO has been shown to be toxic to several fungi (Dahmen et al. 1983), the plugs of *P. clandestina* were washed in sterile deionised water after removal from storage. The washed culture plugs from each thawed vial along with the control plugs were then plated on LBA (20 mL) in 9-cm–diameter Petri dishes and incubated at 25°C for 16 days in darkness. Thereafter, vials were thawed and assayed for survival, growth and pathogenicity of the fungus at monthly intervals.

**Storage on millet seeds in water at 4°C** Millet seeds (*Panicum miliaceum*) were washed twice in deionised water to remove pesticides, then allowed to air dry on paper towelling. Lots of 20 seeds were placed in 28 mL McCartney bottles containing 10 mL of deionised water and sterilised by autoclaving at 121°C for 20 min. The sterilised seeds were inoculated with a 7-mm–diameter plug of a LBA culture of each isolate of *P. clandestina*. The bottles were incubated at 25°C for 14 days to allow colonisation of the millet seeds by the fungus. After colonisation, the lids of the McCartney bottles were tightly screwed down and the cultures transferred to a refrigerator set at 4°C. At monthly intervals for 10 months, a single bottle of seeds was removed for recovery, growth and pathogenicity testing. The test at 0 months of storage was done immediately after the bottle was cooled to 4°C. The bottle was warmed to room temperature and the seeds plated on LBA and incubated at 25°C for 16 days in darkness.

**Assaying recovery, growth and pathogenicity of stored cultures** Recovery, growth and pathogenicity of the stored cultures were assessed at monthly intervals for 10 months. Recovery was the proportion of culture plugs or millet seeds from