Cryopreservation of Strawberry Pathogens in a -95°C Mechanical Ultra-low Temperature Freezer

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The long-term storage of plant pathogens is important for the maintenance of cultures for research and identification/comparison purposes. A popular and successful method for long-term storage of fungal cultures is lyophilization (freeze-drying) (Haskins and Anastasiou, 1953). Cryogenic storage in liquid nitrogen has also been used to preserve the original cultural properties of fungi (Hwang, 1966), including phytopathogenic species (Dahman et al., 1983). Most cryopreservation techniques utilize liquid nitrogen and involve storage either in the liquid (-196°C) or the vapor phase (-150°C). The survival of most eukaryotic microorganisms after cryogenic storage has been improved through the use of cryoprotectants, such as glycerol or dimethyl sulfoxide, and by controlling freezing and thaw rates (Dahman et al., 1983; Kirsop and Doyle, 1991). The need to continuously replace liquid nitrogen can be difficult at small or remote facilities. With the increased availability of ultra-low temperature freezers, their use for storing cultures may be more economical than liquid nitrogen and more readily available than lyophilization. This study evaluates the survival of several strawberry fungal pathogens in a -95°C ultra-low temperature freezer.

Eighteen monoconidial isolates representing four fungal pathogens (*Colletotrichum fragariae* A.N. Brooks, *C. gloeosporioides* Penz. & Sacc. in Penz., *C. acutatum* J.H. Simmonds, and *Phomopsis obscurans* Sutton) of strawberry (*Fragaria xananassa* Duchesne)

were evaluated for viability after long-term storage. They were cultured on potato dextrose agar (PDA) at 25°C . Plugs (6 mm) were cut from the margin of fresh colonies and transferred to five replicate 2-mL cryotubes (Corning part number 430659, Cambridge, Mass.), and 1 mL of sterile 10% glycerol was then aseptically pipetted into each tube before sealing. Freezing was carried out in a prechilled (4°C) cryopreservation module (Stratagene Model 400005, LaJolla, Calif.) in a -95°C freezer. These modules control the rate of freezing ($1^{\circ}\text{C}\cdot\text{min}^{-1}$) to minimize cell damage. After storage at -95°C for up to 21 months, isolates were thawed at room temperature, and the culture plugs were placed onto PDA plates and evaluated for viability and normal culture characteristics after incubation at 23°C for 7 d.

The effect of rapid, uncontrolled freezing on culture viability was also evaluated by placing a different set of prepared cryotubes directly in the -95°C freezer without using the cryopreservation module. After 24 h, they were thawed at room temperature for 2 h, then returned to the freezer for 7 d. This procedure was repeated four more times at weekly intervals. After each thaw event, one tube for each isolate was transferred to PDA, and the viability and colony characteristics of cultures determined after 7 d.

The survival of the cultures stored with and without the cryopreservation modules at -95°C was excellent. Live cultures were recovered from all of the plugs of all 18 fungal isolates evaluated. The freezing rate treatments and storage intervals did not affect the cultural and morphological characteristics of the isolates tested. Colony characteristics of the isolates were not modified by any of the storage treatments.

Preservation of fungal plant pathogens in long-term storage is an important tool for research and regulatory processes. The use of

ultra-low temperature freezers for storing cultures would expand the number of facilities that could store isolates on site, and would reduce the cost of maintaining such collections. In this study, isolates were successfully stored for up to 21 months without noticeable changes in viability or cultural characteristics.

We are currently using this procedure to maintain a culture collection of over 500 fungal isolates from strawberry. Four-year-old cultures from this collection have been successfully retrieved and used in epidemiological and etiological studies, and their pathogenicity and virulence were unaffected. Several other fungi pathogenic to strawberry have also been successfully stored using this method, including: *Verticillium dahliae* Kleb., *Phytophthora* sp., *Gnomonia* sp., *Alternaria* sp., *Pestalotia* sp., *Hainesia* sp., and *Cercospora* sp.

There were no noticeable benefits to using the cryopreservation module to slow the rate of freezing. This finding suggests that for frequently retrieved isolates, a single tube may be thawed, a plug removed, and the tube refrozen for future use. However, the multiple freeze-thaw cycles of this procedure could have caused genetic damage. More detailed studies are necessary to fully determine the effect of repeated freeze-thaw cycles on pathological characteristics of fungi.

Fungi currently preserved in liquid nitrogen at -196°C may also survive storage at -95°C . Although most metabolic activity is suspended below -70°C , ice recrystallization and other biophysical processes that can affect cell survival may still be active at temperatures above -130°C (Jong, 1989). Longer-term survival of cryogenically stored fungi may require the lower temperature provided by liquid nitrogen.

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