LONG-TERM STORAGE OF PLASMOPARA HALSTEDII ZOOSPORANGIA IN LIQUID NITROGEN WITHOUT CRYOPROTECTANTS

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SUMMARY

Zoosporangia of Plasmopara halstedii have been successfully stored in liquid nitrogen without the aid of cryoprotectants for over 4 years. When sunflower seedlings were inoculated with the stored zoosporangia, the infectivity, averaged across 45 stored samples, was 78%, compared to 95% for fresh zoosporangia. The preservation method consisted of harvesting air-dried zoosporangia and immediately placing them in liquid nitrogen. In comparisons with cyroprotectants, only a mixture of glycerol and skim milk, combined with a slow, controlled freezing rate gave results comparable to the dry spore/fast freeze technique. This method eliminates the need for cryoprotectants and elaborate freezing apparatus, and provides a means to conveniently store P. halstedii.

INTRODUCTION

Plasmopara halstedii (Farl.) Berl. & de Toni (PH), the causal agent of sunflower downy mildew, is an obligate fungal parasite which can only be maintained on living sunflowers. Due to the inconvenience of repeatedly growing infected sunflowers, many researchers have sought methods to store this and other downy mildew fungi. Most long-term fungal storage methods rely on freezing spores at temperatures ranging from -10 C, as found in conventional freezers, to -196 C in liquid nitrogen (LN). Most fungi can easily be stored in LN, but the Peronosporales, the class which includes all downy mildews, do not withstand LN storage without some prior treatment (Dahmen et al. 1983). These treatments include the use of cryoprotectants (chemicals which protect cells from freezing injury) and a controlled slow rate of freezing. The objectives of this study were to document the efficacy of the dry spore/fast freeze (DSFF) technique and to compare it with other conventional techniques.

MATERIALS AND METHODS

Zoosporangia of PH for these experiments were produced by a modification of the whole-seedling inoculation technique. Seed of Interstate 7000 hybrid were germinated for 2 day at 24 C. Seedlings with 10-20 mm long radicles were inoculated by immersion in a 2 x 10^4 zoosporangia/ml suspension for 3 hr. Inoculated seedlings were planted in sterile sand/expanded perlite (1:2, v/v) and grown in the greenhouse. Temperatures were maintained at 22 $\pm$ 3 C, with a photoperiod of 16 hr provided by high-pressure sodium vapor lamps yielding 500 $\mu E/m^{-2}/sec$. After 12-14 days growth, at which time the first true leaves were 2 cm long and systemic chlorosis was evident, the plants were moved to a moisture-tight chamber at 100% relative humidity and 15 C, which induced sporulation overnight.

The DSFF method consists of the following steps. Sporulated plants are allowed to air-dry in lighted greenhouses for 4-6 hours. Spores are then collected in

the dry state with the aid of a cyclone separator (Tervet et al. 1951, and Figure 1). The spores are placed in 2 ml screw-cap polypropylene vials (Vanguard, Neptune, NJ, USA) and immediately placed into a LN storage tank (Minnesota Valley Engineering, New Prague, MN, USA). For retrieval the vials are thawed quickly by immersion in 40 C water for 1 min, and spores suspended in distilled water for inoculation.

Forty-five samples of PH zoosporangia which had been in LN for periods ranging from 12 to 57 mo were evaluated for viability. Since infection is the ultimate criterion, viability was measured as percent of plants infected, using the inoculation technique described previously. Two replications of 36 seedlings each were inoculated with 2×10^4 zoosporangia/ml and an additional two replications with 2×10^5.

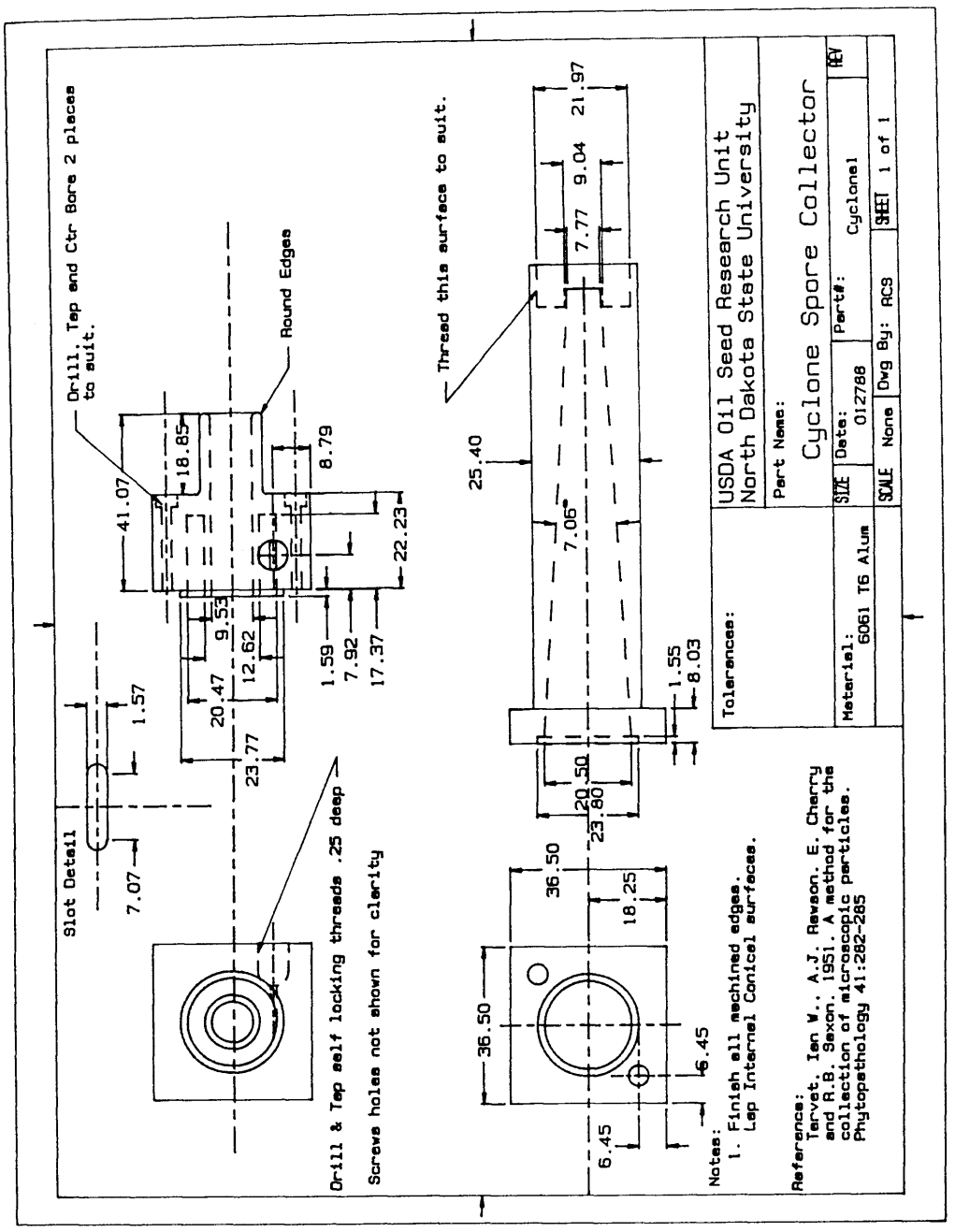
Three cryoprotectants which have been used for LN storage of other downy mildew fungi were compared with the DSFF method. The cryoprotectants were 10% dimethylsulfoxide plus 8% glucose (DMSO) (Smith 1983), 10% glycerol plus 8% skim milk (GLYC) (Dahmen et al. 1983) and 10% polyvinyl alcohol (PVA) (Tetsuka & Datsuya 1983). Approximately 10 mg of fresh zoosporangia were placed in a cryostorage vial with 1 ml cryoprotectant and allowed to equilibrate for 15 min in an ice bath. Four vials of each treatment were prepared, with two vials (replicates) placed immediately in LN and two frozen at a controlled rate of 0.6 C/min from 0 to -40 C, using a Neslab Cryobath CB-80 with an Exatrol temperature controller (Newington, NH, USA). The test was repeated with 5 different isolates. After 3 wk in storage, infectivity was assessed as described previously. Sixty seedlings/treatment/replicate were inoculated at 2×10^4 and another 60 at 2×10^3 zoosporangia/ml.

RESULTS AND DISCUSSION

Infective zoosporangia of PH were recovered from 44 of 45 samples (98%) stored by the DSFF method. The average infectivity was 78% compared to 95% with fresh zoosporangia. Samples stored for varying lengths of time did not differ significantly in infectivity, which ranged from 67% for 10 samples stored 12-19 mo to 74% for 10 samples stored 50-57 mo.

Of the eight cryoprotectant/freezing rate combinations tested, three treatments yielded spores which were as infective as fresh zoosporangia (73%) controls in this experiment. These treatments were the DSFF method (64% infection), dry spore/slow freeze (68%) and GLYC/slow freeze (59%). Spores stored with either PVA or DMSO coupled with the slow freeze produced infection of 12 and 45%, respectively, while all other fast freeze combinations with PVA, DMSO and GLYC yielded infection of 4% or less. In preliminary tests none of the cryoprotectant solutions were toxic to PH at a 1:100 dilution.

These results substantiate that the DSFF method is an effective method for long-term storage of PH zoosporangia in LN, and that infectivity is equal to that offered by the best reported cryoprotectants. The ability to survive an immediate plunge into LN also eliminates the need for costly slow freezing apparatus and for the extra handling required for cryoprotectant addition. The only special equipment needed for this method is a cyclone separator which greatly facilitates spore collection. An additional benefit of the DSFF method is the volume of spores which can be stored, contrasted with storing sporulated leaf


Slot Detail
7.07
1.57
Drill, Tap and Ctr Bore 2 places to suit.
41.07
18.85
Round Edges
8.79
22.23
9.53
12.62
20.47
23.77
1.59
7.92
17.37
Drill & Tap self locking threads .25 deep
Screws holes not shown for clarity
Thread this surface to suit.
25.40
7.06
7.77
9.04
21.97
1.55
8.03
20.50
23.80
36.50
18.25
36.50
6.45
6.45
Notes:
1. Finish all machined edges.
Lap Internal Conical surfaces.
Reference:
Tervet, Ian W., A.J. Rawson, E. Cherry and R.B. Saxon. 1951. A method for the collection of microscopic particles. Phytopathology 41:282-285
Tolerances:
USDA Oil Seed Research Unit
North Dakota State University
Part Name:
Cyclone Spore Collector
Material: 6061 T6 Alum
SIZE
Date: 012788
Part#: Cyclone1
REV
SCALE None
Dwg By: ACS
SHEET 1 of 1

segments (Viranyi 1983, Vear & Tourvielle 1987). A 2 ml cyrovial will easily hold 1 g of dry spores, which is the amount we normally can harvest from 200 seedlings. This is roughly equal to 5.4×10^8 zoosporangia, or enough for 27 liters of inoculum at 2×10^4 spores/ml.

No other downy mildew fungus has been reported to survive storage in LN without both a slow freezing rate and some cryoprotectant. The partial dehydration of the DSFF method, coupled with the absence of any cryoprotectant which would rehydrate the spores may be the factors responsible for the success of this simple technique. Investigations are in process to determine if the DSFF method can be used with other downy mildew fungi.

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