Fungal Genetics Reports

Volume 60 Article 2

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Recommended Citation

Springer, J. C., A.L. Davelos Baines, M.T. Chansler, and A.M. Jarosz (2013) "Evaluating the long-term storage of Cryphonectria parasitica," *Fungal Genetics Reports*: Vol. 60, Article 2. https://doi.org/10.4148/1941-4765.1007

Evaluating the long-term storage of Cryphonectria parasitica

Abstract

Isolates of the Chestnut blight pathogen, *Cryphonectria parasitica*, from six populations in Michigan, were stored in the late 1990s as agar plugs of mycelium in vials of sterile water held at room temperature. Approximately 29% of the fungal isolates were infected with mycoviruses at the time of storage. Each isolate was tested for revivification effectiveness by taking aliquots from vials filled with agar plugs of *C. parasitica* and sterile water and plating onto potato dextrose agar. Average revivification success was 70.5% across populations with a range of 33—84% within populations. In situations where vials had dried out during storage, success was low (4%), while success for vials that retained sterile water averaged 90%. Most importantly however, is the loss of mycoviruses from stored isolates; only 2 of 119 stored mycovirus infected isolates still contained mycoviruses after storage, suggesting that the double-stranded RNA mycoviruses are degraded during storage.

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Evaluating the long-term storage of *Cryphonectria parasitica*

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Fungal Genetics Reports 60: 11- 15
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Isolates of the Chestnut blight pathogen, *Cryphonectria parasitica*, from six populations in Michigan, were stored in the late 1990s as agar plugs of mycelium in vials of sterile water held at room temperature. Approximately 29% of the fungal isolates were infected with mycoviruses at the time of storage. Each isolate was tested for revivification effectiveness by taking aliquots from vials filled with agar plugs of *C. parasitica* and sterile water and plating onto potato dextrose agar. Average revivification success was 70.5% across populations with a range of 33—84% within populations. In situations where vials had dried out during storage, success was low (4%), while success for vials that retained sterile water averaged 90%. Most importantly however, is the loss of mycoviruses from stored isolates; only 2 of 119 stored mycovirus infected isolates still contained mycoviruses after storage, suggesting that the double-stranded RNA mycoviruses are degraded during storage.

Introduction

A well-known example of long-term storage of living organisms is the work of Richard Lenski and colleagues (Lenski & Travisano 1994). In their work, aliquots of *Escherichia coli* bacteria have been stored every 500 generations for greater than 20 years, creating a frozen, revivable equivalent to a fossil record (Woods et al. 2011; Cooper & Lenski 2000). In this way, baseline, ancestral clones have been available for direct comparison with evolved lines at any point their evolutionary history (Lenski et al. 1991) especially to determine average changes in fitness over time (Woods et al. 2011). Collecting and storing living isolates from diverse taxa can allow temporal changes in both phenotype or genotype to be readily assessed. Storage of fungal cultures, for example, in a viable and stable state is important for future studies that relate to pathogen identification, disease control, quarantine, and breeding resistant plants (Abd-Elsalam et al. 2010) and for detecting any changes in virulence.

Fungal isolates are commonly stored for short or long time periods by sub-culturing onto new media filled petri plates, silica gel, or water suspension at 5°C (Richter and Bruhn 1989), or the use of organic substrates such as wood chips or filter paper, or freezing (Nakasone et al 2004). Isolates were stored originally using a method developed by Jones et al. (1991) for use in storing taxonomically diverse fungal species. The method is simple, economical, and does not require freezing or refrigeration. It has not been tested previously with *C. parasitica*, the

pathogen responsible for chestnut blight disease in American chestnuts, *Castanea dentata* (Marsh) Borkh.. Cultures for this study have been in storage since being collected from six populations in Michigan between 1996 and 2000 (Davelos 1999; AL Davelos, unpublished data). This study reports on our attempts to revive these samples for use in a temporal study of vegetative compatibility group patterns across *C. parasitica* populations in Michigan (Springer et al. 2013).

Materials and Methods

Initial storage

Storage in one-dram vials followed the technique of Jones et al. (1991). After growing for about two weeks on Potato Dextrose agar (PDA, Difco: Becton, Dickinson and Company. Sparks, MD). Two to three plugs of *C. parasitica* mycelia were made using a 3-millimeter cork borer or the sterilized end of a glass pipette and placed into 15 x 45 millimeter 1 dram glass vials (Kimble Glass, Inc.), filled with approximately 2mL of sterile water and then sealed with parafilm. The isolates were originally collected from six Michigan populations of *C. parasitica*: Roscommon (RC), Stivers (ST), County Line (CL), Frankfort (FT), Missaukee Diseased (MD) and Missaukee Healthy (MH). All populations are located in the northern half of Michigan's lower peninsula (see Davelos and Jarosz 2004 for detailed locations). Numbers of samples saved and year of storage were variable for each population (Table 1). Isolates from three populations (RC, CL, and FT) were infected with double-stranded RNA (dsRNA) mycoviruses, which reduce both growth rates and conidia production of infected *C. parasitica* cultures. Mycoviruses were not present in isolates from the remaining three sites (ST, MD, and MH).

Revivification tests

In 2010, vials were inspected for water content and filled with sterile water if they had dried out. Sterile water was added to dry vials along the vial shoulder in order to attempt to rehydrate spores or mycelia. All vials were vortexed to homogenize the sample and allowed to rest for approximately one hour before 200µL aliquots were removed and spread onto PDA plates with a sterile glass hockey stick. Growth of *C. parasitica* or contaminants such as bacteria or other fungal species was noted after approximately two weeks; successfully revived isolates were placed back into long-term storage in new vials using initial storage methods. Samples were only tested once.

Results

Stored cultures were considered revived if *C. parasitica* grew from the plated sample. Lack of growth or, growth of bacterial or fungal colonies other than *C. parasitica* were noted as unsuccessful or contaminated, respectively. In rare cases, tiny sub-samples or hyphal tips of contaminated *C. parasitica* could be taken from a contaminated plate and successfully plated onto fresh PDA media. *Cryphonectria parasitica* was successfully revived from 70.5% of the stored vials, but populations differed in their success rate ranging from a low of 33.3% for MD samples to 84.3% for CL samples (Table 1). The success rate for vials that had dried out was very low (3 of 73 = 4%), while 89.7% of the samples with water remaining were successfully revived. Additionally, there was no significant difference in the revivification success for

isolates infected with mycoviruses (71.8%) and isolates not infected with mycovirus (81.1%). Most importantly however, mycoviruses were recovered from only two of 119 stored cultures known to be infected with mycoviruses at the time of storage. Finally, only 4.4% of the vials were contaminated with other microbes.

Table 1. Overview of isolates tested for resurrection success from six Michigan chestnut blight populations. Totals are whole numbers, percentages or averages

Population	Total tested	wet alive	dry alive	% alive	Wet dead	dry dead	% dead	Contaminated	% Contaminated
Roscommon	140	111	1	80.00%	8	18	18.60%	2	1.40%
Frankfort	86	52	0	60.50%	2	30	37.20%	2	2.30%
County Line	89	73	2	84.30%	2	11	14.60%	1	1.10%
Stivers	32	18	0	56.30%	8	4	37.50%	2	6.30%
Missaukee Diseased	36	12	0	33.30%	12	2	38.90%	10	27.80%
Missaukee Healthy	27	20	0	74.10%	1	5	22.20%	1	3.70%
Totals /Averages	410	286	3	70.50%	33	70	25.10%	18	4.40%

Discussion

Storing of biological samples for long time periods is an important aspect of scientific record keeping. Voucher samples of fungi should be stored in a viable state so they can be used for future use in pathogen identification, disease control, quarantine, and breeding resistance. Work by Richter and Bruhn (1989) has shown that viability among fungal species, even after a few months, can vary from 0 to 100% for their 5°C refrigerated water storage technique. Additionally, Borman et al. (2006) have shown that revival of fungal isolates averages 90% for isolates stored since 1983 but that a species effect is seen (Hartung de Capriles et al. 1989). Techniques, such as mineral oil and silica gel were a less successful and freezing at -80°C was the worst (Pumpyanskaya 1964). Our work reviving isolates of *Cryphonectria parasitica* indicates that there are differences in revival success even among populations. For instance, at the Missaukee Diseased population, stored isolates that remained wet were still unable to be resurrected 50% of the time. The County Line population however, had very good resurrection success: 97% of revivable isolates were in tubes that still contained sterile water.

Reviving stored *Cryphonectria parasitica* samples was effective as long as some distilled water remained in the glass vial. If the water evaporated entirely, allowing spores and mycelia to desiccate, re-growth of the culture could not be obtained even when dry material in the tubes was immersed in fresh sterile water. Thus, a tight seal of the cap and wrapping with parafilm are vital for water retention in the vials.

Most important from this study, however, may be the fact that only two isolates out of 119 isolates infected with mycoviruses maintained their mycovirus until 2010. This has important implications for the long-term storage of isolates that are used for biological control of *C. parasitica,* especially if

isolates are stored from year to year and are revived to continue treatment of blight cankers during subsequent field seasons.

If presence of mycovirus in *C. parasitica* mycelia is not important, periodic inspection of water levels in glass vials must be done to determine if additional sterile water should be added to maintain isolate viability. Alternatively, if water has begun to evaporate from tubes, cultures can be grown again and stored immediately in a new water-filled tube. Duplicate or triplicate tubes of isolates should be kept so that the chances of future resurrection are increased, especially if there are inter-tube differences.

Although it is not my intent to completely review techniques for storing and reviving fungal cultures, these data on the chestnut blight fungus, *C. parasitica,* can be added to the list of studies documenting sample storage methods and success of reviving after a relatively long time period. This simple method of storing *C. parasitica* requires minimal effort, is economical, and easy to maintain with no input of electricity and therefore avoids the potential hazard of an electrical outage destroying isolates.

The ability to effectively store and revive a fungal isolate is important for comparative purposes such as confirming the identification of a unknown quarantine isolate, using isolates for comparative purposes in taxonomic studies, determining, determining changes in virulence over time (if the culture stored has remained static during storage), and simply good record-keeping of biological samples.

At present, a test of long-term persistence of mycoviruses is being done. Chestnut blight isolates known to contain mycoviruses have been stored in sterile water and PDA slants at ambient temperature and on filter paper at -20 degrees Celsius. This long-term experiment will test mycovirus persistence over 30 years tested at designated intervals.

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