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Simple techniques for long-term storage of *Plasmopara viticola*

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Summary. Propagules of *Plasmopara viticola* or fragments of *P. viticola* infected host tissue were stored by three techniques, freezing at –25°C, dehydration with $CaCl_2$, and freeze-drying. Viability tests were performed periodically until exhaustion of the testing sample. *P. viticola* samples that were frozen at -25°C remained viable for eight years, whereas the $CaCl_2$ dehydrated samples remained viable for eight and a half years. Freeze-drying was the most successful long-term storage method, being able to preserve samples for almost 21 years.

Key word: preservation, viability, freeze-drying.

Introduction

Correct storage techniques for preserving plant pathogenic fungi or oomycota are very important in biological and epidemiological studies. Proper storage saves time that would otherwise be needed for continuous transfers; it reduces or avoids the risk of contamination caused by repeated transfers; and, most important, it limits or blocks metabolic activity, thus maintaining the original properties of the specimen. When dealing with biotrophic organisms, long-term preservation is obviously of paramount importance.

Freeze-drying and immersion in liquid nitrogen seem to be the most commonly used techniques with which a great number of fungi are preserved (Raper and Alexander, 1945; Hwang, 1966, 1968; Smith, 1982, 1983; Dahmen *et al*., 1983; Hoffmann, 1989; Tan and van Ingen, 2004). However, there is no universal storage technique that can be applied to all fungi and oomycota since several factors play a role in survival of the stored organisms. These factors include the technique itself, the characteristics of the specimen or culture to be preserved, the degree of the storage temperature, the rate of freezing and thawing, the nature of the suspending or protectant medium, the size of the fungal structures and the strength of their attachment to plant tissue, etc. These have all been reported as important factors in ensuring the survival of the preserved material. Moreover, freeze-drying is not satisfactory with a fair number of *Oomycota*, especially the *Peronosporaceae*. *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, in particular, is reported to be very difficult to preserve for a suitable period of time by any such preserving techniques, even with the aid of suitable protectants.

In 1969, when the senior author was engaged in biological and epidemiological studies on *P. vi-*

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ticola, the need arose to preserve samples of the pathogen. Before much of what is now known about different storage methods had become available, therefore, an attempt to preserve *P. viticola* by freeze-drying was undertaken.

Thereafter, to make the process of preserving *P. viticola* faster and easier, two more techniques (dehydration and freezing at –25°C) were also tested.

Materials and methods

Freeze-drying

The *P. viticola* samples to be preserved came from a naturally infected leaf of an unknown grapevine cultivar (*Vitis vinifera* L.) collected near Bari, southern Italy, in June 1969. The pathogen was multiplied and maintained in the laboratory by transferring it from leaf to leaf of grapevine cv. Regina.

Sporangia from a vigorously sporulating lesion of an artificially infected leaf were scraped off and suspended in non-sterilized tap water. The suspension was placed in lyophil ampoules, 2 ml each, and freeze-dried for 24 h in a Speedy Vacuum Edwards mod. 5PS centrifugal freeze-dryer with phosphorus pentoxide as a desiccant. At the end of the freeze-drying process, the ampoules were heat-sealed and divided into two batches that were kept either at room temperature on a laboratory bench, or in a domestic refrigerator at 4–6°C.

Another sporangial suspension prepared as above was left in a shallow container on a bench in an air-conditioned (20±2°C) laboratory for zoospore liberation. After little more than an hour, when most sporangia had liberated their zoospores, the resulting suspension was pipetted from just below the surface, where the suspension was more concentrated, placed in lyophil ampoules, 2 ml each, and freeze-dried without addition of any preservant. At the end of the process, following sealage, the ampoules were divided into two groups, one of which was kept at room temperature and one in a refrigerator.

Aside from the above, young actively sporulating lesions were separated from the surrounding healthy tissue, cut into small fragments (ca. 10 mm^2) and placed in lyophil ampoules (10 fragments per ampoule). Two millilitres of non-sterilized tap water was added to each ampoule before freeze-drying. Following sealage, these ampoules were also divided into two groups and maintained either at room temperature or in a refrigerator. Thirty ampoules were used for each group.

Dehydration

This technique used 4 g of $CaCl_2$ deposited in a test tube. A 7–8-mm-thick layer of bacteriological cotton was laid upon the $CaCl_2$ and a single fragment (ca. 1 cm^2) of a young, actively sporulating lesion was placed upon the cotton. The fragment was further protected by a loose mat of bacteriological cotton completely filling the tube whose opening was sealed with parafilm. Thirty tubes were prepared in this way and stored at 4–6°C. Storage of these samples was initiated in June 1985.

Freezing at -25°C

To test this technique, 15 leaves each with a number of young, vigorously sporulating lesions were selected, placed in empty Petri dishes, and transferred to a freezer set at -25°C as soon as they were collected from the field and without any coadiuvant or pre-storage treatment. Storage of these samples was initiated in June 1985.

Viability tests

The freeze-dried ampoules containing either a sporangial suspension, a zoospore suspension or infected leaf fragments, and that had been stored at room temperature or at 4–6°C in a refrigerator, were broken-open; 2 ml of distilled sterile water per ampoule was added and then the ampoules were gently shaken for 3–4 min. The resulting suspensions were used to inoculate detached leaves of the grapevine cv. Frappato. The leaves, lower face up, and supported on a U-shaped glass rod, were placed in glass Petri dishes (diam. 15 cm) containing 10–15 ml sterile water. Drops of the suspensions were deposited singly in the interveinal space (10–15 drops per leaf). The inoculated leaves were left on a laboratory bench at 20±2°C, exposed to daylight and inspected daily for infection results.

To check the viability of the material dehydrated with $CaCl_2$ and stored at 4–6°C and the material stored at -25°C, samples that were subjected to these techniques were moved to room temperature and left to thaw for 30 min. Infected fragments of leaf tissue were then transferred singly to 10 ml

test tubes, supplemented with 2 ml non-sterilized tap water, and gently shaken for 3–4 min in order to prepare the propagule suspensions for the viability tests. For these two techniques, the inoculation and viability test were performed as described above for the freeze-drying process.

Initially, the viability tests were carried out in triplicate or quadruplicate. As the material began to run out towards the end of the experiment, however, the test was only performed in duplicate.

Results

When an abundant efflorescence of the pathogen was observed on the inoculated leaf, usually 4–8 days after inoculation, indicating that the sample had survived, the viability test was regarded as successful.

As already mentioned, when this study was first started, the sole aim was to check survival after storage, and not how well the material had survived. Therefore, regardless of the degree of retrieval, each test was judged as positive or negative depending only on whether new infections were or were not produced. Besides, since survival was mainly defined as the duration of viability with each type of storage, only the last positive test of those performed for each kind of material stored, was taken into account. It is to be mentioned that all tests were discontinued as the material ran out. They are summarized in Table 1. The first three lines of the Table, dealing with the freeze-drying process, reveal that *P. viticola* when freeze-dried as zoospores or sporangia suspended in water survived well over 16 years of storage, and that when freeze-dried as infected leaf tissue, survival was almost 21 years.

When preserved as infected leaf tissue of grapevine, *P. viticola* easily survived for 8 years when stored at -25°C and for 8 years and a half when dehydrated with $CaCl_2$ and stored at 4–6°C.

Discussion and conclusions

The literature on storage methods (mainly freeze-drying and deep freezing in liquid nitrogen) reports that there are a number of difficulties in preserving members of the *Oomycota* (Smith, 1982, 1983; Hoffmann, 1989; Tan and van Ingen, 2004) and in particular members of the *Peronosporaceae,* even when cryoprotectants are used.

Nevertheless, viable conidia of *Peronospora tabacina* B.D. Adam (Bromfield and Schmitt,1967) and *Sclerospora sorghi* W. Weston & Uppal (Gale *et al*., 1975; Long *et al*., 1978) have been successfully recovered after more than 2 years of storage in liquid nitrogen. *Bremia lactucae* Regel, *Peronospora farinosa* (Fr.) Fr. and *P. parasitica* (Pers.) de Bary were also successfully recovered after one year or more of exposure to -25°C (Kröber, 1981).

Even *P. viticola,* repeatedly reported as being difficult to preserve for long periods of time by any of the commonly adopted techniques, has been successfully stored for 9 years by refrigeration in liquid nitrogen cryoprotected with skim milk+glycerol or with dimethyl sulfoxide (Dahmen *et al*., 1983).

In this investigation no difficulties were experienced in recovering viable and promptly infective propagules (sporangia or zoospores suspended in tap water) of *P. viticola* or infected host tissue when stored without any protectant after 8 years or more if they were preserved by dehydra-

Table 1. Survival of *Plasmopara viticola* after storage by three techniques.

Preservation technique	Material stored	Date of storage[a]	Time of survival[b]
Freeze-drying	Sporangia suspended in water	June 1969	16 yrs 8 mos
Freeze-drying	Zoospores suspended in water	June 1969	16 yrs 8 mos
Freeze-drying	Sporulating leaf tissue	June 1969	20 yrs 10 mos
Dehydration	Sporulating leaf tissue	June 1985	8 yrs 6 mos
Freezing at -25°C	Sporulating leaf tissue	June 1985	8 yrs

[a] Date on which material was first placed into storage.
[b] yrs, years; mos, months.

tion with $CaCl_2$ at 4–6°C or by freezing at -25°C or after16 and even 21 years if they were preserved by freeze-drying.

Since the only reason that the viability tests were discontinued was that the test material ran out, it seems a reasonable assumption that *P. viticola* can be preserved by the techniques here tested, for periods of time even longer than are here reported.

The quite unexpected difference in *P. viticola* survival with and without cryoprotectants (21 years without a protectant *vs*. 9 years with one) prompts some comments.

It is generally accepted that *P. viticola* and other closely related *Peronosporales*, are delicate and easily damaged organisms as long as oospores are not formed. It therefore seems that the storage techniques here tested, which involved fewer steps and did not use any protectants, caused less mechanical damage than other storage techniques, and also less physiological stress such as for example the osmotic shock to which *P. viticola* is subjected when storage technique are employed that require the use of distilled water or cryoprotectants.

Distilled water and cryoprotectants may exert a more or less strong osmotic change which may cause physiological disorder that ultimately leads to mortality.

Even the distilled water used to suspend propagules prior to storage, or to revive them after storage, may cause the tiny zoospore structures to burst. In the present study, the zoospores survived this step, possibly because tap water was used to make the suspension. The distilled water used to revive the sample served only to restore the primary condition, that is, to supply the water lost during the freeze-drying process.

It is known that *P. viticola* infects exclusively by means of zoospores penetrating the stomata. In the present experiment, and the experiments of other researchers, any infection must necessarily involve the participation of the zoospores. In that case it is a legitimate question to understand whether the naked zoospore can withstand the entire freeze-drying process. If the zoospores were unable to withstand the freeze-drying process, all positive samples of the "zoospore suspension" used in the present study must have contained some full sporangia which released the zoospores upon inoculation. Sporangia are certainly more resistant to manipulation and physiological stress than the zoospores, and a number of studies suggest that any sporangia still attached to the matrix or, more generally, any infected tissue, is much more resistant to damage caused by the storage technique, not only in the *Peronosporaceae* (Cohen and Kuć, 1980; Kröber, 1981; Virànyi, 1985) but also in other fungi (Staffeldt and Sharp, 1954; Wester *et al*., 1958; San Antonio and Blount, 1973; Bennet and Wolfe, 1979; Casteldine *et al*., 1982).

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