*Rubber Science*, **27**(2): 202-214, 2014

# RELATIVE EFFICACY OF LONG-TERM STORAGE METHODS ON SURVIVAL AND VIRULENCE OF *CORYNESPORA CASSIICOLA* AND *PHYTOPHTHORA MEADII* PATHOGENIC ON RUBBER (*HEVEA BRASILIENSIS*)

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Received: 22 July 2014 Accepted: 20 September 2014

Roy, C.B.,Srinivas, P. and Jacob, C.K. (2014). Relative efficacy of long-term storage methods on survival and virulence of *Corynespora cassiicola* and *Phytophthora meadii* pathogenic on rubber (*Hevea brasiliensis*). *Rubber Science*, **27**(2): 202-214.

*Corynespora cassiicola* and *Phytophthora meadii,* the economically important pathogenic fungi of rubber (*Hevea brasiliensis*), were preserved with six different storage methods *viz*. continuous growth method, immersion in sterile distilled water, desiccation on filter paper, desiccation in soil, cryopreservation and lyophilization. Survival was evaluated 1, 3, 6, 9, 12, 18, 24, 36 and 48 months after storage. Immersion in sterile distilled water was found to be the best method for long term storage of both the test fungi with a revival rate of 71 and 62 per cent for *C. cassiicola* and *P. meadii*, respectively. *C. cassiicola* also survived well in cryopreservation and desiccation methods. It was significant to note that preservation through continuous culturing eroded the virulence of *C. cassiicola* over a period of time. In contrast, all other preservation methods sustained post-storage virulence of the pathogen, which is of high value for tissue based germplasm screening against Corynespora leaf disease. Immersion in sterile distilled water was the only method which could support the survival of abnormal leaf fall pathogen *P. meadii* and preserve its post-storage virulence. Therefore, this method could be used as efficient and cost effective preservation method for both the pathogens under study, without losing their pathogenicity.

**Keywords:** *Corynespora,* Fungal culture maintenance, Long-term preservation, *Phytophthora*

## INTRODUCTION

Plant pathogen culture collections akin to libraries of genotypic and phenotypic diversity of previously studied pathogens are invaluable resources for advancing future research in plant pathology. Preserving relevant pathogen isolates from past disease epidemics is similar to archiving key documents needed for understanding an important historical event. Further, reference cultures with phenotypic and genotypic identification tags can facilitate the identification and control of new disease outbreaks. The underexplored pathogen diversity in nature highlights the importance of preserving and cataloguing pathogen cultures for future

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reference (Kang *et al*., 2006). Maintaining and preserving fungal cultures are essential for systematics and biodiversity studies (Sharma and Gupta, 2012). Because fungi are a diverse group, several methods of cultivation and preservation are required to ensure the viability and morphological, physiological and genetic integrity of the cultures preserved over time. Storage of fungal cultures in a viable and stable state is also important for future studies that relate to pathogen identification, disease control, quarantine and resistance breeding (Abd-Elsalam *et al*., 2010). Sette *et al*. (2013) reviewed extensively on the importance of culture collections providing knowledge on diversity, conservation and biotechnological exploitation of fungi.

The fungal cultures have been traditionally maintained by repeated transfers on agar slants. Such sub-culturing is labour intensive and time consuming if culture collections are large. Moreover, the cultures may also suffer phenotypic and genetic alterations, besides losing their pathogenicity over several generations of subculture. Fungal cultures can be preserved using a range of well established preservation methods. However, it is imperative to understand that different fungi respond differently to these methods based on their viability, purity, pathogenicity and sporulation over a period of time. Hence, while studying a pathogen in a new agro-climatic scenario it is essential to ascertain suitable preservation methods for newly characterised fungal isolates.

*Hevea brasiliensis*, the para rubber tree, grown extensively in Kerala, parts of Tamil Nadu, Southern Karnataka, Maharashtra and parts of North East India, is prone to several diseases caused by phytopathogenic fungi. Corynespora leaf disease caused by *Corynespora cassiicola* and abnormal leaf fall disease caused by *Phytophthora meadii* are the two major leaf diseases threatening the rubber plantations in southern states of India. Highly virulent isolates of these pathogens are used to identify resistance in the rubber gene pool through screening of wild germplasm and cultivated clones of *H. brasiliensis* adopting artificial inoculations at an early growth stage of plants using *in vitro* screening methods (Joseph *et al*., 2004). Continuous availability of virulent isolates of these pathogens is essential to evaluate the current and future germplasm collections as well as pipeline clones of rubber before their release for cultivation. However, information on suitable preservation methods for *C. cassiicola* and *P. meadii*, pathogenic to para rubber tree is scarce. Hence, the objective of this investigation was to identify and standardise appropriate methods for long term preservation of these two major pathogenic fungi of rubber plants, without radically affecting their regeneration and virulence.

## MATERIALS AND METHODS

### Fungal stock culture preservation:

Following techniques were employed in this study for fungal stock culture preservation:

***i. Continuous growth method***: Cultures were grown on potato dextrose agar (PDA) medium. For sub-culturing, inoculum in form of a bit of mycelium from the periphery of an actively growing fungal culture was transferred aseptically to test tubes containing PDA and maintained at room temperature (Ryan *et al*., 2000). The cultures were regularly checked for contamination and desiccation. To retrieve the fungus, a portion of the culture was removed aseptically and transferred to fresh antibiotic amended PDA medium.

***ii. Immersion in distilled water***: Fungal discs were taken from the edge of actively

growing colony on a culture media, with the aid of a cork borer. The fungal disc along with adhered media was transferred to sterile screw cap test tubes filled with 10 mL of sterile distilled water. The tubes were closed, wrapped with parafilm and stored at room temperature (Smith and Onions, 1994; Burdsall and Dorworth, 1994). To retrieve the cultures, discs were removed aseptically and transferred to antibiotic amended PDA medium.

***iii. Desiccation on sterile soil***: In this method the spores/ mycelium were dried on sterile soil and stored. Spores were harvested from a 10-day-old culture plate using sterile distilled water and concentrated by brief centrifugation. One mL of this spore suspension concentrate was inoculated on 1 g of sterile soil in a microcentrifuge tube, dried and maintained at room temperature (Elliot, 1975; Smith and Onions, 1994). To retrieve the fungus, a few grains of soil loaded with fungal spores were sprinkled onto antibiotic amended PDA medium.

***iv. Desiccation on filter paper***: For storage over filter paper discs, the fungus was allowed to grow over filter paper placed alongside the culture in a petri plate containing PDA media. When the culture overgrew the filter paper, the filter paper was removed from the media, air-dried aseptically and stored in sterile butter paper packets at room temperature (Elliot, 1975; Smith and Onions, 1994). For retrieval of the fungus, the culture laden filter papers were inoculated over fresh antibiotic amended PDA plates and incubated for five days for the fungal growth to be initiated.

***v. Cryopreservation***: For cryopreservation of fungal cultures, six culture discs of 5 mm size were taken from the advancing margins of seven-day-old culture with the aid of a cork borer. These discs were inoculated in micro-centrifuge tubes containing 1 mL of 10 per cent solution of glycerol, frozen in liquid nitrogen and immediately stored in a deep freeze chamber at -80°C (Ryan *et al*., 2000). For retrieval, the tubes were taken out to room temperature, allowed to thaw and the discs were transferred to antibiotic amended PDA media for growth.

***vi. Lyophilization or freeze drying***: The spore suspension from 10-day-old culture was scraped from the culture plate by flooding the surface with sterile water and concentrated by brief centrifugation. The spore suspension was freeze dried using a lyophilizer at a temperature of -60 °C. Evaporating and cooling kept the samples frozen during the drying process (Smith and Onions, 1994; Kolkowski and Smith, 1995). For retrieval, the storage tubes were taken out to room temperature, allowed to thaw and content was suspended in sterile distilled water. Loop full of the suspension was transferred to antibiotic amended PDA media for initiating fungal growth.

**Testing for viability of the preserved culture**

Survival of the culture was evaluated at 1, 3, 6, 9, 12, 18, 24, 36 and 48 months after storage. After these periodic intervals, viability of the culture was tested by placing the preserved culture aseptically on to fresh PDA medium supplemented with 150 ppm streptomycin (Hansen, 1994) for *C. cassiicola* and PARPH (pimaricin, ampicillin, rifampicin, PCNB and hymexazol) for *P. meadii* (Kanwischer and Mitchell, 1978; Abad *et al*., 2011). When the culture started emerging, it was transferred to fresh plates containing PDA.

**Testing for virulence of the preserved culture**

Virulence of the revived culture was assessed at 1, 3, 6, 9, 12, 18, 24, 36 and 48

months after storage. Virulence of *C. cassiicola* was tested *in vitro* by inoculating the spores on healthy leaves of RRII 105, a *Corynespora* susceptible clone of *H. brasiliensis* and maintaining them in moist chamber at room temperature for 72 h. For testing the virulence of *P. meadii* isolates, agar plugs of *Phytophthora* or zoospores released from *Phytophthora* cultures were tested *in vitro* on healthy leaves of RRIM 600, a *Phytophthora* susceptible clone of *H. brasiliensis* and maintaining them in moist chamber at room temperature for 72 h. Virulence was checked by measuring the size of the lesion after 72 h of incubation.

**Purification of *P. meadii* contaminated with bacteria**

Bacterial contamination of *P. meadii* was one of the major constraints encountered during the study, which was detected and purified as per the following tests.

***a. Turbidity test:*** To check for bacterial contamination in *Phytophthora* cultures, discs of *Phytophthora* were made from the advancing margin of the culture and transferred to sterile plates. The agar plugs were submerged in Luria Broth (LB) media overnight at room temperature. Clear media indicated that the culture was free from bacterial contamination and hence processed for long term storage. Turbid media after incubation indicated bacterial contamination, which was eliminated by serially sub culturing on PARPH amended media, before processing for long term storage.

***b. Baiting rescue*:** Purification of *Phytophthora* cultures contaminated with bacteria was done by passing culture through apples used as baits (Braithwaite *et al*., 2007). The apple was surface sterilized, and a "V" or cone shaped cut was made using a sterile blade. An agar plug of *Phytophthora* suspected to be contaminated with bacteria was placed within the incision and covered with the apple tissue. The incision was moistened with a few drops of sterile water, sealed with a tape and incubated in moist chamber for 4-5 days for appearance of disease symptoms. Following incubation, rotting of apple at the point of *Phytophthora* inoculation was observed. Fruit tissues from the edge of the rotten zones were transferred aseptically on to sterile plates containing PDA and incubated at room temperature for growth of *Phytophthora*.

**Investigating genetic variation by RAPD analysis**

To detect for any possible genetic variation in the cultures subjected to long term storage random genome wide checks were conducted using Random Amplified Polymorphic DNA (RAPD) analysis. The fungus was retrieved from the stored condition (at 1, 3, 6, 9, 12, 18, 24, 36 and 48 months after storage) by plating on antibiotic amended PDA media and incubated at room temperature. Genomic DNA was extracted from seven day old fungal mat grown on potato dextrose broth (PDB) from both fresh as well as preserved cultures. Extraction and purification of the total genomic DNA were carried out following the modified CTAB protocol (Saha *et al*., 2000). Genomic DNA was amplified in a thermal cycler using ten arbitrary decamer primers (Operon technology Inc., USA) as per our earlier studies (Roy *et al*., 2006). Amplified products were analyzed along with a DNA size marker by electrophoresis on a 1.4 per cent agarose gel in 1 x TBE buffer. The gels were stained with ethidium bromide (0.5 g $mL^{-1}$) and viewed on a Gel-documentation system (Bio-Rad Laboratories Inc., USA). To check for reliability of the method, amplification reactions were performed twice.

## RESULTS AND DISCUSSION

Long-term storage of plant pathogens is important to maintain cultures for their further characterization and utilization for germplasm screening and fungicide efficacy evaluation. To identify the best method for long term preservation of fungal cultures, six different techniques were employed with two pathogenic fungi of rubber namely *C. cassiicola* and *P. meadii*. The cultures subjected to these treatments were periodically recovered (1, 3, 6, 9, 12, 18, 24, 36 and 48 months after storage) from the preservation treatments to assess their viability and virulence. Tables 1 and 2 consolidate the results obtained with all the techniques studied for *C. cassiicola* and *P. meadii*, respectively.

### Continuous growth method

Viability and virulence of both *C. cassiicola* and *P. meadii* cultures could be sustained for 3 months using continuous sub culturing method (Tables 1 and 2). However both the fungi lost their ability to regenerate after six months of serial transfer. Moreover, regular handling of the cultures also exposed them to contamination. Based on these observations, it was concluded that this method can be a good option for smaller collection of cultures which are in regular use for short periods *i.e.* for less than three months. This technique is not suitable for longer storage periods, as this method is time consuming and labour intensive, which also needs regular strict monitoring for contamination and dehydration of the culture media. Further, the study indicated that there were visible changes in morphology and physiology of the fungi over time probably due to dormancy caused by dryness. The sporulation ability or the ability to infect a host was also low after repeated transfers. Nakasone *et al.* (2004) also reported that the morphology, sporulation ability, virulence and physiology of a sub cultured fungus get altered after repeated transfers. Therefore, this technique was found to be inappropriate for long-term preservation of fungal cultures.

Table 1. **Effect of various storage treatments on the viability and virulence of *C. cassiicola* cultures**

| Sl. No. | Treatment | Viability (after months) | | | | | | | | | Virulence (after months) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 3 | 6 | 9 | 12 | 18 | 24 | 36 | 48 | 1 | 3 | 6 | 9 | 12 | 18 | 24 | 36 | 48 |
| 1 | Continuous growth method: growing the fungus on agar and maintaining by serial transfer | + | + | +* | - | - | - | - | - | - | ✓ | ✓ | - | - | - | - | - | - | - |
| 2 | Immersion in sterile distilled water | + | + | + | + | + | + | + | + | + | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 3 | Desiccation - storage in sterile filter paper discs | + | + | + | + | +* | +* | +* | +* | +* | ✓ | ✓ | ✓ | ✓ | - | - | - | - | - |
| 4 | Desiccation - storage in sterile soil | + | + | +* | +* | +* | +* | +* | +* | +* | ✓ | ✓ | - | - | - | - | - | - | - |
| 5 | Cryopreservation:Storage of agar blocks in glycerol stocks | + | + | + | + | + | + | + | + | + | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 6 | Lyophilization | +* | +* | +* | +* | +* | +* | +* | +* | +* | - | - | - | - | - | - | - | - | - |

\+ - Growth of culture observed ✓ - culture virulent

\- - No growth of culture observed - - culture non-virulent

* - Contamination of culture observed

Table 2. **Effect of storage treatments on the viability and virulence of *P. meadii* cultures**

| Sl. No. | Treatment | Viability (after months) | | | | | | | | | Virulence (after months) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 3 | 6 | 9 | 12 | 18 | 24 | 36 | 48 | 1 | 3 | 6 | 9 | 12 | 18 | 24 | 36 | 48 |
| 1 | Continuous growth method | + | + | +* | - | - | - | - | - | - | ✓ | ✓ | - | - | - | - | - | - | - |
| 2 | Immersion in sterile distilled water | + | + | + | + | + | + | + | +* | +* | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | - | - |
| 3 | Desiccation - storage in sterile filter paper discs | +* | +* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 4 | Desiccation - storage in sterile soil | +* | +* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 5 | Cryopreservation by storage of agar blocks in glycerol stocks | + | - | - | - | - | - | - | - | - | ✓ | - | - | - | - | - | - | - | - |
| 6 | Lyophilization: | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

\+ - Growth of culture observed
\- - No growth of culture observed
* - Contamination of culture observed
✓ - culture virulent
\- - culture not virulent

**Immersion in sterile distilled water**

Based on the growth and virulence of retrieved fungal cultures this technique was highly efficient for storage of both *P. meadii* and *C. cassiicola* for up to 4 years (Tables 1 and 2). However, the percentage of viability of *P. meadii* was comparatively lower than that of *C. cassiicola*, due to the basic nature of their respective composition and morphology. Virulence assay showed that *Phytophthora* cultures were virulent for up to 12 months and after that they had to be passed through the host 2-3 times to induce sporulation. Highly virulent fresh isolates produced larger lesions (Fig. 1a), whereas isolates stored for a longer period either produced no lesion or lesion of very small size (Fig. 1b).

After 12 months of storage, some of the *Phytophthora* cultures were found to be associated with bacterial contamination. This problem was eliminated by adding PARPH directly onto the water tube containing the fungus and allowing it to remain for 10 days. Subsequently, the fungus was transferred to PDA plates supplemented with the same antibiotic mixture. This suppressed the bacterial

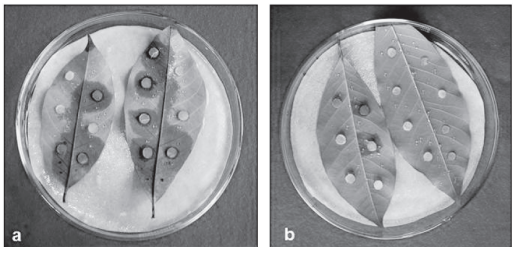


Fig. 1. *In vitro* testing of pathogen for its virulence based on lesion size produced on the surface of leaf. Highly virulent isolate Pm 58 produced larger lesions (a), whereas less virulent isolate Pm 104 either produced no lesion or lesion of very small size (b).

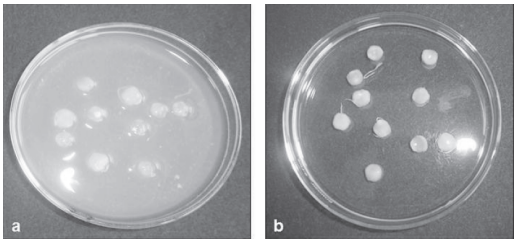


Fig. 2. Turbidity test for testing the purity of *Phytophthora* culture. When the culture was contaminated with bacteria, the media appeared turbid following overnight incubation (a) and when devoid of bacterial contamination, it remained clear (b).

contaminants and allowed revival of *Phytophthora* culture. Further sub culturing of retrieved culture on fresh nutrient media or by passing the culture through a susceptible host (RRIM 600), the cultures could be revived in growth and pathogenicity. This treatment helped to maintain the culture for up to four years. Turbidity test proved purity of the cultures as a culture when contaminated with bacteria turned the media turbid following overnight incubation due to bacterial growth (Fig. 2a) and when devoid of bacterial contamination remained clear (Fig. 2b). Baiting rescue performed to purify *Phytophthora* cultures contaminated with bacteria by passing the culture through green apples was also efficient. *Phytophthora* was cultured from diseased apple tissues at the point of inoculation (Fig. 3 a-c) by transferring them aseptically on to sterile plates containing PDA and incubating at room temperature for 4-5 days. While working with *C. cassiicola* culture, bacterial contamination was avoided by growing them on streptomycin amended media.

Immersion of fungal discs into tubes containing sterile water is a simple and inexpensive method for storing of fungal cultures. The culture remain in an inactive state as water suppresses the growth of the fungi and the culture gets revived when transferred to fresh medium besides avoiding morphological changes in most fungi (Nakasone *et al*., 2004). High recovery rates were reported by Simpfendorfer *et al.* (1996) for cultures of *Phytophthora clandestina* stored in sterile deionised water without loss of pathogenicity. Baskarathevan *et al.* (2009) also reported mycelium plugs in sterile water at 4 °C to be a better method for maintaining viability and preservation of growth rates of *Botryosphaeria* sp. as compared to mineral oil. The water preservation method has been used successfully to maintain several fungi like *Aspergillus fumigatus, A. niger, Cladosporium bantianum, C. carrionii, Fusarium* sp., *Mucor* sp., *Penicillium notatum, Penicillium* sp. and *Rhizopus* sp., for up to one year (Diogo *et al*., 2005). Burdsall and Dorworth (1994) used water preservation method for several fungi under Basidiomycotina for varying periods up to seven years, as they found it a quick, easy, and inexpensive method that may maintain genetic stability. This method had been found to be effective to preserve oomycetes (Clark and Dick, 1974; Smith and Onions, 1983). Evaporation of sterile water may take place over a period of time of

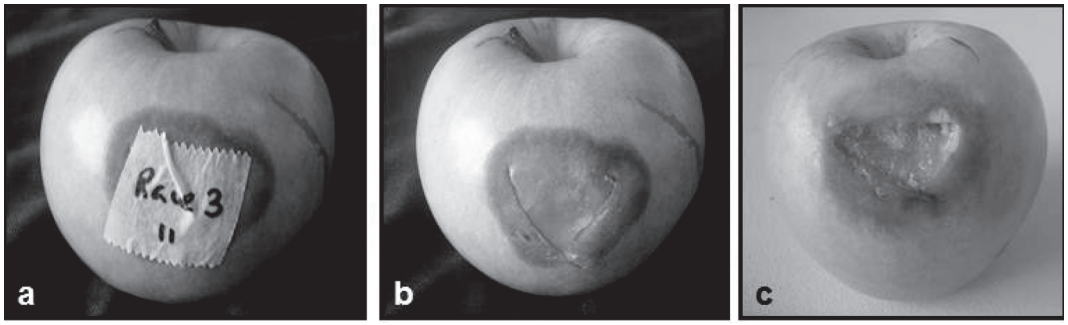


Fig. 3. Baiting rescue of *Phytophthora* contaminated with bacteria by passing through apples. *Phytophthora* culture plug placed within a 'V' shaped incision and sealed (a), rotting of apple at the point of inoculation (b) and culture to be transferred to media for purification (c).

preservation which may need regular monitoring of storage vials for the level of water. Topping up with sterile water may be done to replenish the tubes. Topping the water in vial with a fine layer of sterile oil or liquid paraffin wax can also substantially reduce the loss of water due to evaporation (Nakasone *et al.,* 2000).

**Desiccation**

The two desiccation treatments adopted in the present study *viz*. storage of fungi in sterile filter paper discs and storage of fungal spores in sterile soil, showed that desiccation treatments were partially efficient for *C. cassiicola* but not for *P. meadii* (Tables 1 and 2). Drying is the most useful method of preservation for cultures that produce spores or other resting structures. Oospores and chlamydospores of *P. meadii* are reported to survive in soil for long periods under natural conditions (George and Edathil, 1975). Accordingly, *C. cassiicola* was able to survive for four years in both the desiccation methods, although contamination was observed after one year and six months, respectively, in above two desiccation methods. It has also been reported that fungi such as *Septoria*, *Pseudocercosporella* and *Rhizoctonia* could be preserved successfully for many years in dry, sterile soil or sand although dormancy caused by dryness would take time to develop (Shearer *et al*., 1974; Reinecke and Fokkema, 1979; Nakasone *et al*., 2004).

**Cryopreservation**

Survival of *C. cassiicola* stored by cryopreservation was efficient for all the storage times tested as live cultures were recovered from plugs even up to 48 months of storage (Table 1). In case of fungi surviving under cryopreservation there are several advantages like prevention of increased genetic variability of distributed culture stocks, timesaving, reduced labour requirements compared to other storage methods, elimination of the need for repeated pathogenicity tests, prevention of culture loss from contamination and increased assurance of long term availability of cultures (Meyer, 1955; McGinnis *et al*., 1974; Stalpers *et al*., 1987; Pasarell and McGinnis, 1992). The cryoprotectant glycerol which readily pass through the cell membrane protect both intra- and extra-cellularly, while non-penetrating agents such as dextrose from the media exert their protective effect external to the cell membrane. For all the storage times tested, live cultures were recovered from plugs. Because the rates of mutation in cultured fungi correspond to those of cell division and metabolic activity, storage methods that stop cell division completely and totally arrest metabolism while still retaining viability are best. Freezing the tubes containing the fungal discs using liquid nitrogen at -196 °C is advisable before storing at -80 °C as ice crystals do not form at this temperature and rates of other biophysical processes are too slow to affect cell survival. Almost all stored culture plugs of *C. cassiicola* regenerated to live cultures even up to 48 months of storage (Table 1).

In contrast, cryopreservation was not at all effective for preservation of *P. meadii* (Table 2). A very low percentage of the culture remained viable for a brief period of one month only. These results are in conformity with the observations made by several researchers earlier that damage to fungal spores can occur during freezing and thawing of cryopreserved cultures (Loegering and Harmon, 1962; Davis *et al*., 1966; Smith, 1998). The thin walled sporangia of oomycetes are highly sensitive to freezing step. Microscopic observations of sporangia of oomycetes made by earlier

workers (Dahmen *et al*., 1983) showed extensive deformation, severe plasmolysis and highly granulated plasma when frozen rapidly. Therefore, it is very important to ensure that cultures are completely free from bacterial or other microbial contaminants prior to cryopreservation. Although cryopreservation is a widely acclaimed preservation technique for several fungi, with slight modification of protocol even delicate fungi can be cryopreserved, by encapsulation drying in alginate beads at -130 °C (Lalaymiaa *et al*., 2012).

**Lyophilization**

Lyophilization is not appropriate for all fungi. In the case of *C. cassiicola*, lyophilization of the culture was efficient for storage but contaminants were observed during the revival of the culture. However, this technique did not prove to be suitable for long term storage of *P. meadii*. According to Nakasone *et al*. (2004) lyophilization is preferred for heavily sporulating fungi with spores of 10 µm or less in diameter as larger spores collapse during the lyophilization which is not reversible by hydration. Detrimental effects of the freeze drying process on fungal structures like sporangia of the oomycetes and conidia of the powdery mildew, have also been reported (Fennell, 1960; Hwang, 1966; Bromfield and Schmitt, 1967; San Antonio and Blount, 1973; Gale *et al*., 1975; Long *et al*., 1978).

**Genetic variation by RAPD analysis**

Besides successful survival of the fungi, the genetic and physiological stability is also essential, which needs to be monitored to achieve the basic objective of long term preservation. In the present study the effectiveness of water preservation was confirmed for pathogenic and genetic stability of both the pathogens. The RAPD profiles generated with ten arbitrary decamer primers using four isolates of both *C. cassiicola* and *P. meadii* after subjecting to storage (Figs. 4 and 5) for 48 and 24 months respectively indicated that there were no apparent variation in the genetic makeup of both the fungal pathogens following long term storage. In case of all four isolates of *C. cassiicola* (Cc 1, Cc 8, Cc 24 and Cc 46), genetic variation was not observed following storage in sterile water as well as under cryopreservation for upto 48 months. However, in case of *P. meadii* only storage under sterile water retained the genetic stability of the four isolates tested (Pm 58, Pm 84, Pm 97, Pm 104) after 24 months of storage. It is essential to ascertain the originality of the preserved fungi and its genetic fidelity to fulfil international norms and obligations (Santos and Lima, 2001; Ryan and Smith, 2007). However, there may be potential sources of biochemical mutagens like mutagenic antibiotics amended in media to check contaminants and mutagenic secondary metabolites released by contaminants (Russell *et al*., 2013) which may alter the genetic makeup of preserved fungi. Singh *et al.* (2004) confirmed genetic stability of 11 cryogenically preserved edible mushroom strains using random amplified polymorphic DNA (RAPD) analysis. Ryan *et al.* (2001) and Voyron *et al*. (2009) established vigour and genetic fidelity of different fungi under different methods of preservation. Although Homolka *et al*. (2010) recommended whole genome sequencing for confirming point mutations which may not be detected by RAPD, such an approach will be cost effective only if the candidate organism is of great economic importance.

In the present study it was observed that all the treatments tested for *C. cassiicola* were equally efficient as evidenced by the revival of the culture when inoculated on PDA (Table 1). However, the results indicated that

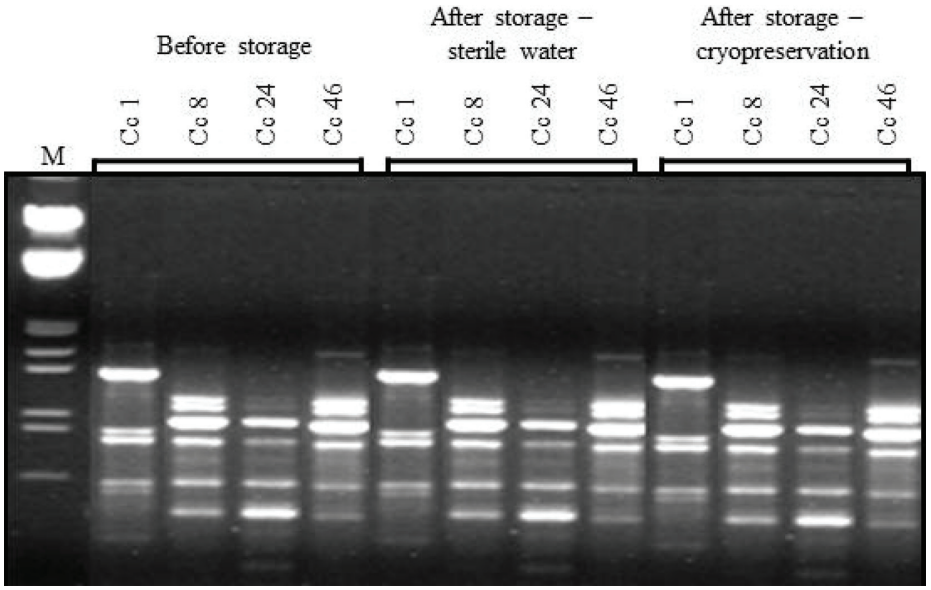


Fig. 4. Representative gel photograph showing RAPD fingerprints of four *C. cassiicola* isolates generated with OPAB-07 indicating no genetic variation in them following long term storage in sterile water and under cryopreservation after 48 months of storage.

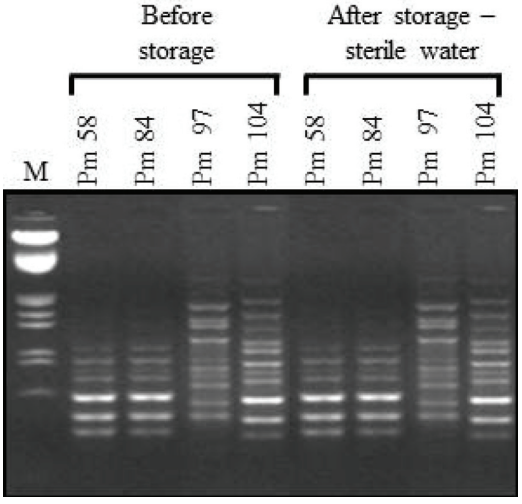


Fig. 5. Representative gel photograph showing RAPD fingerprints of four *P. meadii* isolates generated with OPAC-12 indicating no genetic variation in them following long term storage in sterile water after 24 months of storage.

storage under sterile water and cryopreservation were efficient methods, maintaining their viability and virulence for a period of 48 months. When the cultures were stored under desiccation and lyophilization, bacterial contamination was observed, required extra efforts for purification and these methods were not efficient for long term storage for sustaining virulence. In case of *P. meadii* culture subjected to the various storage treatments, it was observed that out of the six treatments tested only storage in sterile water was efficient in reviving the culture for up to 24 months. Beyond this period contamination was observed and the culture had to be purified. Storage of *P. meadii* in the desiccated treatments showed very low viability, and virulence of the culture was retained only for three months. Cryopreservation method of storage was not efficient for storing *P. meadii* (Table 3). Several factors such as

Table 3. **Revival of cultures subjected to different long term storage treatments**

| No. | Storage treatment | Survival (%)* | |
|---|---|---|---|
| | | *C. cassiicola* | *P.meadii* |
| 1 | Continuous growth method | 82(64.9) | 78(62.0) |
| 2 | Immersion in sterile distilled water | 71(57.4) | 62(51.9) |
| 3 | Desiccation - storage in sterile filter paper discs | 48(43.8) | 15(22.7) |
| 4 | Desiccation - storage in sterile soil | 37(37.4) | 8(16.3) |
| 5 | Cryopreservation | 78(62.0) | 5(12.9) |
| 6 | Lyophilization | 32(34.4) | 3(09.8) |
| | CD (P=0.05) | 2.15 | 2.65 |

* values in parenthesis are arc sine transformed values

economics, labour, infrastructure, and objective of preservation have to be considered when fungi are to be preserved. Continual sub-culture, storage under oil (Kobayashi, 1984; Smith and Onions, 1994), water (Burdsall and Dorworth, 1994; Smith and Onions, 1994), soil (Smith and Onions, 1994) or silica gel (Elliot, 1975; Smith and Onions, 1994) are cost-effective and not much labour intensive. However, their suitability as protocols for long term storage may not be guaranteed. Genetic fidelity of the preserved fungi has also been found to be intact as observed in this study and many earlier studies. From this study, the suitability of storage by immersion in sterile distilled water was found to be efficient for both *P. meadii* and *C. cassiicola*, in terms of viability and virulence. In consideration of the results obtained in the present study and the 'decision based key' (Ryan *et al*., 2000), it is concluded that the economically most important pathogens of natural rubber tree *C. cassiicola* and *P. meadii* could be successfully stored under sterile water. Cryopreservation can also be considered for *C. cassiicola*.

## REFERENCES

Abad, G., Ivors, K.L., Gallup, C.A., Abad, J.A. and Shew, H.D. (2011). Morphological and molecular characterization of *Phytophthora glovera* sp. nov. from tobacco in Brazil. *Mycologia*, **103**: 341-350.

Abd-Elsalam, K.A., Yassin, M.A., Moslem, M.A., Bahkali, A.H., de Wit, P.J.G.M., McKenzie, E.H.C., Stephenson, S.L., Cai, L. and Hyde, K.D. (2010). Culture collections, the new herbaria for fungal pathogens. *Fungal Diversity*, **45**: 21-32.

Baskarathevan, J., Jaspers, M.V., Jones, E.E. and Ridgway, H.J. (2009). Evaluation of different storage methods for rapid and cost-effective preservation of *Botryosphaeria* species. *New Zealand Plant Protection*, **62**: 234-237.

Braithwaite, M., Inglis, C., Dick, M.A., Ramsfield, T.D., Waipara, N.W., Beever, R.E., Pay, J.M. and Hill, C.F. (2007). Investigation of oak tree decline in the Auckland region. *New Zealand Plant Protection*, **60**: 297-30.

Bromfield, K.R. and Schmitt, C.G. (1967). Cryogenic storage of conidia of *Peronospora tabacina*. *Phytopathology*, **57**: 1113.

Burdsall, H.H. and Dorworth, E.B. (1994). Preserving cultures of wood decaying Basidiomycotina using sterile distilled water in cryovials. *Mycologia*, **86**: 275-280.

Clark, C. and Dick, M. W. (1974). Long-term storage and viability of aquatic omycetes. *Transactions of the British Mycological Society*, **63**: 611-612.

Dahmen, H., Staub, Th. and Schwinn, F.J. (1983). Techniques for long-term preservation of phytopathogenic fungi in liquid nitrogen. *Phytopathology*, **73**: 241-246.

Davis, E.E., Hodges, F.A. and Goos, R.D. (1966). Effect of suspending media on the survival of *Puccinia graminis* urediospores during freezing. *Phytopathology*, **56**: 1432-1433.

Diogo, H.C., Sarpieri, A. and Pires, M.C. (2005). Fungi preservation in distilled water. *Anais Brasileiros de Dermatologia*, **80**: 591-594

Elliot, R.F. (1975). Viability of fungal cultures dried and stored over silica gel. *New Zealand Journal of Sciences*, **18**: 577-583.

Fennell, D.I. (1960). Conservation of fungus cultures. *Botanical Review*, **26**: 80-141.

Gale, A.W., Schmitt, C.G. and Bromfield, K.R. (1975). Cryogenic storage of conidia of *Sclerospora sorghi. Phytopathology*, **65**: 828-829.

George, M.K. and Edathil, T.T. (1975). Over summering of *Phytophthora* causing abnormal leaf fall disease of rubber. Rubber Board Bulletin, **12**(3): 112-114.

Hansen, J. (1994). *Corynespora cassiicola* isolated from soybean roots in the red river valley of Minnesota and North Dakota. *Plant Disease*, **78**: 1122.

Homolka, L., Lisá, L., Eichlerová, I., Valášková, V. and Baldrian, P. (2010). Effect of long-term preservation of different basidiomycetes on perlite in liquid nitrogen on their growth, morphological, enzymatic and genetic characteristics. *Fungal Biology*, **114**: 929-935.

Hwang, S.W. (1966). Long-term preservation of fungus cultures with liquid nitrogen refrigeration. *Applied Microbiology*, **14**: 784-788.

Joseph, A., Manju, M.J., Kumar, A., Jacob, C.K. and Nair, R.B. (2004). Aggressiveness of *Corynespora cassiicola* isolates and reaction of some *Hevea brasiliensis* clones to their infection. *National Conference of Emerging Trends in Mycology and Plant Pathology and Microbial Biotechnology,* Osmania University, Hyderabad, date 29-31 Dec., 2004.

Kang, S., Blair, J.E., Geiser, D.M., Khang, C.H., Park, S.Y., Gahegan, M., O'Donnell, K., Luster, D.G., Kim, S.H., Ivors, K.L., Lee, Y.H., Lee, Y.W., Grünwald, N.J., Martin, F.M., Coffey, M.D., Veeraraghavan, N. and Makalowska, I. (2006). Plant pathogen culture collections: It takes a village to preserve these resources vital to the advancement of agricultural security and plant pathology. *Phytopathology*, **96**: 920-925.

Kanwischer, M. E. and Mitchell, D. J. (1978). The influence of a fungicide on the epidemiology of black shank of tobacco. *Phytopathology*, **68**: 1760-1765.

Kobayashi, T. (1984). Maintaining cultures of Basidiomycetes by mineral oil method. *Bulletin of Forestry and Forestry Product Research Institute*, **325**: 141-147.

Kolkowski, J.A. and Smith, D. (1995). *Cryopreservation and freeze drying of fungi*. **In**: *Methods in Molecular Biology 38, Cryopreservation and freeze drying protocols* (Eds. Day J.G. and McLellan M.R. pp. 49-61, Humana Press, NJ, USA ISBN 089603-296-5)

Lalaymiaa, I., Cranenbrouckb, S., Drayec, X. and Declerck, S. (2012). Preservation at ultra-low temperature of *in vitro* cultured arbuscular mycorrhizal fungi via encapsulation–drying. *Fungal Biology*, **116**: 1032–1041.

Loegering, W.Q. and Harmon, D.L. (1962). Effect of thawing temperature on urediospores of *Puccinia graminis* f. sp. *tritici* frozen in liquid nitrogen. *Plant Disease Report*, **46**: 299-302.

Long, R.A., Woods, J.M. and Schmitt, C.G. (1978). Recovery of viable conidia of *Sclerospora philippinensis, S. sacchari,* and S. *sorghi* after cryogenic storage. *Plant Disease Report*, **62**: 479-481.

McGinnis, M.R., Padhye, A.A. and Ajello, L. (1974). Storage of stock cultures of filamentous fungi, yeasts, and some aerobic actinomycetes in sterile distilled water. *Applied Microbiology*, **28**: 218–222.

Meyer, E. (1955). The preservation of dermatophytes at subfreezing temperatures. *Mycologia*, **47**: 664–668.

Nakasone, K.K., Peterson, S.W. and Jong S.C. (2004). Preservation and distribution of fungal cultures. **In**: *Biodiversity of Fungi: Inventory and Monitoring Methods.* (Eds. Mueller G.M., Bills G.F. and Foster M.S.), 37-47 p. Publishers Elsevier Academic Press, San Diego, California.

Pasarell, L. and McGinnis, M.R. (1992). Viability of fungal cultures maintained at -70 °C. *Journal of Clinical Microbiology*, **30**: 1000-1004.

*Reinecke*, P. and *Fokkema*, N.J. (*1979*). *Pseudocercosporella* herpotrichoides: storage and mass production of conidia. *Transactions of the British Mycological Society*, **72**: 329-331.

Roy, C.B., Zachariah, C.A., Jacob, C.K. and Saha, T. (2006). First report of leaf blight caused by

*Alternaria alternata* on *Hevea brasiliensis* in India. *Journal of Plantation Crops*, **34**: 449-454.

Russell, R., Paterson, M. and Lima, N. (2013). Biochemical mutagens affect the preservation of fungi and biodiversity estimations. *Applied Microbiology and Biotechnology*, **97**(1): 77-85.

Ryan, M.J. and Smith, D. (2007). Cryopreservation and freeze-drying of fungi employing centrifugal and shelf freeze-drying. *Methods in Molecular Biology*, **368**: 127–140.

Ryan, M.J., Jeffries, P., Bridge, P.D. and Smith, D. (2001). Developing cryopreservation protocols to secure fungal gene function. *CryoLetters*, **22**: 115-124.

Ryan, M.J., Smith, D. and Jeffries, P. (2000). A decision-based key to determine the most appropriate protocol for the preservation of fungi. *World Journal of Microbiology and Biotechnology*, **16**: 183-186.

Saha, T., Kumar, A., Sreena, A.S., Joseph, A., Jacob, C.K., Kothandaraman, R. and Nazeer, M.A. (2000). Genetic variability of *Corynespora cassiicola* infecting *Hevea brasiliensis* isolated from the traditional rubber growing areas in India. *Indian Journal of Natural Rubber Research*, **13**: 1-10.

San Antonio, J.P. and Blount, V. (1973). Use of liquid nitrogen to preserve downy mildew *(Phytophthora phaseoli)* inoculum. *Plant Disease Report*, **57**: 724.

Santos, I.M. and Lima, N. (2001). Criteria followed in the establishment of a filamentous fungal culture collection—Micoteca da Universidade do Minho (MUM). *World Journal of Microbiology and Biotechnology*, **17**: 215–22.

Sette, L.D., Pagnocca, F.C. and Rodrigues, A. (2013). Microbial culture collections as pillars for promoting fungal diversity, conservation and exploitation. *Fungal Genetics and Biology*, **60**: 2-8.

Sharma, R. and Gupta, M. (2012). The conservation of fungi - an important aspect for industrial microbiology. *Cibtech Journal of Microbiology*, **1**: 43-46.

*Shearer*, B.L., Zeyen R.J. and Ooka J.J. (*1974)*. Storage and behaviour in *soil* of *Septoria* species isolated from cereals. *Phytopathology*, **64**: 163-167.

Simpfendorfer, S., Harden, T.J. and Murray, G.M. (1996). Viability and pathogenicity of *Phytophthora clandestina* after storage in water and liquid nitrogen. *Australasian Plant Pathology*, **25**: 234-239.

Singh, S.K., Upadhyay, R.C., Kamal, S. and Tiwari, M. (2004). Mushroom cryopreservation and its effect on survival, yield and genetic stability. *CryoLetters*, **25**: 23-32.

Smith, D. and Onions, A.H.S. (1983). A comparison of some preservation techniques for fungi. *Transactions of the British Mycological Society*, **81**: 535-540.

Smith, D. (1998). The use of cryopreservation in the *ex-situ* conservation of fungi. *CryoLetters*, **19**: 79-90.

Smith, D. and Onions, A.H.S. (1994). *The preservation and maintenance of living fungi*. Second edition, CAB International, Wallingford, UK. ISBN 085198-902-0.

Stalpers, J.A., De Hoog, A. and Vlug, I.J. (1987). Improvement of the straw technique for the preservation of fungi in liquid nitrogen. *Mycologia*, **79**: 82–89, 122p.

Voyron, S., Roussel, S., Munaut, F., Varese, G.C., Ginepro, M., Declerck, S. and Marchisio, V.F. (2009). Vitality and genetic fidelity of white-rot fungi mycelia following different methods of preservation. *Mycological Research*, **113**: 1027-1038.