

Full Length Research Paper

Availability and morphological characteristics of endophytic fungi held in different methods of preservation

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Maintenance of microorganisms in mycology is of fundamental importance for retrospective and prospective studies that focus on their biology, etiology and epidemiology. The aim of this study was to evaluate the viability, contamination and morphological changes of endophytic fungi maintained under different preservation methods. We evaluated five preservation methods; constant sub-culturing, preservation under mineral oil, under distilled water, preservation at -20°C and storage at -70°C. Every 50 days, we evaluated the viability, purity and macro-micro morphology of the cultures. The main results are as follows: constant sub-culturing and preservation under distilled water have allowed the viability of all strains during the study period. Preservation in mineral oil resulted in the contamination of the strain *Aspergillus* F45 and micro-morphological modification of the *Fusarium* LU5 culture after 100 days of preservation. Preservation at -70°C caused macro-morphological changes in *Fusarium* LU6 after 100 days. Under the experimental conditions and the limited period of study (150 days) it was demonstrated that conservation under distilled water was the most appropriate form of preservation of endophytic microorganisms.

Key words: Endophytes, preservation methods, morphological characteristics.

INTRODUCTION

In fungi preservation, methodologies are used which aim to maintain cell viability and the metabolic properties of

these organisms for long periods. This activity is essential for retrospective and prospective studies that focus

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on the biology, etiology, epidemiology and production of substances which are of biotechnological interest (Ryan et al., 2000). The main preservation methods evaluated can be divided into "simple and cheap", such as constant sub-culturing, storage under oil (Kobayashi 1984; Smith and Onions, 1994), under water (Burdshall and Dorworth, 1994; Smith and Onions, 1994), in soil (Smith and Onions, 1994) and in silica gel (Elliot, 1975; Smith and Onions, 1994), or they may be "complex and expensive", such as lyophilization (Kolkowski and Smith, 1995; Tan, 1997) and cryopreserved in liquid nitrogen (Smith, 1998).

The microorganisms called endophytes coexist inside of higher plants without causing any apparent signs of disease (Petrini, 1991). Currently these microorganisms came to be regarded as an important part of biodiversity. The endophytic mycoflora presents a distribution that differs according to its host. The distribution of endophytic mycoflora differs with the host. The specificity of the endophytes ranges from generalist to highly specific to the host and the environment. These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites (Azevedo et al., 2000; Strobel, 2003). Studies on the potential of endophytes, especially those related to the discovery of new substances, appear relevant both for use in the biological control of pests and diseases and for application in the chemical/biochemical or pharmaceutical industries (Stierle et al., 1995; Azevedo 1998; Azevedo et al., 2000; Stamford et al., 2001, 2002; Suto et al., 2002; Strobel, 2003).

The Laboratory Micobateriologia, CPCS-INPA, has been conducting bioprospecting of substances produced by endophytes which are useful for the treatment and diagnosis of tuberculosis. Some strains of industrial interest have been obtained and need to be preserved. However, the literature is very limited as to the influence of different methods of preservation on morphology and the production of secondary metabolites in fungi of this group.

The aim of this work was to evaluate the feasibility and morphological characteristics of endophytic fungi maintained in different preservation methods and specifically to: a) assess the continued viability and purity of the cultures; b) evaluate the influence of these conservation methods on the micro and macro morphological characteristics of the cultures.

MATERIALS AND METHODS

Microorganisms

Five endophytic fungi belonging to the collection of the National Institute for Amazon Research were used in this study. Four of them were isolated from *Caesalpinia ferrea* Martius (*Fusarium* LU5, *Fusarium* LU6, *Fusarium* LU11, *Aspergillus* F45) and these had been preserved under mineral oil. The fifth strain was isolated from *Himatanthus sucuba* (*Fusarium* H42) and had been preserved in distilled water.

Preservation techniques

Five conventional preservation techniques were evaluated in triplicate:

1) Constant sub-culturing: This technique was performed as described by Lacaz et al. (1991). Every 15 days, a fragment of viable culture was transferred to a new tube containing Potato Dextrose Agar (PDA) which was incubated at 25°C.

2) Preservation under mineral oil: The technique was performed as described by Braz et al. (2009). Glass vials (20 ml) were filled with 2 ml of PDA culture medium and then inoculated with the microorganism. After 7 days, the culture media was covered with 10 ml of mineral oil (autoclaved for two consecutive days). The vials were capped with a rubber stopper and an aluminium seal and stored at 25°C.

3) Preservation under distilled water: The technique was carried out as described by Diogo et al. (2005). Glass vials (20 ml) were filled with 10 ml of distilled water (autoclaved for two consecutive days). Five small fragments (25 mm²) taken from a 7 day culture and transferred to these vials. The vials were closed with a rubber stopper and an aluminium seal and stored at room temperature (28°C).

4) Preservation at -20°C: The technique was carried out as described by Girão et al. (2004). Glass vials (20 ml) containing 8 ml of distilled water, 0.5 ml of dimethyl sulfoxide DMSO (cryoprotectant) and 1 ml of glycerol (cryoprotectant) were autoclaved for two consecutive days. Small fragments (25 mm²) taken from a 7 day culture were transferred to these vials. The vials were closed with a rubber stopper and an aluminium seal and stored at -20°C.

5) Preservation at -70°C: The technique was performed as developed in the Laboratory of Mycobacteriology, CPCS-INPA. In 0.4 ml of distilled water, 0.025 ml of dimethyl sulfoxide DMSO (cryoprotectant), 0.050 ml of glycerol (cryoprotectant) and 10 mg of polypropylenespheres (0.5 mm diameter, with a central hole) were placed into 1.5 ml microtubes. Small fragments (25 mm²) taken from a 7 day culture were transferred to these vials. The vials were closed with a rubber stopper and an aluminium seal and stored at -70°C.

Culture assessments

Each 50 days, during a 150 days period, the preservation techniques were evaluated. The preserved mycelia were transferred to Petri dishes containing PDA medium. Information about the viability, purity, macro-morphology and micro-morphology of the obtained cultures were assessed (Lacaz et al., 2002).

RESULTS

The endophytic isolates selected for the present study belonged to the class of Ascomycetes, specifically from the genera *Aspergillus*, *Fusarium* and *Penicillium*. The first morphological characterization was carried out in the "day zero" and this was used as a standard for evaluating the changes in viability, purity, micromorphology and macro-morphology over 150 days of the experiment. Table 1 show the frequency (%) of cultures that presented viability or that presented contamination, during the 50, 100 and 150 days of the experiment.

The strains subjected to preservations methods must

Table 1. Evaluation of the cultures that presented viability or that presented contamination during the 50, 100 and 150 days of the experiment.

Preservation technique	Viability (n1 /n2)			Contamination (n3/n2)		
	50 days	100 days	150 days	50 days	100 days	150 days
Sub-culturing	5/5	5/5	5/5	-	-	-
Distillated water	5/5	5/5	5/5	-	-	-
Mineral oil	4/5	4/5	4/5	1/5	1/5	1/5
-20°C	4/5	4/5	4/5	-	-	-
-70°C	4/5	4/5	4/5	-	-	-

n1: viable cultures, n2 total cultures, n3 contaminated cultures.

be able to withstand extreme temperatures and environments. In this study the culture of the isolate *Fusarium* L6 presented no growth after the submersion in oil such as *Fusarium* LU11 that did not grow after freezing (-20 and -70°C) (Table 1). The preservation under mineral oil resulted in the fungi contamination of the culture of *Aspergillus* F45 (-20 and -70°C).

In addition, the preservation techniques of constant sub-culturing and mineral oil caused modifications only in the macro-morphology of *Fusarium* LU6 after 100 days of preservation, in both cases this strain lost the ability to produce a red pigment.

DISCUSSION

Regarding the "Constant sub-culturing" preservation, in the experimental conditions all the strains have remained viable. However, previous studies shows that, in the long-term, the loss of viability usually occurs (Roy et al., 2014). Other disadvantages of this method are: a) spent with culture media; b) culture contamination; c) changes in morphology; and d) strains lose the ability to produce secondary substances (Nakasone et al., 2004).

The methods of preservation under mineral oil and at low temperatures (-20 to -70°C) are described in literature as able to maintain the viability of fungal cultures for longer periods, more than 10 years (Braz et al., 2009). However, the strains subjected to preservation must be able to with stand the extreme physical conditions. In the present study the culture of the strains *Fusarium* LU6 and *Fusarium* LU11 became unfeasible after submersion in oil and freezing, respectively. Smith and Onions (1983) reported on the effectiveness of the method of preservation in mineral oil, they assessed preserved cultures and 47 of the 58 have remained viable after 32 years.

Although viability was maintained for all samples tested in this study, the preservation technique for constant sub-culturing causes changes in the macromorphology of a sample. As the mineral oil technique showed the worst results with lower viability, changes in the macro-morphology and contamination. These changes in fungi

macromorphology when preserved for long or short periods have been described in the literature, especially for techniques of constant sub-culturing (Rodrigues et al., 1992; Freitas et al., 2011). Interestingly, described by Roberts et al. (1992) demonstrated that changes in macromorphology such as lost characteristics when kept in constant sub-culturing were recovered after being preserved in distilled water, which is not common.

Universities and research institutes need preservation methods that do not occupy too much space and are inexpensive. The use of low temperatures (-20 and -70°C) with cryoprotectants, primarily glycerol, prevents the formation of ice crystals during freezing, reducing cellular damage caused by their formation (Mata and Pérez-Merlo, 2003). However, even being employed with protective substances, structures in some microorganisms do not support the physical conditions, it happened in the present study and in previous ones (Figueiredo and Pimentel, 1975).

The preservation technique of distilled water has been widely used for maintenance of microorganisms despite having been overcome, in terms of maintenance of viability and genetic changes by lyophilization technique. Studies show that this low cost technique can maintain fungi stored for periods of seven (Burdshall and Dorworth, 1994) to 11 years (Neufeld and Oliveira, 2008) with more than 90% viability and no morphological changes, thus demonstrating the effectiveness of this conservation method. Among the techniques tested in this study, the method of preservation in distilled water was more effective for endophytic fungi tested. There is a need for further studies with a longer preservation and compared to over current methods such as lyophilisation and cryopreservation in liquid nitrogen, and a greater number of samples in order to establish the best preservation criteria for the group of tested microorganisms. The differences in the nature of the microorganism related to the variation in retention time and stress suffered during the reactivation prevent the determination of standard procedure conservation applicable to all microorganisms. This demonstrates the importance of this study for this group of fungi, thus contributing to a better understanding of the conservation methods.

Conflict of Interests

The authors have not declared any conflict of interests.

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