Use of biochemical methods for Ectomycorrhizal fungi spores inoculum improvement

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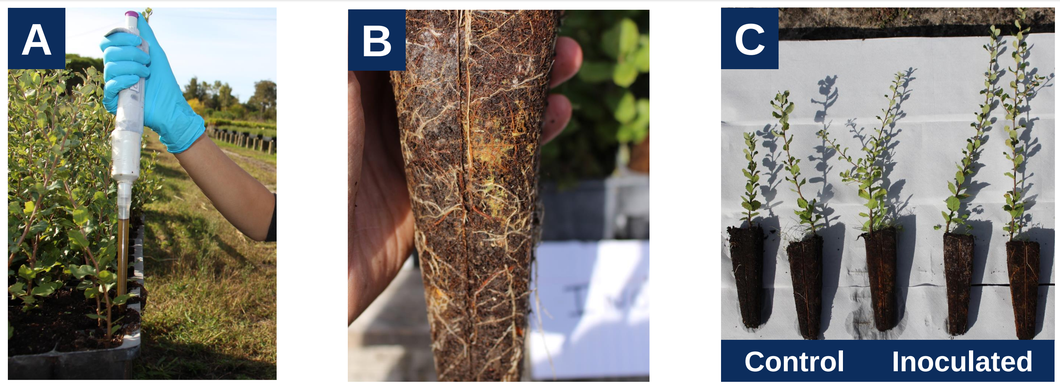
Introduction

Ectomycorrhizal (EcM) fungi are important symbiotic organisms that develop mutualistic relationships with host trees improving its vigour and health status. Their application as inoculants in nursery systems help growth and mycorrhization of plants and promote resistance to abiotic stresses, such as water stress. Despite its benefits, the use of EcM fungi as inoculants is still poorly optimized; the use of mycelium as inoculum can be hardly cost-effective and inadequate in some environmental conditions and the use of spores is sometimes ineffective because of its dormancy state.

This work aims to optimize the use of EcM spores as inoculum by selecting the best individuals using biochemical analysis and staining methods to assess the dormancy and activity of spores. We are testing different methods of conservation as well as the addition of substances to potentially decrease the dormancy and increase the activity of the spores, in order to improve the rate of success of mycorrhization and consequently deliver all the benefits of EcM fungi to plants.



(A) Inoculation of *Quercus suber* with *Pisolithus tinctorius* spores, **(B)** micorrhization of the seedling root and **(C)** improvement of inoculated seedlings growth compared to a non-inoculated control.

Methods

Collection of *Pisolithus tinctorius* fruit bodies for spores dormancy (Acridine orange) and activity (MTT) assessment

Qualitative analysis

Spores wash → NaoH treatment (Increases spore permeability)

Acridine Orange for 20min in the dark at room temperature

Stained spore suspension in slide to observe with fluorescence microscope

Assessment of dormancy

Quantitative analysis

Conservation at **4ºC in sand** for 6 months

Conservation at **4ºC in plastic containers** for 6 months

1. Conservation method

2. Washing and weighting

3. MTT method

MTT in phosphate buffer (PB)

+

PB (without inducer)

PB + **Activated charcoal**

PB + **Glucose**

PB + **Yeast Extract**

Incubation for 24h in the dark at 23ºC

Spores wash → Tubes with **>50mg** of spores → Removal of stained water

Pellet with acidified isopropyl alcohol

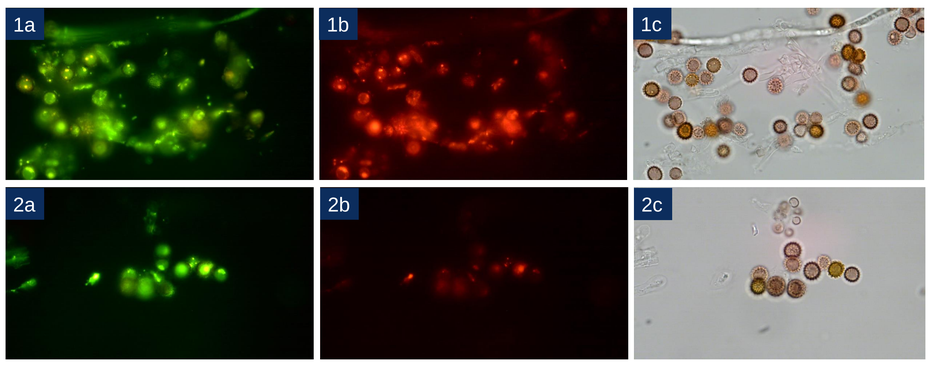
Removal of soluble formazan

Spectrophotometer reading at 570nm

4. Optical density reading

Results and Discussion

Acridine orange



Presence of DNA and RNA was visualized in *Pisolithus tinctorius* spores. DNA-bound acridine orange was visible in images **1a** and **2a** and RNA-bound acridine orange was visible in images **1b** and **2b**. Images **1c** and **2c** were obtained using light. Based on the red fluorescence, the sample 1 which was conserved in sand appears to be less dormant since it is expressing more RNA compared to the sample 2 which was stored in a plastic container. Micrographs were taken at x1000 magnification.

MTT method

Sample	Inducer	Mean A570/Mass
1	Phosphate Buffer	4.40
	Activated Charcoal	4.34
	Glucose	3.45
	Yeast Extract	**6.20**
2	Phosphate Buffer	0.78
	Activated Charcoal	0.40
	Glucose	0.66
	Yeast Extract	**3.10**
3	Phosphate Buffer	3.11
	Activated Charcoal	3.50
	Glucose	3.15
	Yeast Extract	3.43

The table represents the activity of spores measured by the MTT method. The samples 1 and 3 which were stored in sand are more active compared to the sample 2 which was conserved in a plastic container. The addition of yeast extract also appears to promote the spores activity in samples 1 and 2 and in the latter the increase is higher than 3-fold.

Conclusions

The optimization of spores use as a biotechnology tool for mycorrhization of seedlings in nursery context is still ongoing. Different methods of conservation for a long period of time impact differently the dormancy of spores - storing spores in sand was more beneficial and promoted a breaking of dormancy effect, which will improve the success in infection and mycorrhization of plants. Addition of yeast extract also caused an increase in activity of the same spores.

The qualitative and quantitative biochemical methods that were used, namely the staining with acridine orange and the MTT method, were effective in the identification of the best individuals to be used as inoculum and can help assessing its effectiveness for nursery application.

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