

Rooting for Answers: A Reliable Inoculation Method for Difficult-to-Isolate Neotropical Ectomycorrhizal Fungi

Amanda Cristina Zanatta

Universidade Federal de Santa Catarina, Algas e Plantas

Gabrielle Gonçalves

Universidade Federal de Santa Catarina, Algas e Plantas

Maria Eduarda Andrade Borges

Universidade Federal de Santa Catarina, Algas e Plantas

Jéssica Vilvert Klöppel

Universidade Federal de Santa Catarina, Algas e Plantas

Fernanda Maria Cordeiro Oliveira

Universidade Federal de Santa Catarina, Algas e Plantas

Maria Alice Neves

Universidade Federal de Santa Catarina, Algas e Plantas

Paulo Tamaso Mito

`paulo.mito@ufsc.br`

Universidade Federal de Santa Catarina, Algas e Plantas

Short Report

Keywords: Guapiroid ectomycorrhiza, Tomentella sp., inoculum, experimental protocols

Posted Date: November 18th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-8039253/v1>

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Additional Declarations: No competing interests reported.

Abstract

Neotropical ectomycorrhizae (ECMs) have been poorly explored. In Brazilian coastal zones (*restinga*), a new subtype of ECM called guapirioid ectomycorrhizae (gECMs) was described on *Guapira opposita* roots. However, the lack of tools to investigate this association in depth results in significant knowledge gaps. For example, a study model consisting of inoculated plants and their respective controls could open the way to experimentation and deeply increase our understanding of this association. However, traditional methods to isolate fungi (e.g., root tips or basidiome fragments growing in culture medium) were unsuccessful for most of the neotropical fungal species we tested. Here, we present an alternative method to inoculate ectomycorrhizal root tips directly on receiver plants. This method proved to be an efficient and low-cost approach for propagating and manipulating gECM formation in at least one species of neotropical fungus. Root tips of *G. opposita* seedlings grown in *restinga* soil containing a selected gECM morphotype were excised and placed next to the roots of plants grown in sterilized soil. Soil from these receiver plants was also tested as inoculum. Guapirioid ECMs of the same type inoculated were detected on the receiver plants, reaching up to 97.5% and 80% efficiency from root tip and soil inoculation, respectively. Plants without the inoculum showed no signs of gECM formation. DNA barcode analyses confirmed that the formed gECM consisted of the same *Tomentella* sp. inoculated. This is a simple, reliable, and cost-effective method to work with ECMs without the need to isolate fungi in culture medium.

Introduction

Ectomycorrhizae (ECMs) are mutualistic associations between fungi and plant roots that rely on the exchange of resources, such as water, mineral nutrients, and carbohydrates (Becquer et al. 2019). The multiple origins of ectomycorrhizal status (Miyauchi et al. 2020) indicate that there may be differences at anatomical, physiological and genetic levels, especially when considering ECMs that occur outside temperate and boreal regions. Recent studies revealed that ECMs are relatively common in the Neotropics (Corrales et al. 2018) and that some species need their status to be verified, for example, using isotopes (Vanegas-León et al. 2019). The structures of some of the native neotropical ECMs are different from the classic boreal and temperate structures described (Martin et al. 2016). Furtado et al. (2022) described a subtype of ECM, the guapirioid ectomycorrhiza (gECM), on the roots of *Guapira opposita* (Vell.) Reitz (Nyctaginaceae) in southern Brazil. The gECM lacks Hartig net and intraradicular hyphae and the root tips lack an epiderm. *Guapira opposita* plants studied in southern Brazil can be associated with about forty taxa of fungi forming gECMs (Furtado et al. 2023b).

Despite the novelty of the discoveries of ECMs in the Neotropics, it is not yet known how gECMs contribute to fungal or plant performance, mainly because of the lack of tools to study these associations in a controlled way. Ectomycorrhizal fungi (ECMF) are usually isolated in artificial media so they can be inoculated in plants without other fungi to test the effects of the fungi on plant performance. However, despite many trials, we could not isolate any of the gECM-forming fungus using traditional methods (e.g., putting basidiome pieces or root tips on the usual media used to isolate fungi). Here, we

describe an alternative method that would allow researchers to test the impact of gECMs reliably, using a single species and ectomycorrhiza root tips as inocula (Levisohn 1956). This method was inspired by methods used for arbuscular mycorrhizae with the goal of using the soil as a source of the fungus. However, it is rarely mentioned in the literature for the ECMs, and there is no information regarding whether this is an efficient method for ECMs (Levisohn 1956; Ekwebelam 1973; Marx and Kenney 1982; Repák 2011). In this work, we tested using gECM root tips as inoculum for plants growing in sterile soil and evaluated the reliability of the method.

Material and methods

Seeds and plant cultivation

To obtain plants to use as a source of the gECM, seeds of *G. opposita* collected in March 2021 at the Monumento Natural Municipal da Lagoa do Peri in Florianópolis (MONA Lagoa do PERI), Santa Catarina State, Brazil, were depulped and germinated in a substrate made of *restinga* soil and vermiculite at a 1:1 proportion. Additional *G. opposita* seeds were collected (from January 2023 to March 2023 and January 2024 to April 2024) at the same location to germinate in sterile conditions and use as the inoculated plants. The seeds were depulped and disinfected in a 1% sodium hypochlorite solution for 10 min and put in sterilized substrate. The substrate was composed of *restinga* soil and vermiculite (1:1) autoclaved for 40 min at 121°C. The plants growing in the sterilized soil were then inoculated with one morphotype of gECM root tips from the first batch of plants. The plants were kept in a room with controlled light (12 h photoperiod and $80 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of PAR light) at a temperature of $25 \pm 2^\circ\text{C}$, and they were watered weekly with autoclaved distilled water.

Ectomycorrhizal root tip inoculation on *Guapira opposita*

Roots of *G. opposita* growing in non-sterile soil were analyzed using a stereomicroscope (Olympus® SZ61) to recognize the morphotypes. A morphotype that was both abundant and easy to recognize in the root system was selected. The morphotype is brownish at the base and white at the tip (Fig. 1). Root tips with this morphotype were selected and collected with sterile tweezers. Two to three root tips were placed close to the root system of 10 four-month-old plants growing in the sterile substrate (see batch 1 in Fig. 1). Ten four-month-old plants growing in the sterile substrate were used as control, without inoculation. The root systems were checked after one and two months to detect the presence of the gECM on the root tips.

When ECMs were present on root tips, the soil was tested as inoculum. Fifteen grams of the soil from batch 1 were inoculated on 5 plants growing in sterile substrate (see batch 2 in Fig. 1). The root systems were checked after two and four months to detect the presence of the gECM on the root tips. The tips from these plants were collected and sequenced to confirm the identification of the fungus.

Nine plants growing in sterile substrate (batch 3) were inoculated with three root tips from batch 2, following the same protocol described above. Root tips from batch 3 were used to inoculate a new batch

of 40 plants (see batch 4 in Fig. 1), using five root tips per plant to increase the success rate, since the amount of inoculum influences the formation of ECMs (Mortier et al. 1988; Repák 2011). The root systems were checked after five months to detect the presence of the gECM on the root tips. Forty plants were used as controls. After checking for the presence of the gECM, root tips from three random plants were collected to sequence and identify the fungal species and to compare the sequences to the original species identified in batch 2 (Fig. 1).

Plant morphology analysis

Not all plants formed the gECM. We performed some tentative, non-destructive, morphometrical analyses of the plants that formed and did not form the gECM (see the following section for details).

Of the nine plants from batch 3, five formed the gECM after 2 months. The plant height was measured and the number of leaves was counted before the plants were inoculated and again after two months. The differences between the first and second measurements determined the height and leaf gain. The results were statistically evaluated using the Shapiro-Wilk and Levene tests, and Student's T test for independent samples ($p < 0.05$).

DNA extraction, amplification, and sequencing

DNA was extracted from the ectomycorrhizal root tips using the Extraction-N-Amp Equivalent DNA Extraction Protocol (Russell 2024). The primer pair ITS-1F and ITS4 (Gardes and Bruns 1993) was used to amplify the nrITS (nuclear ribosomal internal transcribed spacer region) region, the barcoding region used to identify fungi. Amplification cycling parameters were as follows: initial denaturation step of 94°C for two min., followed by 40 cycles of 94°C for 30 sec., 55°C for 45 sec., and 72°C for one min., respectively, with a final extension at 72°C for 7 min. The PCR (Polymerase Chain Reaction) products were cleaned using polyethylene glycol (PEG 8000–20%). The DNA sequencing used the same pair of primers used in the amplification step, and it was done at the Laboratório Multiusuário de Estudo de Biologia (LAMEB, Universidade Federal de Santa Catarina, Florianópolis, Santa Catarina, Brazil).

DNA sequence analysis

The generated chromatograms were manually edited and assembled in Geneious R7.1.3 (<https://www.geneious.com>). Four sequences were generated during this study: Pf (PX099225), P12 (PX099226), Pi12 (PX099227), and Pi15 (PX099228). The sequences were registered in SISGen (ACA6CAF) and deposited in GenBank as the cited codes. Two sequences most similar to the ones generated in this work were obtained from the GenBank database (Benson et al. 2013) using a BLAST search (Altschul et al. 1990): OR575484—*Tomentella* sp. and OR575504—*Tomentella* sp. The sequences were aligned with ClustalX 2.1 using the default parameters (Larkin et al. 2007). The final nrITS

alignment matrix consisted of 695 characters and was analyzed in Geneious R7.1.3 (<https://www.geneious.com>) to compare the percentage of identity among the sequences (Fig. 2b).

Results and discussion

Guapira opposita seedlings from the first batch formed the gECM two months after being inoculated with root tips of *Tomentella* morphotypes. Three randomly selected plants from the inoculated batch were checked for the presence of the gECM. Two out of ten inoculated plants died and nine out of ten of the non-inoculated control plants died. We do not have evidence on why the plants died, but the presence of the gECM may have improved plant survival. The one non-inoculated plant that survived did not have gECMs on its roots and had abundant root hairs, a characteristic of a root without ECMs (Novero et al. 2008). After seven months, three other plants from the inoculated batch had formed the gECM, totaling six out of ten plants with the gECM (60% of colonization; Fig. 1). No plants from the non-inoculated batch had the gECM on their roots.

Substrate from the first inoculated batch was added to five pots (batch 2) with seedlings that were germinated and grown on sterile substrate. After three months, four plants had the gECM in their roots. The one plant that did not have the gECM in their roots died. The formation of the gECM happened in two plants after two months and in another two plants after 4 months. The difference in time could be due to the different amount of mycelium in the portions of substrate used. The success rate of inoculation using portions of substrate from the pots with inoculated plants was 80%. However, due to the low number of samples used, further experiments are needed to support this result.

Nine plants were inoculated with three root tips each from plants from the second batch (batch 3; Fig. 1). After three months, five (55%) had formed the gECM in the roots. The plants with and without the gECM were compared based on the height and the number of new leaves formed. Plants with the gECM had an average of 5.6 leaves versus 1.5 leaves in the plants without the gECM ($p < 0.05$; Fig. 2a). There were no significant differences regarding plant height. Further analysis and more experiments are needed to assess how the presence of gECMs affects plant metabolism.

The last part of the experiment included the inoculation of 40 seedlings with 5 root tips each (batch 4; Fig. 1) plus 40 control seedlings. Thirty-nine (97.5%) of the inoculated seedlings formed the gECM with the *Tomentella* morphotype after five months. The 40 non-inoculated control plants did not form the gECM. Inoculation of plant seedlings using the gECM root tips as inocula is a viable approach that will allow future research to be done to assess the impact of ectomycorrhizal fungi on plant hosts.

To confirm the identity of the morphotypes selected for the experiments, the ITS region of the DNA was amplified and sequenced from plants from the second batch and from the fourth batch. The molecular results had 99.70% to 100% similarity (Fig. 2b). The sequences were compared with other sequences in the GenBank database using the Blast search, limiting the search to type sequences, resulting in a match of 97% similarity with *Tomentella* sp. sequences (Fig. 2b). To confirm the identity of the species of the fungus, it is necessary to use an integrated analysis that includes anatomical, taxonomic, and

phylogenetic studies with more samples, as well as basidiomes. Comparing the obtained sequences with sequences from basidiomes collected in *restinga* will help to identify the species in the future. Furtado et al. (2023a), based on molecular studies, found *Tomentella* as the most common genus associated with *G. opposita* in *restinga*. *Tomentella* is frequently cited in studies about the ectomycorrhizal community in forest soils in tropical regions (Tang et al. 2024; Truong et al. 2024).

This study was an attempt to use ectomycorrhizal root tips as a source of ECMF on *G. opposita*. Levinsohn (1956) described similar methods for *Fraxinus excelsior* and *Pinus contorta* and had success with the mycorrhization of the plant hosts. Marx and Kenney (1982) and Repác (2011) cited the possibility of using this method to inoculate plants but without mentioning any specific experiments. Repác (2011) mentioned that the presence of harmful microorganisms could interfere with the results when root tips are used as a source of ECMs. However, the protocol presented here is a suitable and cost-effective alternative that will allow experimentation in cases when the ECM fungus is difficult to isolate in culture.

Conclusion

Our results show that root tips can be used as inocula to promote interactions between plants and *Tomentella* sp., reaching up to 97% efficiency. This strategy is especially relevant when dealing with fungal species that are not easily isolated in culture. We hope that this work provides an alternative method to study plant-fungus interactions when traditional protocols cannot be used. Further studies about gECMs will help us understand their role in ecosystems and widen our view on plant-fungus interactions.

Declarations

The authors declare that they have no conflict of interest.

Authorship

A. C. Z.: conceived and designed the study, performed research, analyzed data, wrote the paper; G. G.: performed research, figure design; M. E. A. B.: analyzed data, contributed new methods, wrote the paper; J. V. K.: performed research; F. M. C. O.: wrote the paper; M. A. N.: wrote the paper; P. T. M.: conceived and designed the study, analyzed data, wrote the paper. All authors reviewed the manuscript.

Author Contribution

A. C. Z.: conceived and designed the study, performed research, analyzed data, wrote the paper; G. G.: performed research, figure design; M. E. A. B.: analyzed data, contributed new methods, wrote the paper; J. V. K.: performed research; F. M. C. O.: wrote the paper; M. A. N.: wrote the paper; P. T. M.: conceived and designed the study, analyzed data, wrote the paper. All authors reviewed the manuscript.

Acknowledgement

We are thankful to the Universidade Federal de Santa Catarina for its laboratories that were useful for these experiments: Plant Physiology Laboratory (LAFISIO), Mycology Laboratory (MICOLAB), and Multiuser Laboratory of Biology Studies (LAMEB). We are also thankful to Willian da Silva Oliveira and João Paulo Ernzen for their contributions to this work.

Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

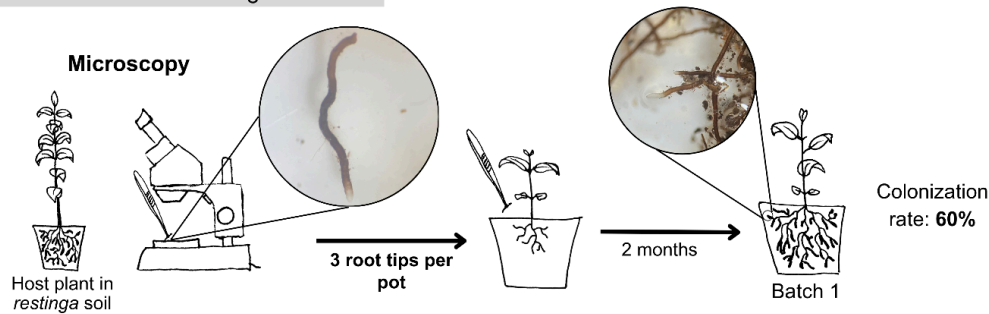
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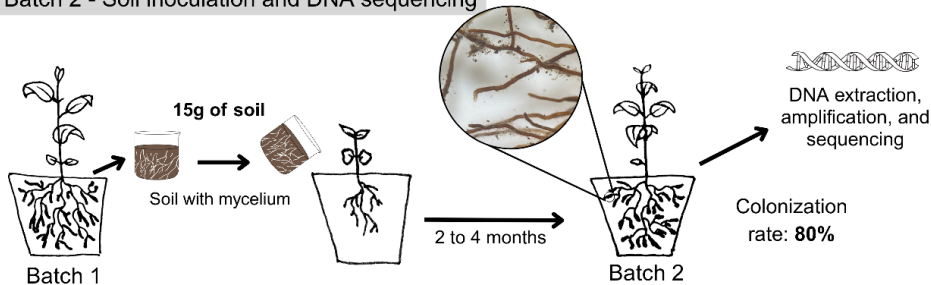
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Figures

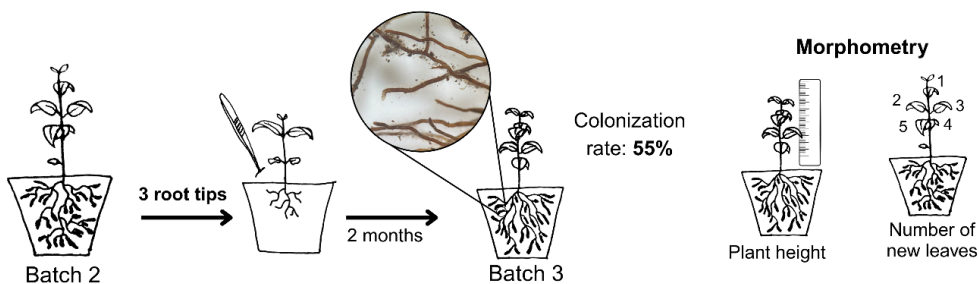
Batch 1 - Inoculation using natural soil



Batch 2 - Soil inoculation and DNA sequencing



Batch 3 - Root tip inoculation and plant morphometry



Batch 4 - Root tip inoculation and DNA sequencing

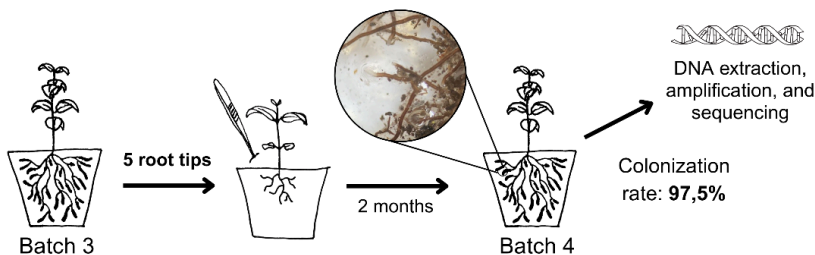


Figure 1

Experimental design. Batch 1: *Tomentella* root tip morphotype was inoculated on *G. opposita* seedlings. Root tips were cut and put in the pot near the roots of seedlings growing in sterile substrate. Colonization happened after two months. Batch 2: Portions of soil with mycelia from batch 1 were inoculated on *G. opposita* seedlings. Colonization formed after two or four months. DNA samples were collected to confirm the identification of the *Tomentella* morphotype. Batch 3: Root tips from batch 2

were inoculated on *G. opposita* seedlings, and the height and the number of leaves were measured. Batch 4: Five root tips from batch 3 were inoculated on 40 *G. opposita* seedlings. DNA samples were collected to confirm the identification of the *Tomentella* morphotype. Drawings by Gabrielle Gonçalves.

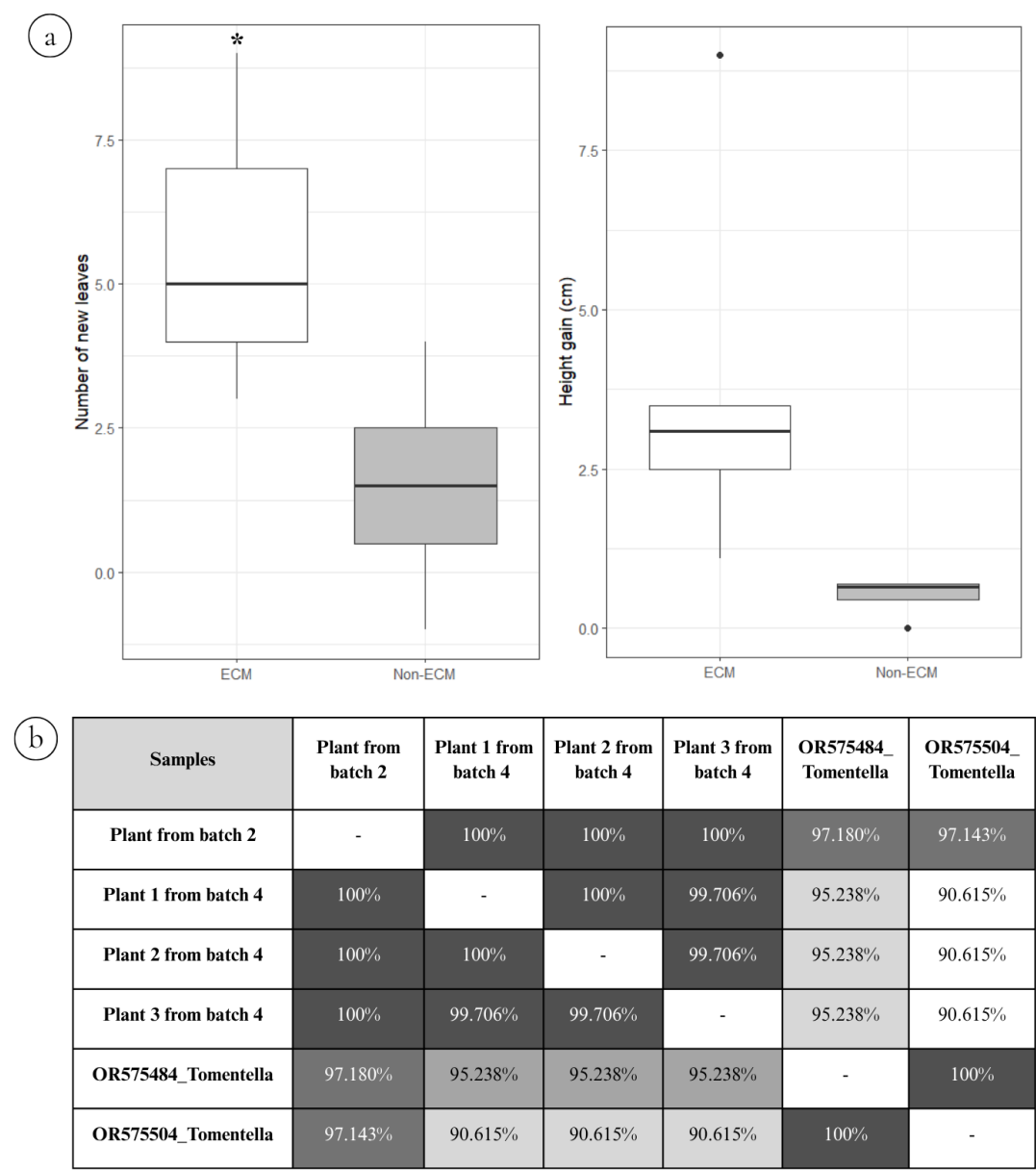


Figure 2

a) Plant morphometry. Number of new leaves and height gain of plants in the third batch after two months (*p <0,05). b) Molecular identification using the ITS barcoding region. The table shows the

percentage of identity among sequences that corresponds to the rate of positions where the sequences have an identical base or residue.