

Pre-inoculation Tissue Physiological Induction Promotes In Vitro Mycelial Recovery of *Russula griseocarnosa*: A Two-Stage Framework of “Pre-inoculation Tissue State–Post-inoculation Nutritional Support”

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Abstract

Tissue isolation and host-free cultivation of ectomycorrhizal (ECM) fungi are often constrained by low rates of mycelial recovery and by browning or loss of viability of excised tissues. Previous studies have largely focused on post-inoculation medium composition and nutritional optimization, whereas the influence of the physiological state of tissues before inoculation has received comparatively little systematic evaluation. Here, we tested a methodological hypothesis that, for difficult-to-culture ECM fungi, pre-inoculation physiological treatment may be an important factor influencing whether excised tissues resume mycelial growth, whereas medium composition may primarily affect growth after recovery. *Russula griseocarnosa*, an economically valuable ECM fungus that is difficult to isolate and cultivate, was used as the model. Two independent fresh wild fruiting bodies (fruiting bodies 1 and 2) were examined. Ten internal tissue blocks were excised from each fruiting body and allocated to a pre-inoculation induction treatment (1A and 2A) or an untreated control (1B and 2B), yielding five plates per subgroup and 20 plates in total. Tissues were inoculated in parallel onto PDA, MMN, and three experimental media (N5, N8, and N11) and incubated at 30°C in darkness for 7 days. Mycelial recovery was observed in all induced tissues from both fruiting bodies (1A, 5/5; 2A, 5/5; pooled, 10/10), whereas no recovery was observed in the corresponding untreated controls (1B, 0/5; 2B, 0/5; pooled, 0/10). A two-sided Fisher's exact test using plates as the analytical unit yielded $P = 1.08 \times 10^{-5}$; because tissue blocks originating from the same fruiting body are not fully independent biological replicates, this test is used only to describe plate-level differences within the present experimental system. Within the treatment group, an observational trend in recovered mycelial abundance was $N8 > N11 > N5 > MMN > PDA$. Previous Oxford Nanopore Technologies (ONT) ITS sequencing data (NCBI BioProject accession PRJNA1536671) provided molecular support for assignment of the recovered mycelium to *Russula* and for close sequence matching to *R. griseocarnosa*: 207 of 217 high-quality reads (95.39%) were assigned to *Russula*, and the ITS consensus sequence showed 99.718% similarity to *R. griseocarnosa* reference sequence OM760493.1 and 99.444% similarity to MN275663.1. These results support a strong association between pre-inoculation tissue treatment and in vitro mycelial recovery of *R. griseocarnosa* and suggest a two-stage experimental framework of “pre-inoculation tissue state–post-inoculation nutritional support” for studies of difficult-to-culture ECM fungi.

1. Introduction

Ectomycorrhizal (ECM) fungi form intimate symbiotic associations with host-plant roots and play important roles in nutrient acquisition, carbon cycling, and plant adaptation in forest ecosystems (Smith and Read, 2008). Unlike many saprotrophic fungi that grow readily on artificial substrates, species of *Russula*, *Cortinarius*, and several bolete lineages can be difficult to isolate and maintain under host-free conditions (Hintikka and Niemi, 1999).

Russula griseocarnosa is an edible ECM fungus of substantial economic value. Genomic and molecular ecological studies suggest that its repertoire of carbohydrate-active enzyme (CAZyme)-related genes differs markedly from that of typical saprotrophic fungi and is consistent with a relatively strong

dependence on host-associated nutrition (Liu et al., 2022). Subsequent work has also reported difficulties in obtaining verified target cultures through conventional tissue isolation, basidiospore germination, or mycorrhiza-derived isolation, with isolates potentially affected by endophytic fungi or other co-occurring microorganisms (Liu et al., 2024).

Research on in vitro cultivation of difficult-to-culture mycorrhizal fungi has traditionally emphasized medium composition, including optimization of carbon and nitrogen sources, mineral nutrients, vitamins, and other organic supplements (Ohta, 1997). Such optimization, however, implicitly assumes that the inoculated tissue remains physiologically capable of responding to exogenous nutrients and resuming growth. In fresh wild fruiting bodies, excision, cleaning, transfer, and removal from the original ecological context may rapidly alter tissue physiology. Maintaining or improving tissue recoverability before inoculation may therefore be as important as nutritional support after inoculation.

In our previous work (Chen and Lin, 2026), tissue isolation was combined with Oxford Nanopore Technologies (ONT) ITS sequencing to molecularly evaluate the taxonomic identity of recovered mycelium. Of 217 high-quality reads, 207 (95.39%) were assigned to *Russula*. The recovered ITS consensus sequence showed 99.718% similarity to *R. griseocarnosa* reference sequence OM760493.1 and 99.444% similarity to MN275663.1.

Based on this framework, the present study examined whether pre-inoculation tissue treatment was strongly associated with mycelial recovery. The same treatment-control design was applied independently to two fruiting bodies, while differences in post-recovery growth among media were also observed. The objectives were to (1) compare mycelial recovery between pre-inoculation induction and untreated controls; (2) describe preliminary differences in post-recovery growth among media; and (3) establish a two-stage experimental framework for subsequent independent replication and testing in other difficult-to-culture ECM fungi.

2. Materials and Methods

2.1 Fungal material and aseptic tissue excision

Fresh, morphologically intact wild fruiting bodies of *R. griseocarnosa* were selected. Surface cleaning and handling followed the aseptic procedures established in our previous work. Under a laminar-flow hood, sterile dissection instruments were used to excise internal tissue blocks of approximately 5 × 3 × 2 mm. Tissue was preferentially sampled from internal regions not directly exposed to the external environment to reduce the risk of surface microbial contamination.

2.2 Experimental design and pre-inoculation tissue treatment

The same treatment-control design was applied to two independent wild fruiting bodies, designated fruiting body 1 and fruiting body 2. Ten internal tissue blocks were excised from each fruiting body (20

tissue blocks in total) and allocated to a pre-inoculation induction group (A) or an untreated control group (B).

Fruiting body 1 (10 plates): five tissue blocks received the pre-inoculation physiological induction treatment (group 1A, five plates), and five received only the routine pretreatment and served as controls (group 1B, five plates).

Fruiting body 2 (10 plates): five tissue blocks received the pre-inoculation physiological induction treatment (group 2A, five plates), and five received only the routine pretreatment and served as controls (group 2B, five plates).

Routine soaking and washing pretreatment (disclosed step): before inoculation, all tissue blocks received the same surface-disinfection and soaking/washing pretreatment. Tissues were soaked in a combined antibiotic solution containing ampicillin sodium and streptomycin and then thoroughly rinsed with sterile water. In addition to suppressing bacterial contamination, this process may have produced some physical leaching and dilution. We hypothesize that it may reduce locally accumulated soluble wound-associated compounds after tissue excision; however, the chemical composition of the wash solution, phenolic metabolites, and polyphenol oxidase (PPO) activity were not directly measured. This interpretation therefore remains a hypothesis for future testing.

Induction groups (1A + 2A, 10 plates): after routine antibiotic soaking and washing, tissue blocks received an additional proprietary pre-inoculation physiological induction treatment. Because this step involves undisclosed technical details, the present manuscript describes only its experimental position and intended purpose and does not disclose the core formulation, concentrations, treatment duration, or operational parameters. The treatment was intended to improve the recoverable state of excised tissue before culture; its physiological mechanism was not directly measured in this study.

Untreated control groups (1B + 2B, 10 plates): tissue blocks received only the routine antibiotic soaking/disinfection and sterile-water washing described above, without the additional pre-inoculation physiological induction step, and were then inoculated directly onto the corresponding media.

2.3 Media and culture conditions

Five pairs of tissue blocks from fruiting body 1 (1A vs 1B) and five pairs from fruiting body 2 (2A vs 2B) were inoculated in parallel onto five solid media. Each medium therefore contained two treatment plates and two control plates, with one treatment-control pair derived from each fruiting body. The media were: PDA (potato dextrose agar); modified Melin-Norkrans (MMN) medium; N5, an experimental medium characterized by a relatively high glucose level and complex nutritional supplementation; N8, an experimental medium characterized by a relatively high sucrose level and complex nutritional supplementation; and N11, an experimental medium characterized by a relatively high sucrose level and complex nutritional supplementation.

All plates were incubated at 30°C in darkness for 7 days. Tissue browning, appearance of mycelium, mycelial extension, and morphology were observed periodically. Microscopy was used to assist morphological documentation. The primary binary endpoint was the presence or absence of discernible newly extending mycelium.

2.4 Molecular evidence and taxonomic assignment

Taxonomic assignment of the recovered target mycelium was supported by previous ONT ITS sequencing. The sequencing data were archived under NCBI BioProject accession PRJNA1536671, associated with SRA Submission ID SUB16519719. Of 217 high-quality reads, 207 (95.39%) were assigned to the genus *Russula*. At the species level, the ITS consensus sequence showed 99.718% similarity to *R. griseocarnosa* reference sequence OM760493.1 and 99.444% similarity to MN275663.1.

2.5 Statistical analysis

Mycelial recovery on day 7 was recorded as a binary outcome, using the plate as the analytical unit. Overall recovery rates for the treatment group (1A + 2A, $n = 10$ plates) and untreated control group (1B + 2B, $n = 10$ plates) were compared using a two-sided Fisher's exact test ($P = 1.08 \times 10^{-5}$). Because plates originating from the same fruiting body are not fully independent biological replicates, this test is used only to describe plate-level treatment differences and is not treated as an independent basis for population-level inference across fruiting bodies.

3. Results

3.1 Pre-inoculation physiological induction and mycelial recovery

After 7 days at 30°C, a clear separation between treatment and control outcomes was observed for both fruiting bodies. For fruiting body 1, recovery occurred in 5/5 plates in group 1A and 0/5 plates in group 1B. For fruiting body 2, recovery occurred in 5/5 plates in group 2A and 0/5 plates in group 2B. Thus, newly extending mycelium was observed in 10/10 treatment plates and in 0/10 untreated control plates (Table 1).

Mycelium in the treatment groups generally began to appear 3–5 days after inoculation and showed discernible extension by day 7. In contrast, untreated control tissues predominantly showed browning, shrinkage, or loss of viability. The two-sided Fisher's exact test indicated a marked plate-level difference between the pooled groups (10/10 positive vs 0/10 positive; $P = 1.08 \times 10^{-5}$). No mycelial recovery occurred in untreated tissues on any of the five media, whereas recovery occurred on all five media in treated tissues, further supporting a highly consistent experimental association between pre-inoculation treatment and mycelial recovery.

Table 1
Mycelial recovery of *R. griseocarnosa* tissues under pre-inoculation treatment and different media on day 7 (N = 20)

Medium	Treatment (1A + 2A)	FB1 (1A)	FB2 (2A)	Control (1B + 2B)	FB1 (1B)	FB2 (2B)	Post-recovery observation
N8	2/2 (100%)	1/1	1/1	0/2 (0%)	0/1	0/1	Strongest in this experiment; marked aggregation
N11	2/2 (100%)	1/1	1/1	0/2 (0%)	0/1	0/1	Relatively abundant; stronger growth
N5	2/2 (100%)	1/1	1/1	0/2 (0%)	0/1	0/1	Moderately abundant
MMN	2/2 (100%)	1/1	1/1	0/2 (0%)	0/1	0/1	Less abundant; relatively sparse
PDA	2/2 (100%)	1/1	1/1	0/2 (0%)	0/1	0/1	Weakest in this experiment; very sparse
Overall	10/10 (100%)	5/5	5/5	0/10 (0%)	0/5	0/5	Observational trend: N8 > N11 > N5 > MMN > PDA

3.2 Post-recovery growth on different media

Within the induction groups (1A + 2A), all five media supported mycelial recovery, but observational differences in mycelial density, growth vigor, and apparent abundance were present on day 7. The overall observational trend was N8 > N11 > N5 > MMN > PDA. Mycelial aggregation and cord-like growth were most evident on N8; N11 and N5 supported relatively strong branching and extension; mycelium was comparatively sparse on MMN; and growth was weakest on PDA.

Because only two treatment plates were available for each medium, this ranking is considered exploratory and is not interpreted as a statistically demonstrated hierarchy of medium performance. Future experiments should include additional independent replication and quantitative measures such as colony diameter, mycelial dry weight, image area, or other standardized growth metrics.

4. Discussion

4.1 Pre-inoculation tissue state and the treatment-response pattern

The most important observation in this study was not that one medium outperformed another, but that the pre-inoculation treatment and untreated control groups showed a highly consistent treatment-response pattern across two independent fruiting bodies, with a pooled separation of 10/10 versus 0/10 plates. This finding suggests that the physiological state of *R. griseocarnosa* fruiting-body tissue before

it enters the culture medium may be an important variable influencing whether mycelial growth can resume.

Regarding possible mechanisms, antibiotic soaking followed by sterile-water washing may, in addition to suppressing bacterial contamination, have produced some physical leaching and dilution. We hypothesize that this process may reduce locally accumulated soluble wound-associated compounds after tissue excision, while the subsequent induction step may improve the physiological state of excised cells. However, the chemical composition of the wash solution, phenolic metabolites, reactive oxygen species, PPO activity, membrane integrity, and gene expression were not directly measured. These mechanistic interpretations therefore remain hypotheses rather than demonstrated conclusions.

These findings do not diminish the importance of medium optimization. Instead, they support dividing the isolation process into two sequential stages: a first stage involving maintenance or induction of the pre-inoculation tissue state, which may determine whether recovery is possible, and a second stage of post-inoculation nutritional support, which may influence continued growth, growth rate, density, and morphology after recovery.

4.2 Relationship to previous *R. griseocarnosa* isolation studies

Previous studies of *R. griseocarnosa* indicate strong host-associated characteristics and report difficulties in verifying the target fungus in isolates obtained by conventional tissue isolation or other approaches, partly because of interference from co-occurring fungi (Liu et al., 2022; Liu et al., 2024). Building on the previous ONT ITS evidence (Chen and Lin, 2026), the present study shifts experimental attention upstream from medium screening alone to the physiological handling of tissue before inoculation.

The current ONT data support a close relationship of the recovered mycelium to *Russula* and *R. griseocarnosa*. However, stronger terms such as “pure culture,” “first pure isolate,” or “first host-free pure culture” would require additional evidence, including single-hyphal-tip purification, repeated molecular identification, long-term stable subculture, and, where appropriate, independent verification. Accordingly, the present manuscript uses more cautious terms such as “mycelial recovery” and “molecularly supported recovery of the target fungus.”

4.3 Mechanistic interpretation and scope

This experiment directly tested only *R. griseocarnosa*. It therefore does not establish that the method is generally effective across all ECM fungi, and the findings should not be extrapolated directly to arbuscular mycorrhizal (AM) fungi. Future independent testing in *Tricholoma matsutake*, bolete lineages, additional *Russula* species, and ECM fungi representing different ecological strategies will be required to define the method’s boundaries and reproducibility.

4.4 Limitations and next steps

This study is a controlled methodological proof-of-concept experiment. Its main limitations include the small number of replicates per medium, reliance on observational growth measures for medium comparisons, the absence of direct physiological measurements of the pre-inoculation treatment mechanism, and the need for additional molecular and cultivation evidence regarding culture purity and long-term stability.

Future work should prioritize additional independent biological replication across different fruiting bodies and collection batches, prespecification of criteria for mycelial recovery, quantitative image analysis or biomass-based measures for medium comparisons, and repeated ITS or multilocus identification of independently recovered cultures. If the present results are reproduced, the proposed “pre-inoculation tissue state–post-inoculation nutritional support” framework would gain stronger methodological significance.

5. Conclusion

Across the two independent fruiting bodies examined in this experiment, pre-inoculation induction was consistently associated with mycelial recovery (1A, 5/5; 2A, 5/5; pooled, 10/10), whereas no recovery was observed in the corresponding untreated controls (1B, 0/5; 2B, 0/5; pooled, 0/10). These findings indicate a strong association between pre-inoculation tissue treatment and in vitro mycelial recovery and suggest that the physiological state of the inoculum before placement on culture medium may be an important and underappreciated variable in the isolation of difficult-to-culture ECM fungi. Differences among media were observational, with the present experiment showing a trend of N8 > N11 > N5 > MMN > PDA. Given the limited replication per medium and the nested biological structure of the experiment, these findings should be regarded as a strong methodological signal rather than definitive evidence for a universal mechanism or cross-species applicability. Additional independent biological replication, quantitative growth measurements, repeated molecular identification, and cross-species validation will be required to further test the stability and broader value of this two-stage cultivation framework.

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