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# Mycelial growth of *Scleroderma sp.* as Affected by Culture Media

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## Abstract

The prevailing problem towards commercial cultivation of ectomycorrhizal mushrooms including *Scleroderma sp.* is the lack of protocol and information on the culture medium to produce inoculant, which is required in producing the mushroom alongside with pine. *Scleroderma* can be tapped as an inoculant for pine trees to increase the production of *Scleroderma* at the mountains which not only helps the pine trees to absorb water and nutrients from the soil but also accelerates the shoot growth and rehabilitates degraded land. To attain this, a protocol needs to be developed along with the appropriate culture medium process that may result in the mass production of *Scleroderma* mycelia to serve as inoculant for pine seedlings resulting in increased pine areas and production of *Scleroderma*. Thus, a study to compare the mycelial growth of *Scleroderma sp.* on two kinds of media was conducted. Results revealed that the use of Murashige and Skoog medium promoted faster mycelial growth and had the heaviest mycelial biomass. It is recommended that further studies be conducted on the optimal conditions of *Scleroderma sp.* as well as its inoculation to pine seedlings and testing the same media on other ectomycorrhizal fungi to strengthen and validate the study's method as protocol on culture medium preparation.

## Introduction

In the past decades, cultivating edible ectomycorrhizal mushrooms has garnered a lot of interest with the reality that there has been a dramatic decline in the production of ectomycorrhizal mushrooms while demand is high. Commercial cultivation of these mushrooms especially in the Cordillera Administrative Region (CAR) can also speed up reforestation with pine trees as the intimate relationship between pine and the said mushrooms is inevitable. Despite this, only few edible ectomycorrhizal mushrooms have been cultivated with less degree of success and definitely not in volumes. This is due to the fact that ectomycorrhizal mushrooms need to be associated with a host plant

for nutrition (Declerk, Strullu & Fortin, 2005).

The fungal genus *Scleroderma* is an ectomycorrhizal fungus belonging to the Phylum *Basidiomycota*, Order *Boletales*, Family *Sclerodermaceae* (Binder & Hibbet, 2006). The mushroom is commonly found at pine areas in the Cordillera Region and is utilized as food in indigenous communities (De Leon, Kalaw, Dulay, Undan, Alfonso, Undan, & Reyes, 2016). Ectomycorrhizal fungi can form a mutualistic relationship with root. These modified roots enhance nutrient and water uptake, increase tolerance to environmental stresses, promote plant growth, and protect against pathogens (Brundrett, Bougher, Dell, Grove, & Malajczuk, 1996; Chung, Kim, Cho, & Lee, 2002; Brundrett, 2004) and mycorrhizal formation is the consequence of a mutualistic interaction between certain soil fungi and plant roots that helps in overcoming nutritional limitations faced by the respective partners (Nehls, 2008). Van der Heijden, Martin, Selosse, and Sanders (2015) characterized ectomycorrhizal mushroom (ECM) symbiosis by a distinct root shape, formation of hyphal mantle encasing root tips and mycelia extending into the surrounding soil, and fungal penetration between cortical cells.

In symbiosis, the ectomycorrhizal fungi obtain carbon source from their host plants and, in return, the fungi help the plants to absorb water and nutrient from the soil leading to accelerated shoot growth and degraded land rehabilitated (Almsjah, Husin, Santoso, Putra, & Alamsjah, 2015). In 2002, Kim et al. concluded that carbon-nitrogen (C:N) ratio is an important factor affecting mycelial growth, exopolysaccharide production, and fruiting body formation in mushroom cultures. Also, the influence of bioelements such as $Ca^{2+}$, $Co^{2+}$, $Fe^{2+}$, $K^{+}$ and $Mn^{2+}$ are favorable to the mycelial growth and exopolysaccharide production of *Paecilomyces sinclairii*. Lee et al. (2004) reported that the optimum culture C:N ratio for *Phellinus linteus* is 10:1. Likewise, the optimum culture C:N ratio for mycelial growth of *Coriolus versicolor* is 2:1, 5:1, and 10:1 (Jo et al., 2010). Jargeat et al. (2003) characterized and analyzed the nitrate transporter and nitrite reductase genes of the symbiotic basidiomycete *Hebeloma cylindrosporum*. Saha, Mandal, Dasgupta, and Saha (2008) on the other hand, concluded that glucose and sucrose containing media showed highest mycelial growth on *Lasiodiplodia theobromae*. Hamad, Alma, Ismael, and Gogeri (2014) found out that in *Aspergillus niger*, fructose and sucrose were suitable sources of carbon while glucose and maltose were good carbon sources to have a higher affinity.

*Pinus kesiya*, commonly known as Baguio pine, is found in the elevated areas of Baguio City, Benguet, and Mountain Province. Its prevalence in Baguio has earned the name "The City of Pines." One of the most widely distributed pines in Asia, it is also found in Khasi Hill in India, Thailand, Burma, Cambodia, Laos, China, and Vietnam (Stuart, 2017). It is a host plant for ectomycorrhizal fungi, which forms a mutually beneficial symbiosis.

Many ectomycorrhizal fungal genera such as *Amanita, Astraeus, Boletus, Hebeloma, Lactarius, Rhizopogon, Pisolithus, Scleroderma,* and *Tricholoma* can be cultivated in pure culture and most culturable ectomycorrhizal fungi rely on specific requirements. Therefore, many studies have attempted to define the optimal physical conditions and the suitable medium for their growth (Brundrett et al., 1996; Dazaet al., 2006; Sanchez, Honrubia & Torres, 2001; Siri-in, Kumla, Suwannarach, & Lumyong, 2014; Xu et al., 2008).

Many studies were done to inoculate ectomycorrhizal mushrooms; however, only a few can be seen for culturing *Scleroderma species*. Malajczuk and Hartney (1986) inoculated *Scleroderma paradoxum* and *Scleroderma verrucosum*, *Pisolithus microcarpus* and *Pisolithus tinctorius* —all under family *Sclerodermataceae*— and other ectomycorrhizal fungi on plantlets and seedlings of *Eucalyptus camaldulensis*, ectomycorrhizal formation were observed in both root initiation medium and *Modified Melin-Norkrans* (MMN) agar; however, in seedling inoculation, following that of Molina (1979) only few exhibited ectomycorrhizal development. Further, Brundrett et al. (1996) inoculated different ectomycorrhizal fungi on Eucalyptus seedlings using inoculum from fruiting bodies in Ferry and Dass medium, Pachlewski medium, and MMN medium, within surface sterilized mycorrhizal root tips, spore germination, and seed surface sterilization were done. Siri-in et al. (2014) studied on the optimal physical conditions and suitable medium of *Scleroderma sinnamariense* in which fungus host agar was the best medium for mycelial growth and biomass yield followed by Modified Murashige and Skoog (Kalmiş & Kalyoncu, 2008) and MMN (Langer, Krpata, & Peintner, 2008). Also, 20 isolates of *Pisolithus*, a genera under *Sclerodermataceae* were compared on their ability to form mycorrhiza in vitro with *Eucalyptus grandis* and stimulated seedling growth in vivo (Burgess,

Dell & Malajczuk, 1994). Kumla, Suwannarach and Lumyong (2016) characterized *Pisolithus orientalis* in culture and its *in vitro* mycorrhization with *Eucalyptus camadulensis* and *Pinus kesiya* which resulted in Murashige and Skoog exhibiting both the largest fungal colony diameter and biomass production.

This research is important in identifying knowledge about the culture medium as appropriate for *Scleroderma sp.*, which can be useful in the mass production of inoculant to speed up pine tree reforestation with *Scleroderma*. Further, inoculating pine seedlings can be of vital scientific research area. The medium may also serve as basis for other ectomycorrhizal mushrooms researches. Thus, the study is seen vital in two contexts: (1) pine reforestation with *Scleroderma* inoculant; and (2) culture medium protocol foundation

It is possible to mass produce *Scleroderma* inoculated pine seedlings with resulting products including reforestation with pine and *Scleroderma* mushroom. Consequent to such inoculation, it may be possible to reforest in faster and wider scale areas in CAR deforested areas that were originally pine areas.

With such undertaking at hand, the study compared mycelial growth of *Scleroderma sp.* on two kinds of media, Murashige and Skoog media (MS) and *Modified Melin-Norkrans* media (MMN).

## Materials and Methods

Techniques in producing pure cultures of ectomycorrhizal mushrooms are available (i.e., Brundett et al., 1996); however, such techniques may not necessarily work for the local *Scleroderma sp.* in the Philippines. As such, the research made root sterilization modifications to suit the local condition.

### Pine seedling inoculation with *Scleroderma sp.*

Mountain soil derived from pine areas were sterilized, cooled, and placed in 16 pots (5x10 inches). Two seeds of pine (*Pinus kesiya*) were sown unto each pot and cared for four months. Eight pots were then inoculated with *Scleroderma* spores while the other potted seedlings were not inoculated to serve as control. After two months, roots from both pots were obtained for mantle observation. As well as to be a source of tissues for in vitro culture for the inoculated roots (Figure 1).

### Filter Sterilization

Stock solutions of Benomyl and Streptomycin (Table 1) were prepared separately, filtered in sterilized amber bottles using a 0.22 micron syringe filter, and sealed to be added to the isolation medium to help control microbial contamination.


*Figure 1.* Pine seedling inoculation (a) Pine seedling; (b) Obtaining roots of pine; (c) Roots of inoculated pine with mycorrhiza; (d) White mycorrhiza

Table 1

*Antibiotic and Fungistatic Components*

| Antibiotic | Concentration (mg/l) | Susceptible organisms | Reference |
|---|---|---|---|
| Streptomycin Fungicide | 10 | Bacteria | Tommerup and Malajczuk (1993) |
| Benomyl | 1 | Ascomycete fungi | Erland and Sodestrom (1990) |

Table 2

*Composition of the Media to be Used in Isolating the Mycorrhizal Fungi*

| Ingredient | MS[1] mg/L | MMN[2] mg/L |
|---|---|---|
| $(NH_4)_2HPO_4$ | 250 | |
| $KNO_3$ | 1,900 | |
| $KH_2PO_4$ | 170 | 500 |
| $MgSO_4•7H_2O$ | 370 | 150 |
| $CaCl_2•2H_2O$ | 440 | 50 |
| $CaCl_2$ | 332.2 | |
| $NH_4NO_3$ | 1,650 | |
| NaCl | | 25 |
| $FeSO_4•7H_2O$ | 27.80 | 20 |
| Na2EDTA•$2H_2O$ | 37.3 | |
| $H_3BO_3$ | 6.20 | |
| $ZnSO_4•7H_2O$ | 8.60 | |
| $MnSO_4•H_2O$ | 22.30 | |
| $Na_2MoO_4•2H_2O$ | 0.25 | |
| $CuSO_4•5H_2O$ | 0.25 | |
| $CoCl_2•6H_2O$ | 0.25 | |
| Myo-inositol | 100 | |
| Thiamine HCl | 0.1 | 0.1 |
| Pyridoxine HCl | 0.5 | |
| Nicotinic acid | 0.5 | |
| Glycine | 2.0 | |
| Agar | 13 g | 13 g |
| Sucrose | 30 g | 10 g |
| pH | 5.6 | 5.6 |

*Notes:*
[1] Murashige and Skoog (Purohit & Mathur, 1999)
[2] *Modified Melin-Norkrans* (Marx, 1969)

**Preparation of the media**

Murashige and Skoog (MS) and *Modified Melin-Norkrans* (MMN) media were prepared following the usual kind and concentration of macro and micro minerals (Table 2). The stock solution of macro, micro, iron, and vitamin sources were prepared for both MS and MMN. From the stock solution, 1 L each of the MS and one-half strength MMN were separately prepared. The pH for both media was then adjusted to 5.6. Sucrose was added at 30 g and 10 g for MS and MMN media, respectively. The MS and MMN were separately simmered while mixing agar at 13 g/l. Fifteen (15) ml of each medium was then transferred to each petri plate, sterilized at 15 psi for 30 minutes. After sterilization, 0.1 ml each of benomyl and streptomycin was pipetted into both media then cooled for future isolation.

**Root Sterilization**

Two months after inoculating *Scleroderma* spores, root samples were obtained from inoculated and uninoculated pots of pine seedlings, around the root zones. The roots were sieved through a (25) mesh sieve and washed thoroughly until free from soil particles. Following that of Brundrett et al. (1996), selected mycorrhizal roots of uniform appearance were then pre-treated with a wetting agent by immersion in 0.2% aqueous solution of tween 80 (v/v), washed with detergent for 30 minutes followed by several changes of tap water until free from bubbles and was finally rinsed in distilled water. The root samples were sterilized in 30% aqueous $H_2O_2$ (v/v) for 20 seconds, were transferred immediately into 1 L of sterile distilled water for further rinsing, and blot-dried onto sterilized tissue paper.

**Isolation of Root Inoculum**

Root tips dissected 1-2 cm long using sterilized

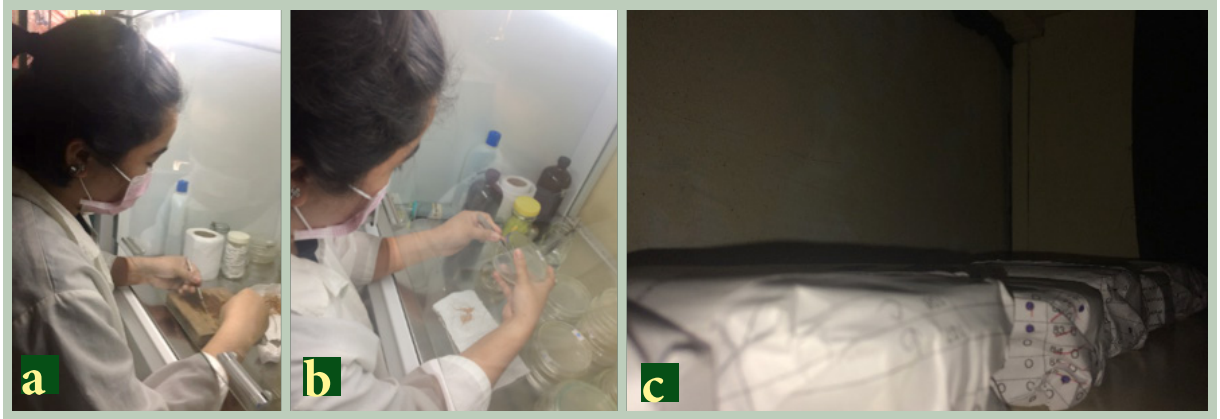


*Figure 2.* Isolation (a) Dissecting pine roots; (b) Isolating roots of pine; (c) Isolated in darkness.

fine pointed tweezers were obtained from segments of root and fungus tissue from the inner mantle (Figure 2). The pieces were individually arranged on plated MS and MMN media.

### Experimental Design

The two treatments, MS and MMN Media with three replicates and two petri dish per replicate, were laid out in the incubation box following the Complete Randomized Design. The plates were incubated at 22°C in darkness and examined weekly for growth of fungal colonies. Growing mycorrhizal fungal colonies recognized by their appearance under a dissecting microscope were then transferred onto fresh MS and MMN media.

### Data Gathered

**Number of days from isolation to mycelial colonization of 5 cm.** This data was gathered by counting the days from isolation of the root explant until a mycelial colonization of 5 cm was obtained.

**Mycelial growth diameter.** This data was obtained by measuring the colony diameter of the mycelia.

**Mycelial fresh weight.** The protocol of Day and Hervey (1946) was followed with modification in obtaining the mycelial fresh weight. The agar-mycelial contents of each petri dish was removed by steaming it on a rapidly boiling distilled water for 4 minutes. The mycelial mat was then lifted using a tweezer and was aspirated for 30 seconds to remove excess media and was weighed.

**Mycelial dry weight.** The protocol of Day and Hervey (1946) was followed with modification in obtaining the mycelial dry weight. Dry weights were obtained by placing the samples in an 80°C forced-air-drying oven for 48 hours. The dry samples were then weighed. The dry weight of the fungus was calculated by using the formula:

Dry weight = (weight of filter paper + mycelium) - (weight of filter paper)

## Results and Discussion

### Presence of Dichotomously Short Roots

*Scleroderma*, an ectomycorrhizal mushroom (ECM) forms a symbiotic relationship with pine trees which helps increase growth of pine trees and pines tolerance to environmental stresses resulting in lower mortality rate of seedlings without the mushroom, pine trees are left susceptible to diseases leading to stunted growth. As evidence of mycorrhizal relationship between *Scleroderma sp.* and pine roots, short dichotomous roots covered with mantle was found evident in the inoculated pine seedlings vis-à-vis absence of mantle from uninoculated roots (Figure 3). Mycorrhizal fungi produce dichotomously short roots as a result of symbiosis, these short roots are covered with mantle which provides a physical barrier from other pathogens, along with the mantle are hyphae and rhizomorphs which extends over a larger volume of soil than roots can which increases the plant nutrient absorptive surface area resulting in better growth of pine seedlings as compared to

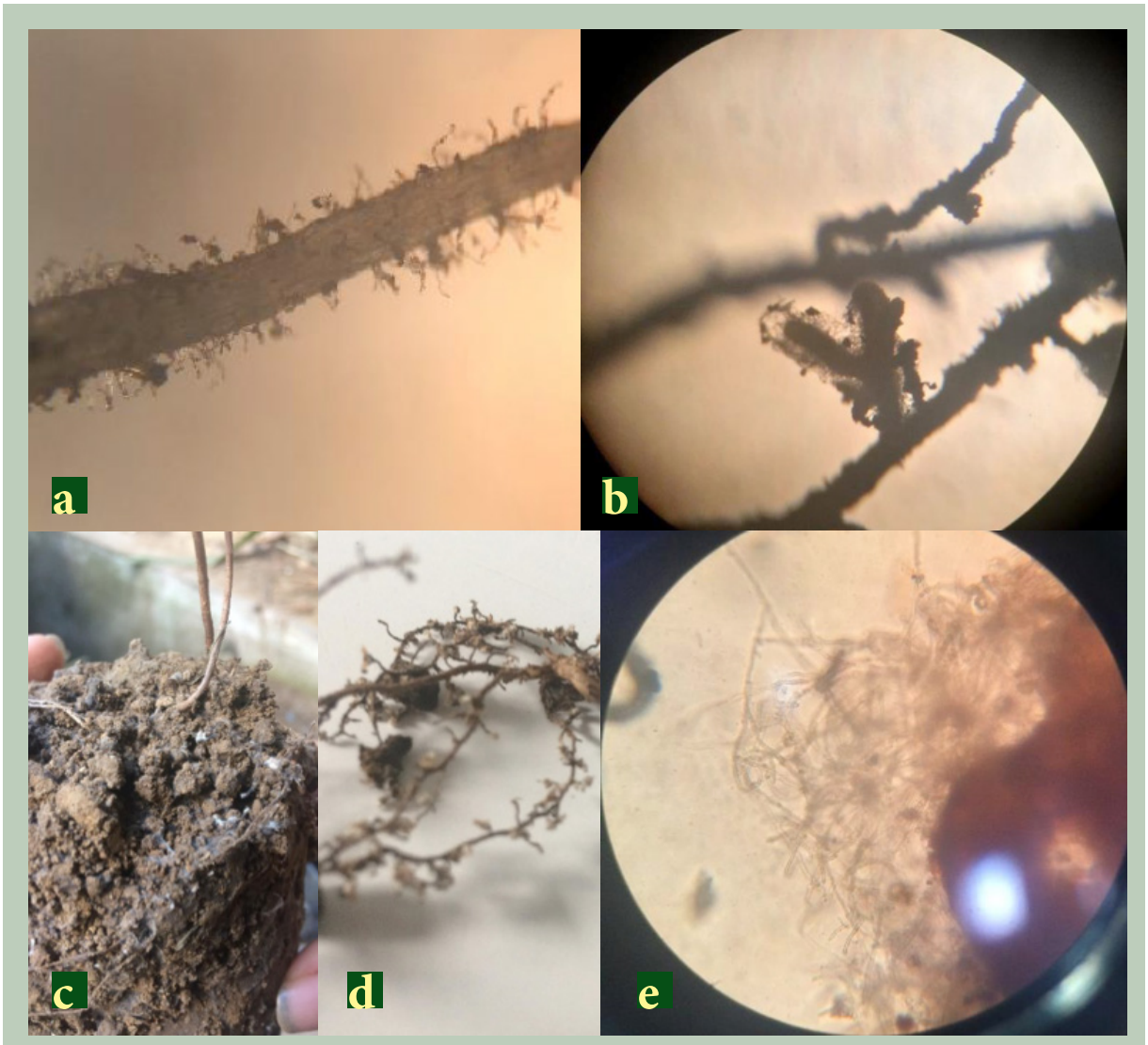


*Figure 3.* Pine roots (a) uninoculated root (10x) (b) inoculated root with dichotomously short root covered in mantle (10x) (c) inoculated root with white mycorrhizae (d) inoculated root (e) hyphae penetrating among the cortex tissue of pine (40x)

that of the uninoculated pine. Van Der Heijden et al. (2015) characterized ECM symbiosis by a distinct root shape, formation of hyphal mantle encasing root tips and mycelia extending into the surrounding soil, and fungal penetration between cortical cells.

**Mycelial Growth of *Scleroderma sp.***

The use of Murashige and Skoog (MS) medium resulted in the highest mycelial growth starting at two weeks of incubation. Such growth trend between the two media continues to differ with period (Table 3). Figures 4 and 5 show that the growth of *Scleroderma* on the MS medium was 14% higher in mycelial growth compared to *Modified Melin-Norkrans* (MMN) medium at 14 days and was 20% higher at 21 days. Higher mycelial growth leads to more seedlings inoculated, lower mortality rate resulting in more areas to reforest by planting pine trees on cleared land which leads to tree cover establishment resulting in the alleviation of carbon dioxide emission.

The difference in the amount of carbon-nitrogen (C:N) sources between the two media may have contributed to the faster mycelial growth of *Scleroderma sp.* Kim et al. (2002) concluded that

C:N ratio is an important factor affecting mycelial growth of mushroom cultures. It is possible that the C:N ratio of MS at 10:1 is enough to support faster mycelial growth of *Scleroderma sp.* as compared with MMN at 40:1. Lee et al. (2004) indicated that 10:1 is the optimum culture C:N ratio for mycelial growth of *Phellinus linteus*.

As to carbohydrate source, mycelial growth of *Scleroderma sp.* may be affected by the amount of sucrose in the medium as high as 20 g (67%) in MS when compared to MMN. According to Saha et al. (2008) sucrose containing media tend to exhibit higher mycelial growth resulting in a heavier mycelial dry weight.

Jargeat et al. (2003) found out that when nitrate is assimilated into the fungi, it is transported across the plasma membrane by a high-affinity nitrate transporter and is converted into ammonium via nitrate and nitrite reductase. Furthermore, according to Nehls (2008) nitrate is delivered directly to the fungal or plant interface without processing the mineral N, avoiding carbon drain; thus, the fungus receives additional C resulting to increased growth and greater fruit body production. Also, potassium nitrate, which is present in the MS medium was found to encourage maximum radial mycelial growth in *Alternaria carthami* by Taware, Gholve, Wagh, Kuldhar, Pawar, and Chavan (2014) while Pekşen and Kibar (2016) found out that ammonium nitrate is

Table 3

*Mean Mycelial Growth Diameter (cm) of Scleroderma sp.*

| Treatment | Mean Mycelian Growth Diameter (cm) in 7 days | Mean Mycelian Growth Diameter (cm) in 14 days | Mean Mycelian Growth Diameter (cm) in 21 days |
|---|---|---|---|
| MS | 2.493 | 4.067[a] | 5.160[a] |
| MMN | 2.387 | 3.567[b] | 4.293[b] |

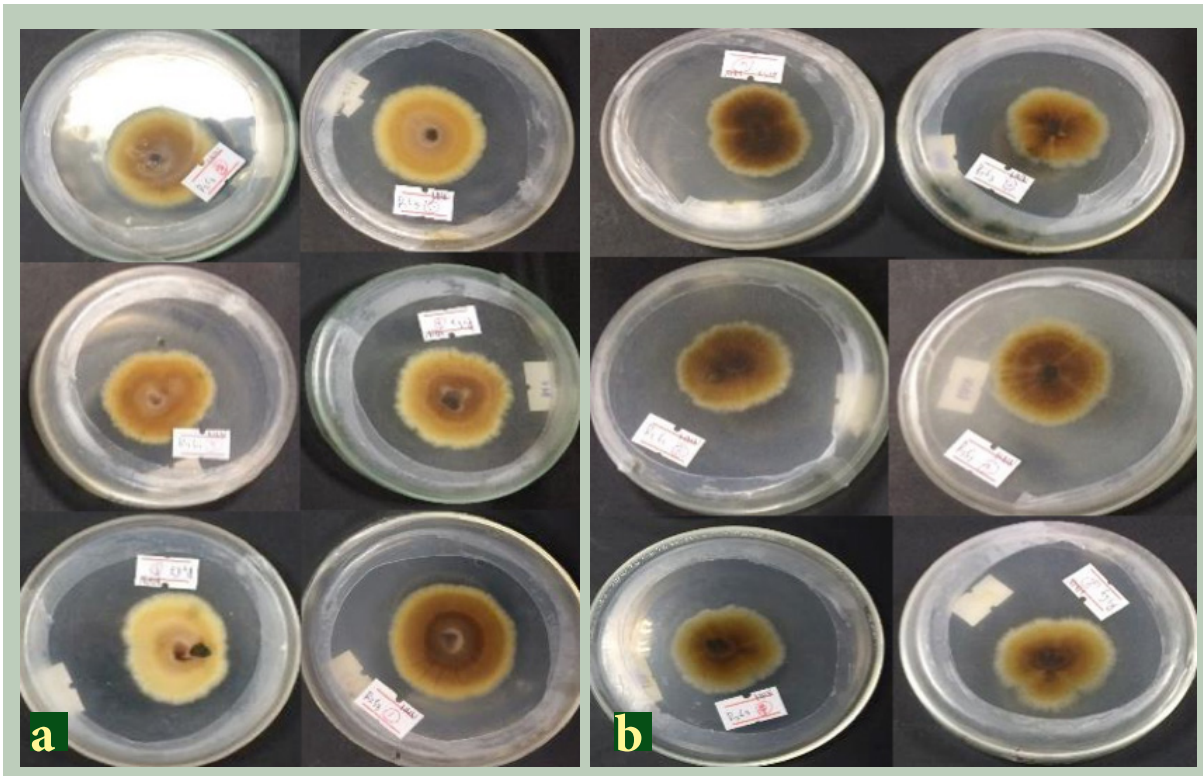


*Figure 4.* Mycelial growth of *Scleroderma sp.* on (a) MS medium at 14 days (b) MMN medium at 14 days

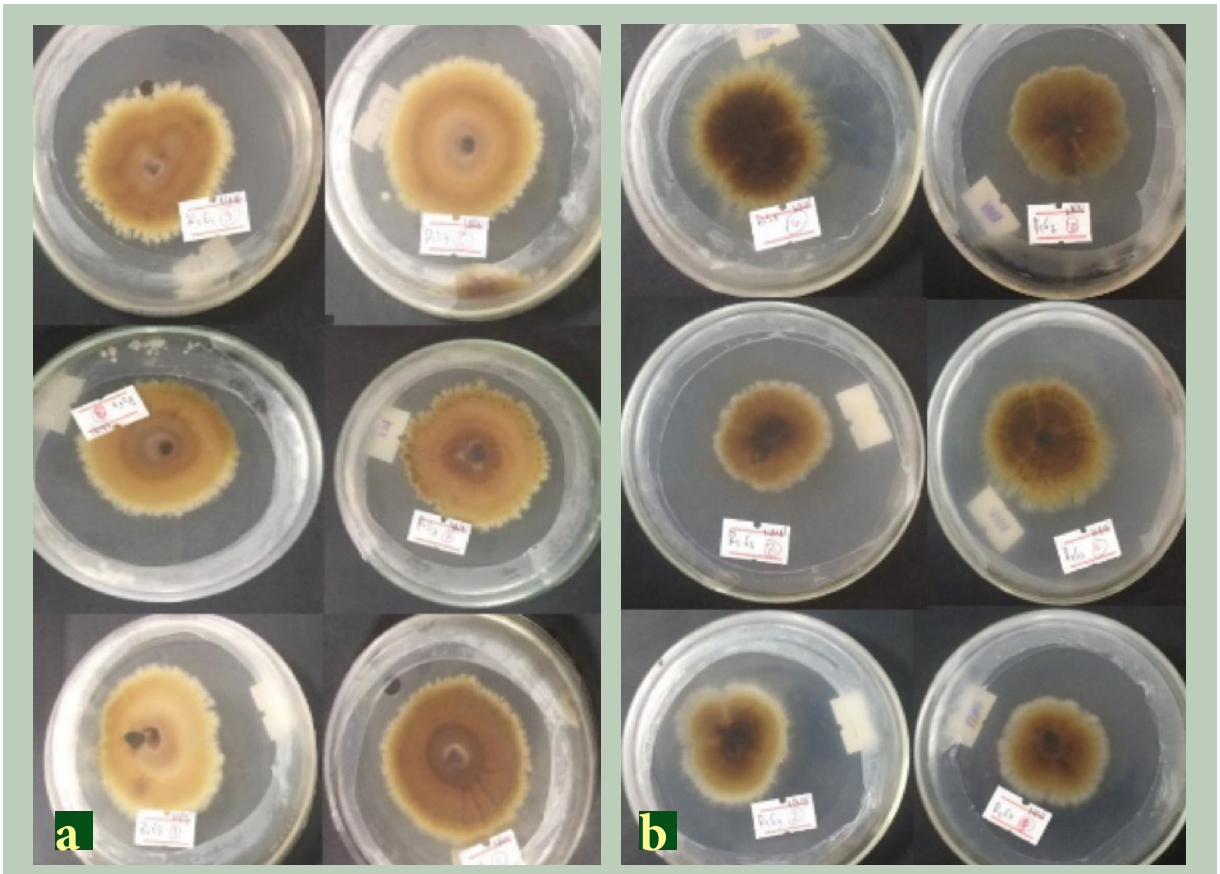


*Figure 5.* Mycelial growth of *Scleroderma sp.* on (a) MS medium at 21 days (b) MMN medium at 21 days.

good for biomass production.

In addition, Siri-in, Kunla, Suwannarach, and Lumyong (2014), found out that MS medium is one of the good media for *Scleroderma sinnamariense*. Further, Kumla et al. 2016 also found that the mycelial growth of *Pisolithus orientalis* in MS medium exhibited both the largest fungal colony diameter and biomass production.

**Mycelial Weight of *Scleroderma sp.***

After three weeks of incubation, the mycelial dry weight on MS medium was twice heavier with 700 mg as compared to MMN medium with 317 mg (Table 4). Likewise, the mycelial dry weight from the MS medium was significantly higher than that of the MMN medium. More pure cultures of *Scleroderma sp.* can be derived from the MS medium than from the MMN medium leading to more seedlings inoculated, lower mortality rate resulting in more areas to reforest by planting pine trees on cleared land which leads to tree cover establishment resulting in the alleviation of carbon dioxide emission.

Table 4

*Mean Mycelial Weight (mg) of Scleroderma sp. after 21 days of Incubation at 22°c*

| TREATMENT | MYCELIAL FRESH WEIGHT (mg) | MYCELIAL DRY WEIGHT (mg) |
|---|---|---|
| MS | 1,113 | 700a |
| MMN | 567 | 317b |

The greater amount of sucrose (20 g) in MS medium may have contributed to the difference in weight. Saha et al. (2008) concluded that MS medium containing sucrose tend to exhibit higher mycelial growth; thus, a higher fresh and dry weight.

Furthermore, Nehls (2008) concluded that sucrose breaks down into glucose and fructose,

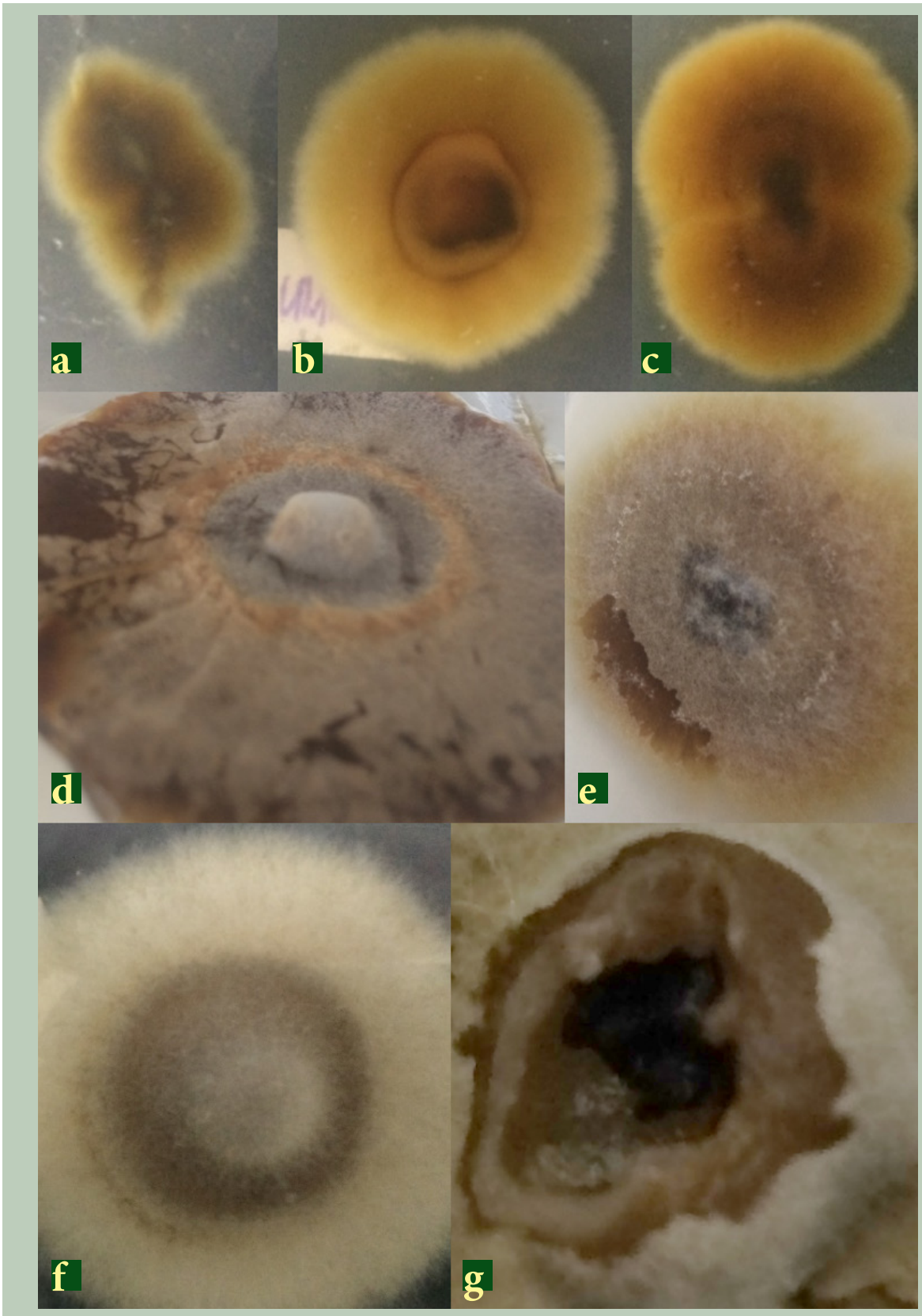


*Figure 6. Scleroderma sp.* on (a) growth from root inoculum (b) growth on MS (c) growth on MMN (d) Mycelial mat on MS (e) Mycelial mat on MMN (f) growth of fruiting body on MS (g) gleba (arrow)

which are imported by the fungal hyphae for ATP generation, amino acid biosynthesis, and formation of carbohydrate storage compounds. He further discussed that as the glucose is absorbed, fructose concentration increases, which triggers fungal physiology in symbiosis. As earlier stated in Table 3, Hamad et al. (2014) found out that during glycolysis, as the glucose is absorbed, the glucose is converted into fructose forming fructose-6-phosphate; thus, the increase of fructose, which enhances fungal growth.

In addition, it is possible that the C:N ratio of MS medium is available enough to induce fruiting body formation of *Scleroderma sp.* which added to the mycelial fresh and dry weight. Kim et al. (2002) stated that C:N ratio is an important factor affecting fruiting body formation of mushroom cultures.

## Conclusions

The technologies developed brought out the possibility of using *Scleroderma* as an ectomycorrhizal inoculant. Based on the results of the study, *Scleroderma sp.* grows faster and heavier on Murashige and Skoog medium than in *Modified Melin-Norkrans* medium. The establishment of a protocol in producing pure cultures of *Scleroderma* gives confidence towards inoculant production of the mushroom to be used in pine tree reforestation.

## Recommendations

Based on the findings, it is recommended that further studies be conducted to determine the best optimal physical growth conditions of *Scleroderma sp.*, substrates for inoculant production and inoculation protocol on pine seedlings and testing the same media on other ectomycorrhizal fungi.

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