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FORESTRY

RESEARCH NOTE

Effect of the geographical origin, culture media, and pH on the growth dynamic of the edible ectomycohrrizal mushroom *Suillus luteus*

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Abstract

R. Santelices, S. Espinoza, N. Brunel, and G. Palfner. 2012. Effect of the geographical origin, culture media, and pH on the growth dynamic of the edible ectomycohrrizal mushroom *Suillus luteus*. Cien. Inv. Agr. 39(2): 369-376. *Suillus luteus* is the most important wild edible ectomycorrhizal mushroom harvested and exported in Chile. This introduced species forms mycorrhizal symbiosis with *Pinus radiata*, the most important exotic forest tree. To obtain optimized protocols for the controlled infection of *P. radiata* trees with this species, it is important to determine the appropriate culture conditions. Therefore, we studied the effect of the geographical origin (three localities from the Maule Region of Chile), culture media (Modified Melin and Norkrans (MMN) and Malt Extract Agar (MEA) 2%), and pH (5.0, 5.8, and 6.5) on the *in vitro* growth dynamics of *S. luteus* over 37 days. The results showed significant differences in the growth rate and colony diameter as a consequence of its geographical origin. However, no differences were found for either pH or culture medium. Our results confirm that a non-specific, economical culture medium, such as MEA 2%, may be used to obtain a suitable amount of mycelium for both medium- and large-scale assays of inoculation with this species.

Keywords: Ectomycorrhizal, edible fungi, fungi, *Pinus radiata*, *Suillus luteus*.

Introduction

Mycorrhizal fungi are primarily known as a natural form of fertilization because they improve the mobilization and absorption of nitrogen and phosphorus in different tree species; they also help to reduce the biotic and abiotic stresses, such as those caused by pathogens or drought (Smith and Read, 2008). This effect is reciprocated by the tree to the fungus in the form of carbohydrates that it cannot synthesize, thus creating a mutual relationship in which ectomycorrhizal fungi contribute to several ecosystem functions, including the carbon cycle, the mobilization of mineral soil nutrients and by connecting the trees through common mycorrhizal networks (Courty *et al*., 2010). In addition, edible mycorrhizal fungi have a high social and economic value to rural communities by generating sources of food and work (Barroetaveña *et al*., 2008). Similar to many fungi, *Suillus luteus* (L.) S. F. Gray is characterized by genetic variations among populations, especially when growing in soils

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contaminated with heavy metals (Colpaert *et al*., 2000; Muller *et al*., 2007). Therefore, the source of fungal material can play an important role in the development of the species and, consequently, in the symbiotic association. In this context, an appropriate selection of symbiont mycorrhizal fungi species and their subsequent handling, in both the laboratory and the nursery, can be a key aspect for the successful establishment of many species in the field (Honrubia *et al*., 1992). The ecological requirements may vary among members of the same fungal species, and the reaction to culture media may differ, meaning that the culture medium pH may play an important role in fungal development. Aside from influencing the distribution of the mycorrhizal fungi population, it also affects the availability of nitrogen, phosphorus, calcium and magnesium, among others (Bockheim, 1991; Smith and Read, 2008). Consequently, the pH of the culture media may also affect the growth of ectomycorrhizal fungi (Zak, 1973), and each species has an optimum pH level for both *in vitro* growth and to establish symbiosis with the roots of its host (Hung and Trappe, 1983). Several previous studies have investigated the role of pH and suggested an ideal range from acidic to slightly alkaline levels (Hung and Trappe, 1983; Pereira *et al*., 2007; Chávez *et al*., 2007). For example, a strain of *S. luteus,* sampled from a sandy loam soil of the Biobío Region (Chile), was grown in BAF culture media adjusted to a pH between 4.8 and 7.8 and displayed a greater rate and area of *in vitro* growth at pH 4.8 and 5.8 (Pereira *et al*., 2007). Although the *in vitro* culture of species of *Suillus* is simple and does not require expensive or complex culture media that other ectomycorrhizal fungi require (Curguz *et al*., 2010), a large amount of inoculum is required to carry out mycelial inoculations over a greater number of plants of interest. It is therefore essential to optimize the *in vitro* culture system and understand the specific development of the fungus in these conditions to obtain the maximum amount of mycelium possible within a given time and with minimal resources.

The objective of this study was to examine, under controlled laboratory conditions, the effect of the geographical origin of fruiting bodies, the culture medium, and the pH of the culture medium on the *in vitro* growth of *S. luteus*, the most harvested wild ectomycorrhizal fungal species in Chile (which accounts for approximately 90% of fungal exports) and to determine optimized protocols for the controlled large-scale infection of *Pinus radiata* D. Don, the most important forest species in the country.

Materials and methods

Fungal material, growth conditions and testing

Basidiomes that were fresh, healthy and smaller than 3 cm were collected from different geographical origins from plantations of *P. radiata* between 12 and 18 years in age located near the towns of Empedrado (35°33' S; 72°11'W), Chanco (35°36' S; 72°31'W) and Gualleco (35°15' S; 71°57'W) in the Cordillera de la Costa of Maule Region (Table 1).

Table 1. Geographic location and general climatic and soil features of the collection sites (Santibáñez and Uribe, 1993).

Site collection (Town)	Location (geographic coordinates)	Soil acidity (pH)	Average annual temperature (°C)	Average annual rainfall (mm)	Average rainfall in April (mm)
Las Risqueras (Empedrado)	35°33' S; 72°11'W	5.1-5.5	13.3	926	51.2
San José (Chanco)	35°36' S; 72°31'W	5.1-5.5	11.7	837	46.8
Gualleco (Gualleco)	35°15' S; 71°57'W	5.6-6.0	13.9	709	36.7

At least 50 basidiomes of each geographical origin were collected from the field. Once collected, the fruiting bodies were quickly moved into coolers for transport to the laboratory. For laboratory cultivation, ten basidiomes of each geographical origin were selected, soil particles and plant material were removed and the basidiomes were cut in half. Under sterile conditions in a ESCO® laminar flow cabinet (ESCO, Canada), approximately cubic pieces of a few millimeters in length were cut from the tissue located immediately above the hymenium, a dormant growth area, using a sterilized scalpel. The pieces were placed into petri dishes containing Modified Melin and Norkrans (MMN) medium, adjusted to pH 5.8 (Marx, 1969) and incubated until an active growth of mycelia was obtained (stock cultures). Subsequently, mycelial discs of approximately 5 mm in diameter were chosen randomly from the stock culture and transferred into new petri dishes containing culture medium MMN and Malt Extract Agar 2% (MEA 2%) at pH 5.0, 5.8 and 6.5. These petri dishes were incubated at a temperature of ± 24ºC for a period of 37 days in a Memmert™ incubator (Memmert, Germany). To evaluate colony development, the diameter (mm) of the colony was measured every three days until the end of the trial (37 days) using Mitutoyo™ digital calipers (Mitutoyo Corporation, Japan). The average rate of growth (mm day^{-1}) was then calculated according to Santiago-Martínez *et al*. (2003).

Data analysis and experimental design

The experiment consisted of five repetitions of all three treatments (geographical origin, medium, and pH of the culture medium). The data were analyzed using analysis of variance, and when significant differences were found, Tukey's test was used. The General Linear Model procedure (GLM) of the statistical program SPSS V. 18 was used for analysis (SPSS Inc., Chicago, Illinois, USA).

Results

Average growth rate

Significant differences in the growth rate were only observed when comparing the geographical origin of the fungal material (Figure 1); San José colonies differed from colonies from Gualleco and Las Risqueras. It was also noted that the strains of Las Risqueras, although they took approximately three to four days to form the first hyphae, recovered starting from day 13, and the final growth rate was greater than the strains from other sources. The early growth of strains from San José was faster, forming hyphae within two to three days; however, toward the end of the experiment, the strains had achieved a smaller colony diameter. Additionally, neither the culture medium nor the pH influenced the growth rate of the *S. luteus* colonies (Figure 1), with an average growth rate of 1.09 mm day^{-1} for both culture medium and pH.

Average growth area

The results showed that there were significant differences in the final colony diameter between the cultures from the origins of Gualleco and Las Risqueras compared with San José (close to the coastal range). However, no significant differences in the average growth area were observed as a result of differences in the nutrient medium or pH (Figure 2). The average values were relatively similar, ranging from 37.8 to 41.6 cm^2 for the pH levels studied and from 39.8 to 40.5 for the two culture media.

Discussion

Populations of *S. luteus* can be genetically differentiated according to the environmental conditions where they develop (Colpaert *et al*., 2000; Muller *et al*., 2007). In this study, strains from San José, a more coastal environment, displayed less efficient growth than strains from

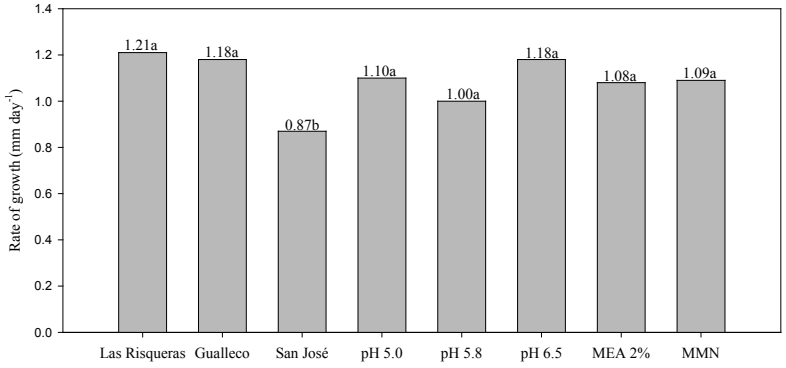

Figure 1. Effect of the geographical origin, pH, and culture media on the rate of growth of *Suillus luteus*. Mean values with the same letter are not significantly different, P>0.05. (MMN: Modified Melin Norkrans, MEA 2%: Malt Extract Agar 2%).

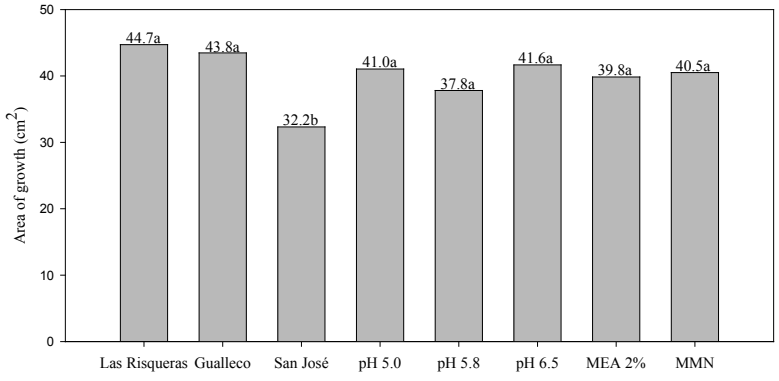

Figure 2. Effect of the geographical origin, pH, and culture media on the final area growth of *Suillus luteus*. Mean values with the same letter are not significantly different, P>0.05. (MMN: Modified Melin Norkrans, MEA 2%: Malt Extract Agar 2%).

Gualleco and Las Risqueras, which suggests that edaphoclimatic conditions play an important role in the growth of *S. luteus*. Significantly, the climatic characteristics of the Gualleco and Las Risqueras origins differed from those of the San José origin, a coastal area with different rainfall patterns, possibly affecting the observed results. The three geographical origins have differences in both temperature and rainfall (Santibáñez and Uribe, 1993). When Ruiz-Diez *et al.* (2006) analyzed *S. luteus* from different geographical areas in Spain (north, southeast, northwest and central regions), they found that strains from the north had the largest colony diameter.

Numerous studies have highlighted the importance of culture medium on *in vitro* fungi development (Hung and Trappe, 1983; Brundrett *et al*., 1996; Vaario *et al*., 2002). However, the results reported by several authors for different species of *Suillus* are inconsistent. For example, Torres and Honrubia (1991) found a correlation between culture medium and growth for six types of fungi (*S. collinitus*, *S. granulatus*, *Rhizopogon roseolus* (Corda) Th Fr, *Rhizopogon luteolus* Fr. & Nordholm, *Amanita muscaria* (L.: Fr) Lam., *Lactarius deliciosus* (L.) Fr.) grown in different culture media (MMN, MMN + glucose, MEA 2%, PDA, Raper and Hagem), with a pH range between 5.5 and 7.5. They determined that *S. collinitus* and *S. granulatus* displayed the most efficient growth in PDA and MMN media, respectively. These results are in agreement with results from Coleman *et al*. (1989), who observed the largest colony diameter for several species of

the genus *Suillus* (including *S. luteus*) in MMN medium. The results observed in this study are also similar to those reported by Sánchez *et al*. (2000), who observed a final colony diameter of 4 cm of *S. luteus* from Spain cultivated in MMN. Torres and Honrubia (1991), Coleman *et al*. (1989), and Curguz *et al*. (2010) observed the largest colony diameter for different species of *Suillus*, including *S. luteus*, when the strains were grown in MMN. Ruiz-Diez *et al*. (2006) observed an average colony diameter in the range of 30 to 40 cm^2 for strains of *S. luteus* grown in MMN medium, which is slightly lower than the results obtained in this study.

The pH of the culture medium has been shown to be an important factor in the development of mycorrhizal fungi, although the results observed are variable (Torres and Honrubia, 1991; Sánchez *et al*., 2000; Pereira *et al*., 2007). In this study, the *in vitro* growth of strains of *S. luteus* responded similarly to the different values of pH tested (5.0, 5.8 and 6.5), which is inconsistent with the results from other studies. For example, Pereira *et al*. (2007) and Chávez *et al*. (2007) found that *S. luteus* displayed efficient growth in MMN and BAF media (biotin-aneurin-folic acid-agar) that were adjusted to pH values between 4.8 and 5.8. Curguz *et al*. (2010) obtained the same result for MMN and MEA media at pH 6.0. Torres and Honrubia (1991) collected six species of ectomycorrhizal fungi from *Pinus spp*. forests in Spain and found no differences in the colony diameter for *S. collinitus* and *S. granulatus* when the pH of the culture medium was varied. Taken together, the results from previous studies suggest that there is a relationship between pH and increases in colony diameter for *S. collinitus* but that the results are more variable for *S. granulatus*; the greatest mycelium development was obtained at a pH value between 5.5 and 7.5. Sánchez *et al*. (2000) observed, a final colony diameter of *S. luteus* grown in MMN medium similar to the results observed in this study. In a similar study involving strains of *Rhizopogon* spp., Vázquez-García *et al*. (2002) observed optimal growth at pH 6.0, with an average rate of 1.28 mm day^{-1}. The differences seen in the studies described above could be caused by aggressive strains colonizing the medium by the pH of the site where the fruiting bodies grew or by the laboratory growth conditions, which may be altered by factors such as temperature and light.

The differences observed in these studies suggest that a wide range of strains of different sources should be tested before large-scale inoculum production is initiated because the geographical origin, soil pH and natural hosts may significantly affect the culture. The findings of this study indicate that *S. luteus* from all three geographical origins grew the same under all conditions tested and suggest that samples from several provenances should be isolated to determine which has the best growth and which should be used at an operational level in forest nurseries.

The typical growth pattern of *S. luteus* (lag phase, exponential growth, *plateau*, and finally growth cease) could not be observed; however, a phase of exponential growth was observed, which was followed by a phase of slow growth at approximately day 35 before growth arrested. This may be because the colonies originated from a stock culture free of contaminants that could eventually affect the normal development of the colony. Coleman *et al*. (1989) reported the same exponential growth model without the lag phase, but when they grew the colonies under water stress (with polyethylene glycol in the culture media), they observed a lag phase followed by an exponential growth phase, and finally, a *plateau* until the colony inactivated its growth. In this study, there was a slight lag phase between days 10 to 15, but after this time and until day 35, the colony continued to grow exponentially. The analyses of the pH values of culture media show that colonies from all three origins cultivated grew well in the nutrient media because the pH levels of culture media were similar to those recorded in the soils where they naturally grow. This finding indicates that the pH conditions of the sample collection

area of fungal material should be considered to optimize the culture and propagation of the fungi in the laboratory and improve the production of mycorrhization in nursery plants. It should be noted that the climatic characteristics of the site of geographical origins might have some influence on the observed results.

The pH of the culture medium had no significant effect on the *in vitro* growth of the vegetative mycelium of *S. luteus*. Of the three geographical origins studied, colonies from Las Risqueras displayed the greatest growth. The culture medium had no influence on the rate of growth of this species, indicating its potential for cultivation in a more economic medium, such as Malt Extract Agar 2% (MEA 2%).

Resumen

R. Santelices, S. Espinoza, N. Brunel y G. Palfner. 2012. Efecto de la procedencia, del medio de cultivo, y del pH, en la dinámica de crecimiento del hongo ectomicorrícico comestible *Suillus luteus*. Cien. Inv. Agr. 39(2): 369-376. *Suillus luteus* es la especie silvestre de hongo micorrícico comestible más cosechada en Chile, que tiene una importancia económica y silvícola. Es una especie silvestre introducida, que se asocia con *Pinus radiata*, la especie forestal económicamente más importante del país. Si se quisieran desarrollar metodologías de infección controlada en árboles de *P. radiata* con este hongo ectomicorrícico, es importante estudiar condiciones apropiadas para su cultivo. Por ello, se estudió el efecto del origen geográfico (tres localidades de la Región del Maule de Chile), del medio de cultivo (Melin y Norkrans modificado (MMN) y extracto de malta agar (MEA) al 2%), y del pH (5,0; 5,8; y 6,5), en la dinámica de crecimiento *in vitro* de *S. luteus* durante 37 días. Los resultados indicaron diferencias significativas en la velocidad de crecimiento y diámetro de la colonia, como consecuencia de la procedencia del material fúngico. Sin embargo, no hubo diferencia tanto en el medio de cultivo como en el pH. Esto confirma, para las cepas chilenas analizadas, que es posible emplear un medio de cultivo económico como MEA 2%, con el propósito de producir una cantidad adecuada de biomasa miceliar para futuros programas de micorrización con esta especie.

Palabras clave: Ectomicorrícico, hongo, hongo comestible, *Pinus radiata*, *Suillus luteus*.

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