In vitro GROWTH OF ECTOMYCORRHIZAL FUNGI ASSOCIATED WITH *Pinus radiata* PLANTATIONS IN CHILE

CRECIMIENTO *in vitro* DE HONGOS ECTOMICORRÍCICOS ASOCIADOS CON PLANTACIONES DE *Pinus radiata* EN CHILE

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SUMMARY

A comparative study of *in vitro* growth of three species of ectomycorrhizal fungi (ECMF) (*Rhizopogon luteolus*, *Suillus granulatus* and *Suillus luteus*) was performed. Fungal material was collected in adult *Pinus radiata* plantations. Isolation and purification of the strains were performed on potato-dextrose-agar medium and the evaluation of the radial growth rate and the increase in mycelial biomass, under different culture conditions, was performed on the Modified Melin Norkrans growth medium. The effects of temperature (24, 28 and 32 ºC) and pH (4.8, 5.3, 5.8, 6.3 and 6.8) of the growth medium were tested for the three fungal species in two independent assays. The results indicate that the temperature had a significant effect on the radial growth rate (RG) and mycelial biomass increase (MB) in all of the evaluated fungal species. The highest RGR and MBI were recorded in *R. luteolus*, and the lowest values for these variables were registered in *S. luteus*. *Rhizopogon luteolus* had the highest sensitivity to pH changes. Meanwhile, there was no pattern in *S. granulatus* and *S. luteus* growth response under different pH conditions. When cultivated *in vitro*, the three studied species of ECMF presented adaptation, exponential, declining and stationary growth phases. The *in vitro* growth responses recorded in the present study showed the great potential of *R. luteolus* to be used in future programs using mycorrhizal inoculation in the production of *Pinus radiata* trees in nurseries in Chile.

Index words: *Rhizopogon, Suillus*, biomass, pure culture, radial growth rate.

RESUMEN

Se hizo un estudio comparativo del crecimiento *in vitro* de tres especies de hongos ectomicorrícicos (ECMF) (*Rhizopogon luteolus, Suillus granulaus* y *Suillus luteus*). El aislamiento y purificación de las cepas se hizo en medio de cultivo papa-dextrosa-agar (PDA), y la evaluación de la velocidad de crecimiento radial (RG) y del incremento en biomasa micelial (MB), bajo diferentes condiciones de cultivo, se hizo en el medio de crecimiento Melin Norkrans Modificado (MMN). Los efectos de la temperatura (24, 28 y 32 ºC) y pH (4,8, 5,3, 5,8, 6,3 y 6,8) del medio de crecimiento fueron probados para las tres especies fúngicas en dos ensayos independientes. Los resultados indican que la temperatura tuvo un efecto significativo en la tasa de crecimiento radial (RG) y en el ncremento de biomasa micelial (MB), en todas las especies fúngicas evaluadas. Los más altos valores de GR y MB fueron registrados en *R. luteolus*, y los valores más bajos fueron registrados en *S. luteus*. *Rhizopogon luteolus* presentó la mayor sensibilidad a los cambios de pH del medio de cultivo. En cambio, no existió un patrón en la respuesta de crecimiento de *S. granulatus* y *S. luteus* bajo diferentes condiciones de pH. Cuando se cultivaron *in vitro*, las tres especies de ECMF estudiadas presentaron en su crecimiento micelial las fases de adaptación, crecimiento exponencial, declinación y estacionaria. Las respuestas de crecimiento *in vitro* registradas en el presente estudio mostraron el gran potencial de *R. luteolus* para ser utilizado en futuros programas de micorrización controlada para la producción en vivero de árboles de *Pinus radiata* en Chile.

Palabras clave: *Rhizopogon, Suillus*, biomasa, cultivo puro, tasa de crecimiento radial.

INTRODUCTION

Around 95 % of higher plants naturally establish mutualistic symbiotic relationships with mycorrhizal fungi whose external mycelium can explore up to 1000 times more soil than the roots (Brundrett *et al.*, 1996; Honrubia *et al.*, 1992). Therefore, this symbiosis improves the nutrition of the associated plants by mobilizing nutrients and water (Honrubia *et al.*, 1992; Quoreshi, 2008; Skinner and Bowen, 1974). Quality of plants successfully mycorrhized by ectomycorrhizal fungi (ECMF) is improved in terms of vigor, drought tolerance, increase in nutrient uptake and resistance to post-transplant stress in the field (Bücking *et al.*, 2012; Dulmer *et al.*, 2014). The inoculation with ECMF is a recommended practice to produce plants of high quality in nurseries (Díaz *et al.*, 2009).

The selection of the appropriate ectomycorrhizal fungal species as symbionts and their subsequent manipulation, both in the laboratory and in the nursery, can be a key factor for the successful establishment of many tree species in the field (Chávez *et al.*, 2007; Honrubia *et al.*, 1992; Marx *et al.*, 1991). One criteria of great relevance in the selection of the ECMF is the use of species which

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are growing naturally associated with the selected plants in the sites of interest (Honrubia *et al.*, 1992; Pereira *et al.*, 2007). Different types of mycorrhizae have been described depending on the structures that they form, although in the case of tree species relevant in forestry in temperate climates, the ectomycorrhiza is the most important type of mycorrhizae (Harley and Smith, 1983; Marks and Kozlowski, 1973; Smith and Read, 2008).

The ECMF is an important component of microbial soil communities in boreal, temperate, mediterranean and some tropical forests, where they play an important role in nutrient recycling processes (Allen *et al.*, 1995). In nature, ECMF growth depends on factors such as temperature, pH, humidity, physical and chemical soil properties and nutrient availability, mainly carbon and nitrogen sources (Bowen, 1973; Harley and Smith, 1983). The ECMF are usually mesophilic; the temperature directly influences their growth because it affects the enzymatic production; and thus, can cause the denaturation of proteins and other macromolecules of the cell membranes (Frioni, 1999). Additionally, the soil pH affects the microbial activity through the solubility and the ionization of the inorganic and organic compounds in the soil solution (Voroney, 2007).

In vitro pure culture studies contribute to the study of the ECMF in order to understand their behavior in different cultivation conditions (Pirt, 1975). Growth differences of ECMF under *in vitro* conditions have been considered a guide for prescreening potential fungal species (strains or ecotypes) to be used in nursery mycorrhization programs. In the present study, the biomass production in different *in vitro* conditions was analyzed in order to understand the optimal temperature and pH conditions for the evaluated ECMF (Eng *et al.*, 2003; Lazarević *et al.*, 2016; Pereira *et al.*, 2007; Sánchez *et al.*, 2001). The objective of the present study was to analyze the effect of three temperatures and five different pH levels on the *in vitro* growth of the species of ECMF *R. luteolus*, *S. granulatus* and *S. luteus* naturally associated with *Pinus radiata* plantations growing at low productivity sites in Chile, assuming that each strain or ecotype has its own ecological optimal conditions.

MATERIALS AND METHODS

Fungal material. The ectomycorrhizal fungi (ECMF) *Rhizopogon luteolus* Fr., *Suillus granulatus* (L.) Roussel and *Suillus luteus* L. Gray were harvested in an adult plantation of *Pinus radiata* D. Don growing on a representative site of a large forest area with sandy soils of low productivity (pH 5.6; organic matter: 2.39 %; N:1.3 %; P:6.7 %; K:47.9 %) in the province of Biobío, Region VIII, Chile (37º 20'42.58 "S, 72º 17'55.15 "W) during June to September 2015 (Figures 1A, B, C). The species identification was performed by using a macro- and micro-morphological characterization of their sporomes (Calonge, 2009; Gerhardt *et al.*, 2000; Honrubia *et al.*, 2010; Lazo, 2016). In laboratory, under aseptic conditions in a laminar flow cabinet (ESCO®, USA) the sporomes were dissected and small parts of the pilea context located immediately above the hymenium in epigeous mushrooms; and in the central area of the gleba in hypogeal fungi, were respectively extracted (Brundrett *et al.*, 1996; Honrubia *et al.*, 1995; Kumar y Satyanarayana, 2002).

The tissue fragments were placed in 50 mm diameter Petri dishes with 10 mL of potato-dextrose-agar medium (HiMedia®, India) with pH adjusted to 5.8. For each species, five strains were collected from the collection site, and from them, the strain with the best initial growth was studied in detail. The plates were incubated at 24 ± 1 ºC (BINDER® incubator, Germany) until an active growth of the mycelia (stock cultures) was obtained. Later, agar-mycelial discs of 5 mm in diameter of the margin of the isolated strains (Díaz *et al.*, 2009), were transferred to new Petri plates with 20 mL of potato-dextrose-agar medium and pH adjusted to 5.8, in order to complete the processes of isolation and purification. These plates were incubated at 24 ± 1 ºC for 18 d, obtaining pure strains of the three ECMF species of *R. luteolus*, *S. granulatus,* and *S. luteus* (Figures 1A, B, C).

Experimental approach. Two independent experiments were set up. In the first experiment, the effect of the temperature was evaluated. The three ECMF strains were cultivated at pH 5.8 and the effect of three different temperatures was evaluated. In the second experiment, the effect of pH on mycelial growth was evaluated for the three isolated strains. The strains were cultivated at 24 ºC and the effect of five different pH values was evaluated.

Culture conditions tested. In the first experiment, the evaluated temperatures were 24, 28 and 32 ºC (Andrino *et al.*, 2011; Arana-Gabriel *et al.*, 2014; Bran *et al.*, 2015; Lazarević *et al.*, 2016). From growing active colonies (stock cultures), 5 mm diameter agar-mycelial discs were transferred to 90 mm diameter Petri dishes containing 20 mL of agar Modified Melin Norkrans (MMN) as a culture medium, with a pH adjusted to 5.8. This pH value was used because it has been successfully employed in the cultivation of other ECMF. Five plates per species and three temperature values were set up (n = 45). Cultures were grown in three separate incubators (Memmert®, Germany). In the second experiment, five different pH values were evaluated: 4.8, 5.3, 5.8, 6.3 and 6.8 (González *et al.*, 2015; Pereira *et al.*, 2007; Sundari and Adholeya, 2003; Vázquez-García *et al.*, 2002). Prior to sterilization, the pH adjustment of the culture medium was performed with a PH 21 Hanna Instruments electrode by applying HCl to acidify the medium and KOH to basify

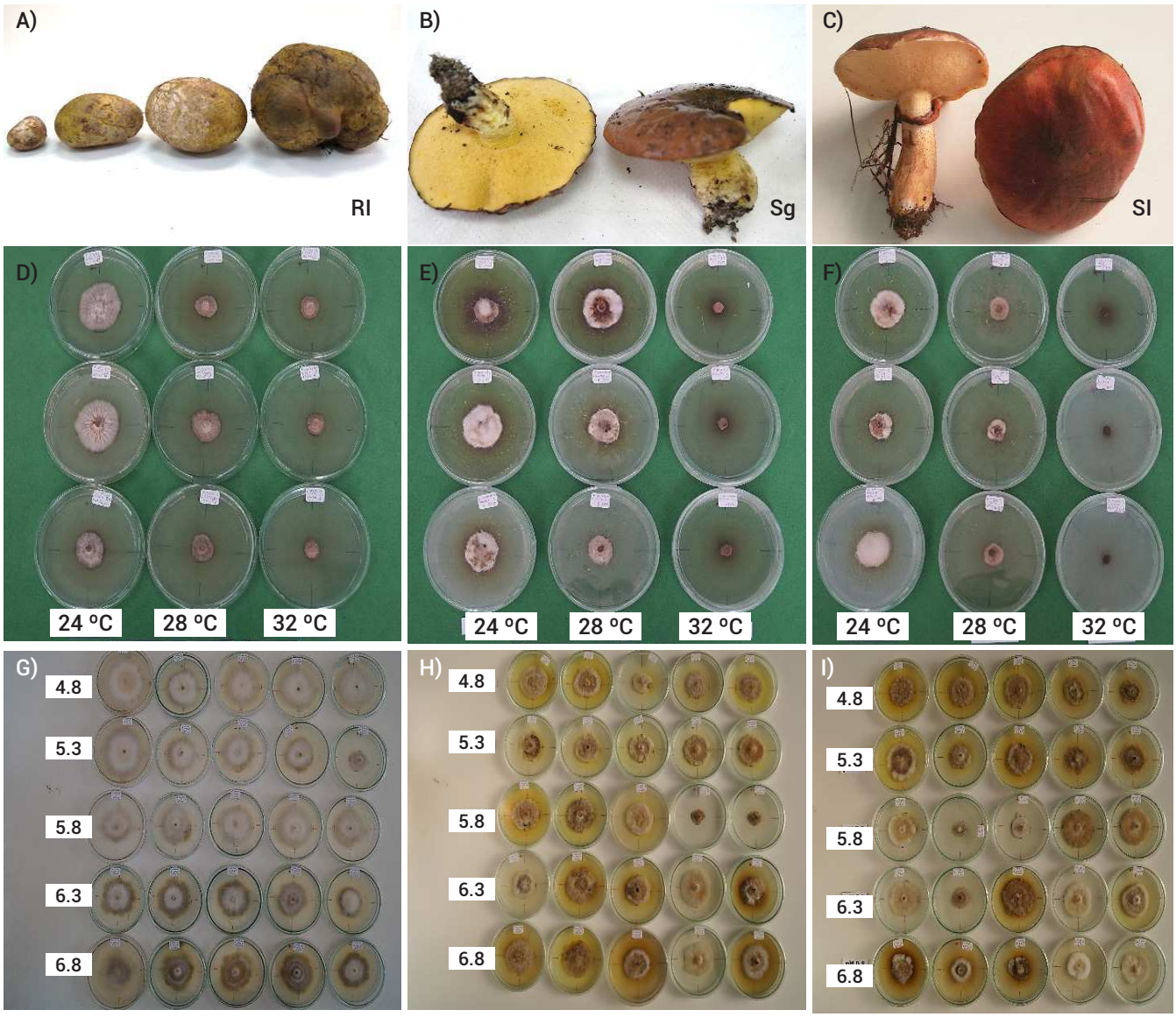

Figure 1. Sporomes of the ectomycorrhizal fungi *Rhizopogon luteolus* (A), *Suillus granulatus* (B), *S. luteus* (C) and *in vitro* mycelial growth at three temperatures (D, E and F) and five pH values (G, H and I) in Modified Melin Norkrans culture medium.

it. In this experiment, 5 mm diameter agar-mycelial discs were transferred to 90 mm diameter Petri dishes containing 20 mL of agar MMN culture medium, with each level of pH tested. Five plates for each pH value were set up (n = 75), which were incubated in darkness at 24 ± 1 ºC based on the fact that this was the optimal temperature in terms of fungal growth in the first experiment.

Growth traits. The average radial growth (RG) of the evaluated ECMF strains was recorded for each of the evaluated temperatures and pH values. The plates were incubated for 21 d and the radial growth of the colonies was measured every 3 d on the back of the plates (in four directions that passed through the center of the colonies) with a digital caliper (Mitutoyo, Mod. CD-6, Japan) (Murrieta-Hernández *et al.*, 2014). The non-accumulated radial growth (NARG) was the average of the growth distance of four radial points for each measurement, to which the average growth of previous measurements was not added.

These data were adjusted by a linear regression equation to calculate the slope of the growth curve corresponding to the average growth rate of each fungal species and expressed in mm d^{-1} (Santiago-Martínez *et al.*, 1995; Vázquez- García *et al.*, 2002). After the culture period, the increase in mycelial biomass (MB) was evaluated for both experiments by recovering the biomass produced in Petri dishes with vacuum filtration (Filtering Pump K, model SU

660, Germany). The filtering of the growth medium previously melted in a microwave was carried out by using filter paper, previously oven dried and weighed in an analytical balance (RADWAG®, USA). The mycelial mass retained on the filter paper was oven dried (Memmert, model BE-400, Germany) at 60 ºC during 48 h in order to determine the constant dry weight (Duñabeitia *et al.*, 2004; Pereira *et al.*, 2014).

Statistical analysis. In every experiment, a completely randomly statistical designs with factorial arrangements, 3x3 in the first experiment and 3 x 5 in the second experiment, was used. In the first experiment the factors were: ECMF species and culture temperatures, giving a total of 45 experimental units. In the second experiment, the factors were the ECMF species and the pH of the culture medium, giving a total of 75 experimental units. In order to determine the effect of the studied factors on the RGR and MB, the tests of homogeneity of variances and normality through the Shapiro-Wilk and Levene Test were previously performed. When the distributional assumptions were not verified, Box-Cox powers for data normalization (Olivier and Norberg, 2010) were applied. In both experiments a two-way analysis of variance was performed with a 95 % confidence level and because the interaction was significant, contrasts were performed by LSD Fisher ($\alpha = 0.05$) for the analysis of significant differences. Tukey ($P < 0.05$) mean comparison tests (Steel and Torrie, 1989) were carried out among treatments. Statistical analysis of the data was performed by using the statistical program STATGRAPHICS®.

RESULTS

Experiment I (temperature)

Radial growth (RG). The results showed that the temperature had a significant effect on the RG for the three ECMF evaluated species, with a negative effect when this variable increased (Figures 1D, E and F; Figure 2A). The highest RG was obtained at 24 ºC and the lowest at 32 ºC, regardless of the ectomycorrhizal fungi (ECMF), having differences ($P < 0.05$) among species. It was observed that *R. luteolus* and *S. luteus* had greater sensitivity to the temperature change, expressed in the differences between their treatments ($P < 0.05$). *Rhizopogon luteolus* had the highest RG in the three evaluated temperatures being 2.49, 1.81, 1.10 mm d^{-1}, respectively. These RG were different ($P < 0.05$) to those recorded for *S. luteus* and *S. granulatus* in the three evaluated temperatures. The last two species did not show differences ($P < 0.05$) in RG between them, independently of the tested temperatures.

Mycelial biomass (MB). The MB of the three evaluated ECMF was also affected by the tested temperatures (Figure 2B). The highest ($P < 0.05$) MB was registered at 24 ºC and the lowest at 32 ºC for all species. *Rhizopogon luteolus* showed the highest sensitivity, expressed as differences ($P < 0.05$) between each of the tested temperatures. *Suillus granulatus* and *S. luteus* only showed differences ($P < 0.05$) between extreme temperatures (24 and 32 ºC). *Rhizopogon luteolus* had the highest MB with 143.5, 101.8 and 60.7 mg, and *S. luteus* had the lowest MB with 28.5, 17.3 and 1.9 mg for the three evaluated temperatures, respectively. At the most extreme temperatures (28 and 32 ºC) there were greater MB differences ($P < 0.05$) in the three ECMF.

Experiment II (pH)

Radial growth (RG). The pH had a different effect on the RG of the evaluated fungal species, and there was not a defined pattern (Figures 1G, H, I, and Figure 3A). *Rhizopogon luteolus* reached its maximum ($P < 0.05$) RG at pH 4.8 (1.52 mm d^{-1}) and the lowest at pH 6.3 (1.00 mm d^{-1}), with significant differences between pH 4.8 compared to 5.8, 6.3 and 6.8. This species showed the highest RG in all of the evaluated pH values compared with the other two fungal species. In the case of *S. granulatus* and *S. luteus*, the RG only differed ($P < 0.05$) at pH 4.8, 5.3 and 6.8.

Mycelial biomass (MB). *Rhizopogon luteolus* showed only MB differences ($P < 0.05$) between the extreme pH treatments (4.8 and 6.8) (Figure 3B). *Suillus granulatus* and *S. luteus* did not show a pattern in terms of MB when grown in the different evaluated pH values. The pH of 4.8 yielded a higher ($P < 0.05$) MB in *R. luteolus* (104.76 mg) compared to *S. granulatus* (63.26 mg) and *S. luteus* (64.74 mg).

Experiments I and II (temperature and pH)

Accumulated radial growth (ARG) and non-accumulated radial growth (NARG). The three evaluated ECMF showed the four cultivation growth phases: culture medium adaptation, exponential, declining and stationary phases. In general, this trend was observed in all of the evaluated temperatures and pH values (Figures 4B and 4D). The adaptation phase was longer (approximately 6 d) in the experiment where the influence of temperature was evaluated compared with that of different pH values (approximately 3 d) (Figures 4A and 4C). *Rhizopogon luteolus* showed the highest ARG in both the temperature and pH experiments (Figures 4A and 4C).

In *R. luteolus*, the period of the exponential phase of NARG was dependent on the temperature. This phase concluded on day 12 at 24 ºC and on day 9 at both 28 ºC and 32 ºC (Figure 4B). After this phase, the decline of NARG in

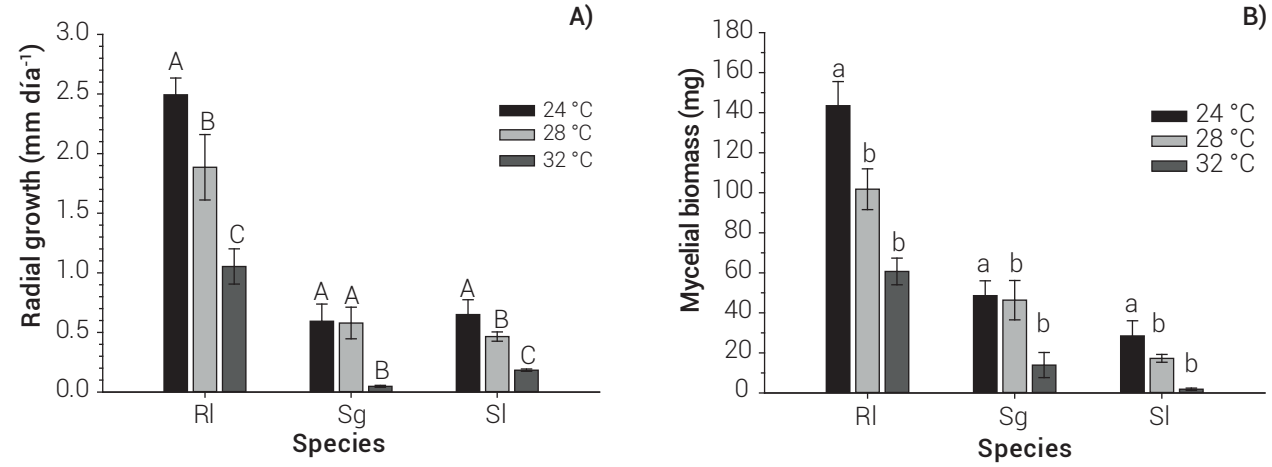

Figure 2. (A) Radial growth (mm d^{-1}) and (B) mycelial biomass (mg) of three fungal ectomycorrhizal species at three culture temperatures. Values are means ± standard error of the mean (n = 5). Bars with the same letter, for each species, are not different according to the Tukey test (P < 0.05). Rl = *Rhizopogon luteolus*; Sg = *Suillus granulatus*; Sl = *Suillus luteus*.

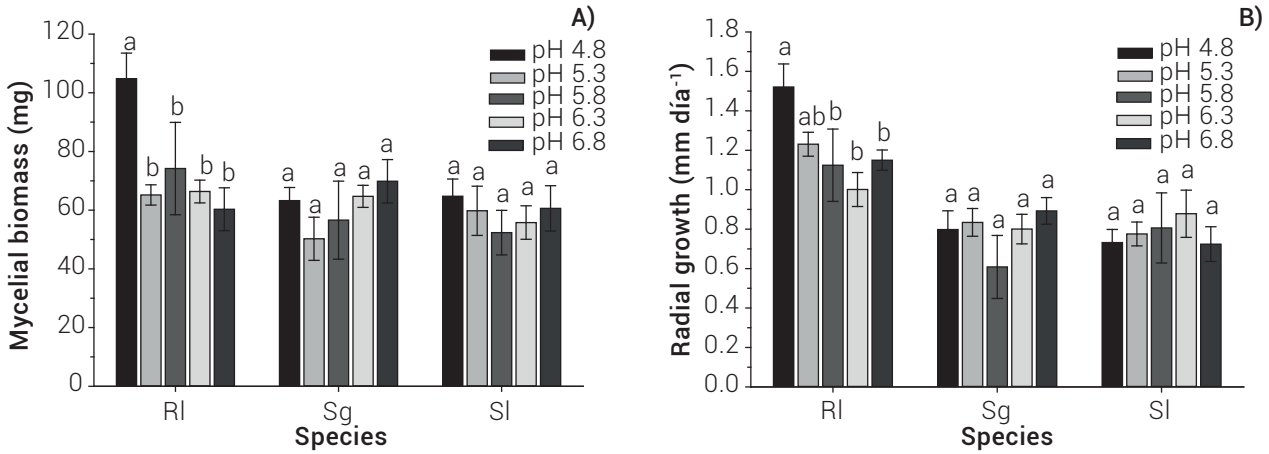

Figure 3. (A) Radial growth (mm d^{-1}) and (B) mycelial biomass (mg) of three fungal ectomycorrhizal species at five pH. Values are means ± standard error of the mean (n = 5). Bars with the same letter, for each species, are not different according to the Tukey test (P < 0.05). Rl = *Rhizopogon luteolus*; Sg = *Suillus granulatus*; Sl = *Suillus luteus*.

R. luteolus continued until day 21, and then the stationary phase started. In the case of the experiment were different pH values were evaluated, in general the exponential phase of the NARG was shorter, finishing in between days 6 and 9 depending on the ECMF species. Then, the exponential period was recorded between days 15 and 18, and after that the declining phase began until day 24, to conclude with the stationary phase of NARG (Figure 4D).

DISCUSSION

The knowledge of the biological, physiological and symbiotic characteristics of ECMF, as well as the specificity that they have with certain hosts, are fundamental requirements in order to choose the most appropriate fungal species in the production of ectomycorrhizal tree seedlings. Environmental factors, including temperature and pH, largely influence the formation and the development of mycorrhizal structures (Smith and Read, 2008). In general, most ECMF are considered to grow well at moderate temperatures between 11 ºC and 28 ºC (Hutchison, 1990). However, there are differences in the fungal growth among isolates of ECMF at different environmental temperatures (Dames *et al.*, 1999; Hutchinson, 1990).

Some studies have shown that the ectomycorrhizal mycelium might be very sensitive to temperature variations (Lazarević *et al.*, 2016; Marx *et al.*, 1970; Sánchez *et al.*, 2001). If the soil dramatically cools, the metabolic activity of the fungi and the roots decrease, with negative consequences for mycelial growth and nutrient availability for both the fungi and the associated plants (Lazarević *et al.*,

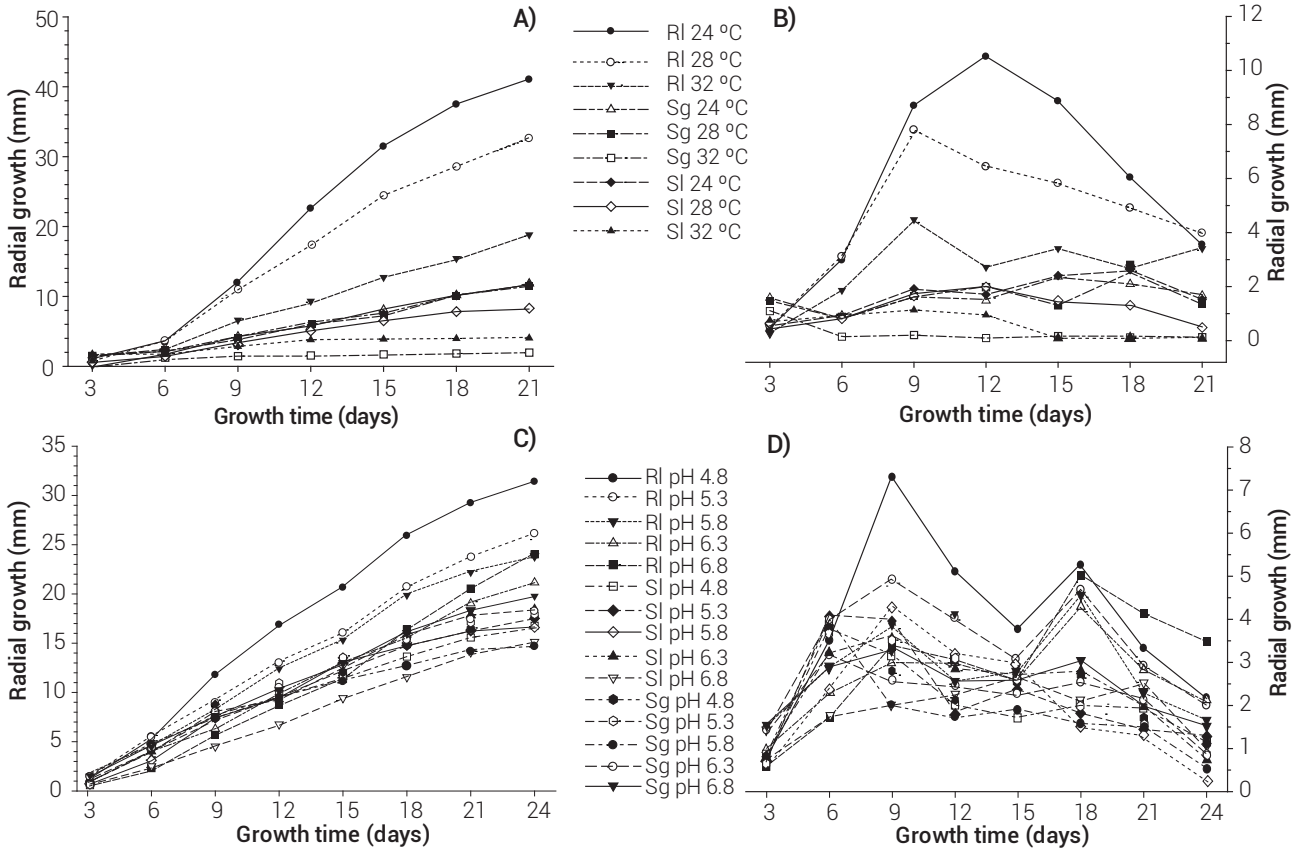

Figure 4. Average (A and C) accumulated and (B and D) non-accumulated radial growth of three fungal ectomycorrhizal species at three temperatures (at pH 5.8) and under five pH values (at 24 ºC). Values are means (n = 5). In order to have more clarity in the graphs the ± error bars are not shown. Rl = *Rhizopogon luteolus*; Sg = *Suillus granulatus*; Sl = *Suillus luteus*.

2016). The results of the present study showed that the culture temperature had an effect on the *in vitro* growth of *R. luteolus*, *S. granulatus* and *S. luteus* species. The best growth, both in terms of RG and MB, was reached at 24 ºC for the three species in the evaluated ECMF, and when the temperature was increased to 28 and 32 ºC, a negative effect was observed.

Different authors have reported similar findings to those that we found here. Jha *et al.* (2006) reported optimal growth for *R. luteolus* in temperatures ranging from 20 to 25 ºC. Sánchez *et al.* (2001) recorded the highest growth values for *R. roseolus*, *Suillus collinitus*, *S. granulatus* and *S. luteus* at temperatures of 23 ºC; similarly to our own findings, these authors registered significant decreases with increasing temperatures. Dennis (1985) reported optimal growth for *S. granulatus* at 25 ºC and for *S. luteus* in the interval of 20 to 25 ºC. Lazarević *et al.* (2016) recorded the best growth for *S. collinitus* and *S. granulatus* at 22 ºC. Cline *et al.* (1987) reported the best growth for *S. granulatus* at 27 ºC, and for *Suillus* spp. optimal mycelial growth between 16 and 32 ºC.

In the present study, *R. luteolus* showed the best mycelial growth among the three studied ECMF species independently of the temperatures tested. Sánchez *et al.* (2001) found similar results for *Rhizopogon* and *Suillus* species. The maximum radial growth rate reached by *R. luteolus* (2.49 mm d^{-1}) in the present work was above those previously reported 1.15, 1.98 and 1.28 mm d^{-1} by Chávez *et al.* (2007), Pereira *et al.* (2007) and Vázquez-García *et al.* (2002), respectively. However, the maximum values of RG obtained here by *S. granulatus* and *S. luteus* are below those registered by Santelices *et al.* (2012) for *S. luteus*, under the same culture conditions.

The pH can biologically determine the type of organism able to develop on a soil or a substrate, due to its significant influence on the nutrient availability (Pereira *et al.*, 2007). The pH can strongly affect the microbial activity through nutrient availability, ionization of the constituents of the inorganic and organic soil solution, and therefore can have a paramount importance in the enzymatic activity of the soil (Voroney, 2007). In the present study, a different behavior of the evaluated ECMF species was recorded at the different

pH values. *Rhizopogon luteolus* presented the greatest sensitivity of RG and MB to changes in pH, showing a decrease in these variables when the pH was increased. Jha *et al.* (2006) and Pereira *et al.* (2007) found similar trends for *R. luteolus*. Vázquez-García *et al.* (2002) reported optimal growth for *Rhizopogon* sp. in BAF medium at pH 6.0, and significant growth decreases when pH was either increased or decreased. Duñabeitia *et al.* (2004) did not find changes in terms of colony diameter and mycelial growth biomass of *R. luteolus* when the pH was modified.

In the present study, *S. granulatus* and *S. luteus* did not show a clear trend when the pH changed. Similar responses were found for *S. luteus* by Santelices *et al.* (2012) and for *S. bellinii* by Pereira *et al.* (2007). In their studies with several species of *Suillus*, Murrieta-Hernández *et al.* (2014) and Vázquez-García *et al.* (2002) reported different behaviors in terms of growth when the pH of the culture medium was modified. In contrast, Dennis (1985) found significant differences in radial growth when *S. granulatus* and *S. luteus* were grown in ranges of pH from 3 to 6. Barros *et al.* (2006), and Pereira *et al.* (2007) recorded differences in terms of RG for *S. luteus* when the pH was modified. In our work, *R. luteolus* showed the highest values of RG at pH 4.8 (152 mm d^{-1}) and at pH 6.3 the lowest one (1.0 mm d^{-1}). The results showed that, in general, *R. luteolus* tends to grow better under acidic than alkaline conditions. This finding agrees with the trends mentioned by Hung and Trappe (1983), Lazarević *et al.* (2016) and Willenborg *et al.* (1990), who mentioned that ECMF have in general, an acidophilic nature when grown under pure culture conditions.

However, it is important to mention that there are some exceptions either specific or intraspecific, i.e. Sánchez *et al.* (2001) found that *S. granulatus*, *S. luteus* and *S. collinitus* had their best biomass increase responses at a pH of 8.5. *In vitro* mycelial growth of a fungus changes according to the culture medium used and the experimental culture conditions (Pereira *et al.*, 2014; Santelices *et al.*, 2012). The lack of significant trends obtained in the pH experiment could probably be explained because no biological buffers were added to growth media. Such buffers are essential to stabilize the pH variable. According to García-Rodríguez *et al.* (2006) and Hung and Trape (1983) during the development of *in vitro* mycelium, ECMF produce a series of organic acids, through the absorption of ions, acidifying the growth medium, and negatively affecting the growth and development of the fungi. Additionally, conventional culture media such as Modified Melin Norkrans have low buffering capacity and therefore the effects of pH on fungal growth in such media are difficult to evaluate (Child *et al.*, 1973, Hung and Trappe, 1983; Yamanaka, 2003).

Organic and inorganic acids, such as acetates, citrate, phthalate and phosphate, have been used as alternative buffers to stabilize pH. Guerin-Laguette *et al.* (2000) showed the importance of biological buffers in pH experiments with fungal growth, they found very strong evidence of the influence of pH on the *in vitro* mycelial development of two edible ectomycorrhizal mushrooms. The growth of *Lactarius deliciosus* and *L. sanguifluus* was optimal at pH 5.5-6.0 and 5.5-6.5, respectively. They used 5X agar medium to which the biological buffer MES (2-(*N*-morpholino) ethanesulfonic acid was added and the pH was adjusted with 4 M NaOH prior to autoclaving. Although some of these acids may inhibit the growth of fungi (Giltrap and Lewis, 1981; Hilger *et al.*, 1986; Inoue and Ichitani, 1990), MES and other buffers such as piperazine-N and N'-bis (2-ethanesulfonic acid) are considered to be physiologically inert (Good *et al.*, 1966). It has also been shown that these buffers can stimulate the growth of some ectomycorrhizal fungi (Yamanaka, 2003).

The fungi present a growth kinetics that usually involves the adaptation, exponential, declining and stationary phases (Manero *et al.*, 2012; Sánchez and Royse, 2002). The three species of evaluated ECMF in the present work showed these four well-defined phases in our *in vitro* culture conditions. It was observed that the adaptation phase was more conspicuously marked in the first experiment where temperatures were evaluated, compared to the second experiment where the effect of pH conditions was studied. Santelices *et al.* (2012) reported only the adaptation, exponential and stationary phases for *S. luteus*. Coleman *et al.* (1989) reported the same growth pattern for *S. luteus* in Modified Melin Norkrans culture medium, with absence of the initial adaptation phase; however, when water stress treatments were involved in their trials, the initial phase of adaptation became more conspicuous.

In our study the exponential phase of *R. luteolus* was strongly dependent on the culture temperature. When the pH of the medium varied, the period of the exponential phase was in general short, producing in some species a second period of exponential growth. In the kinetics study of the ECMF, the growth phases involved in their cultivation are fundamental to analyze and understand because they provide important information related to the particular growth dynamics of each ECMF species. These studies can show the exact moment when the mycelium of a particular fungus is physiologically active in culture, and has the maximum potential to be used as an inoculum in possible programs of inoculum production in order to produce mycorrhizal plants. The understanding of the optimal *in vitro* conditions to grow native strains will allow the generation of ectomycorrhizal inoculum in enough quantities to be applied to *P. radiata* tree production under greenhouse conditions. Such work has previously been carried

out in other pine species such as *P. pinea* in Spain with *Rhizopogon* spp. (Rincón *et al.*, 2005). Afterwards, if there is successful and abundant ectomycorrhizal colonization the establishment of plantations with the local plant and the studied fungal genotypes might be favoured.

CONCLUSIONS

The results indicated that the mean radial growth rate and the biomass increase of strains of *R. luteolus*, *S. granulatus* and *S. luteus* were significantly affected by increasing the temperature. The optimum cultivation temperature for all of the evaluated species was 24 ºC. There was a significant decrease with temperature increases.

Under the present culture conditions without biological buffers, the pH variability of the medium, in the range of 4.8 to 6.8, did not significantly affect the mycelial growth of *S. granulatus* and *S. luteus* species. However, *R. luteolus* decreased its growth when pH was increased.

Among the three evaluated fungal species, *R. luteolus* showed the greatest mycelial production. This species also shows the greatest potential for the large-scale production of mycelial inoculum at a larger scale, useful in the production of ectomycorrhizal *Pinus radiata* trees.

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