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Optimization of Biomass Production of Two Ectomycorrhizal Fungi (*Lactarius quieticolor* and *Rhizopogon roseolus*) for the Future *Pinus radiata* Nursery Inoculation

Daniel Chávez[1*], Ángela Machuca[1] and Carolina Aguirre[2]

[1]Department of Plant Sciences and Technology, School of Sciences and Technology, Campus Los Ángeles, Universidad de Concepción, Chile
[2]Environmental Chemistry Department, Faculty of Sciences, Universidad Católica Ssma. Concepción. P.O. Box 297, Concepción, Chile

Corresponding author

Abstract

The growing interest in mycelial biomass for its application in nurseries has led to the creation of inoculum production programs in New Zealand, Australia, the United States, and Chile, where the advantages of mycorrhizal inoculation have been demonstrated in plant morphological and physiological parameters. However, the mycelial biomass yields produced with these fungi through traditional submerged culture are generally low. Therefore, the aim of this study was to optimize the variables pH, temperature, and carbon source concentration required for maximum biomass production by the ectomycorrhizal fungi *L. quieticolor* and *R. roseolus* in liquid culture medium. The Box-Behnken (BB) design and response surface methodology (RSM) were used to establish the optimal conditions in the experimental area. Increases in biomass production of 1.2 g L^{-1} for 3.25 g L^{-1} for *L. quieticolor* (pH 5.5, 24°C and 20 g L^{-1} glucose) and 3.02 for 8.6 g L^{-1} for *R. roseolus*(pH 5.5, 28°C and 30 g L^{-1} mannitol) were found in comparison to the control culture (pH 5.5, 24°C and 10 g L^{-1} glucose) for both fungi. This optimization is the first step required to scale biomass production by *L. quieticolor* and *R. roseolus* in bioreactors.

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Biomass optimization,
Carbon sources,
Ectomycorrhiza,
P. radiata.

Introduction

Mycorrhizal symbiosis improves the health of plants, increasing protection against biotic factors (e.g. attack by pathogens) and abiotic factors (e.g. drought, salinity, metals), and improves the structure of the soil, promoting its aggregation (Karaki, 2006; Barea *et al.*, 2011; Sousa *et al.*, 2012; Trocha *et al.*, 2016). It has also been verified that ectomycorrhizal fungi encourage plant growth in nurseries and help establish plantations in the field, facilitating interplant transfer of water as well as producing fungus of high economic value such as the truffles demanded in the European market (Sebastiana *et al.*, 2013; Sánchez-Zabala *et al.*, 2013; Chávez *et al.*, 2014; Prieto *et al.*, 2016; Iotti *et al.*, 2016). *Pinus radiata* D. Don is planted on more than 1.5 million ha of Chilean territory, being one of the most important lumber species (Mead, 2013). There are approximately 32 fungal species in Chile associated with pine plantations found at different stages of growth (Garrido, 1986; Palacios *et al.*, 2012). Among the important ectomycorrhizal fungi in the central southern zone of Chile associated with exotic

plantations are the species *Lactarius quieti color* Romagn. And *Rhizopogon roseolus* (Corda) Th. Fr., both of which play important ecological roles in *P. radiata* plantations. Both species naturally form associations with *P. radiata*; however, *R. roseolus* does so during the young stage of the plants and *L. quieticolor* in the more adult stage. Currently, different inoculum types have been developed for the application of these species in nurseries, with the highest percentages of mycorrhization being obtained through the application of liquid mycelial inoculum (Chávez *et al.*, 2009; Sánchez-Zabala *et al.*, 2013; Pereira *et al.*, 2014). But mycelium yields obtained by traditional submerged culture are relatively low, thus explaining the growing interest in improving mycelial biomass production through the study of culture variables such as pH, temperature, nutrient concentration, and stirring rate, among others. Statistical experimental designs such as the Box-Behnken (BB) design and response surface methodology (RSM) particularly help in achieving this goal. As a statistical tool and mathematical, RSM aids in constructing models that identify effective factors, evaluate interactions, select the optimal conditions for the study variables, and quantify the relationships between one or more response measures, as well as allowing a consistent response with a limited number of trials (Avishek and Arun, 2008; Liyana-Pathirana and Shahidi, 2005). In this context, the purpose of this study was to determine the optimal conditions of pH, temperature, and carbon source concentration for the mycelial biomass production of the ectomycorrhizal fungi *L. quieticolor* and *R. roseolus* in liquid culture medium. Prior to optimization, the best culture medium and type of carbon source were selected for each of the fungal species. Optimization of biomass production is the first step required for the future scaling of these fungal cultures in a bioreactor for the controlled mycorrhization of a large number of nursery plants.

Materials and Methods

Experimental design

RSM was used for the optimization(Chacín, 2000; Montgomery, 2002), making it possible to predict the system's response to each of the factors considered in the experimental area. The optimization process essentially involves three main steps: i) conducting the statistically designed experiments, ii) estimating the coefficients of a mathematical model and predicting the response, and iii) adequately verifying the model. The variables in this study were pH, temperature, and carbon source concentration (glucose, mannitol, or sucrose, depending on the fungal species used), whereas the observed response was the total biomass produced after the incubation period. The response predicted (Y) by the model can be generally expressed from Equation(1).

$$Y = B_0 + \sum_{i=1}^{k} B_i X_i + \sum_{i=j=1}^{k} B_{ij} X_i X_j$$

In this study, *k* takes the value of 3as there are three variables (pH, temperature, and carbon source concentration), and thus Equation (2) becomes:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{23}X_2X_3 + B_{11}X_1^2 + B_{22}X_2^2 + B_{33}X_3^2$$

Where X_1, X_2, andX_3 are the input variables, pH, temperature, and carbon source concentration, respectively; B_0 is a constant; B_1, B_2, and B_3 linear coefficients; B_{12}, B_{13}, and B_{23} cross product or interaction coefficients; B_{11}, B_{22}, and B_{33} are quadratic coefficients. The low, medium, and high values of each variable (pH, temperature, and carbon source concentration) were coded as -1, 0, and 1, respectively (Figure 1) and are shown together with the BB experimental design in Table1.The data were obtained from the program Modde 4.0 (Umetri, Umeå, Sweden). The statistical method of squared minimums was used to determine the regression coefficients of each of the terms of the functions that describe the behavior of the "biomass" response for *L. quieticolor* and *R. roseolus* in the intervals established for the multifactorial planning variables. The determination coefficient (R^2) is the fraction of response variation explained by the model. The prediction coefficient (Q^2) is the fraction of response variation that can be predicted by the model and provides the best summary of model fit. R2-Q2 is an underestimate of the goodness of fit of the model.

Fungal species

Two ectomycorrhizal species belonging to the culture collection of the Fungi Biotechnology Laboratory at the Universidad de Concepción, Los Ángeles Campus were used: *Rhizopogonroseolus* (Corda) Th. Fr. and *Lactariusquieticolor* Romagn (Chávez *et al.*, 2015). Mycelial agar pieces from the stock cultures were transferred to dishes with modified Melin-Norkrans

(MMN) medium (Marx, 1969) and Biotin-Aneurin-Folic acid agar (BAF) (Moser,1960) and incubated for 20 days at 24°C to obtain active mycelia for the following trials.

Selection of culture medium and carbon source

The effect of the MMN and BAF media as well as of the carbon sources mannitol, glucose, and sucrose (10 g L^{-1}) on the growth of the ectomycorrhizal fungi were assessed in order to choose the most suitable culture medium and carbon source for the subsequent optimization analysis of the culture variables. Erlenmeyer flasks (100 mL) containing 40 mL of liquid culture medium were inoculated with two mycelial agar discs (5 mm diameter) in triplicate and incubated at 24ºC for 30 days. Once the incubation period was finished, the biomass was determined by the dry weight of the mycelia (mg).

Growth kinetics

Once the culture medium and carbon source were chosen for each fungal species, kinetic growth was tested at pH 5.5 and 24°C for 35 days under static and agitated (120 rpm)conditions, and the specific growth rate (μ), time of cell duplication (Tdup) (time of duplication of biomass)and culture time when the stationary growth phase began were determined. Erlenmeyer flasks (100 mL) containing 40 mL of liquid medium was inoculated in triplicate with two mycelial agar discs (5 mm diameter) of each fungal species. Every 5 days, the mycelium grown in each flask was recovered by filtration and dried at 60°C until constant weight.

Growth optimization of *L. quieticolor* and *R. roseolus*

Growth optimization of the fungal species in liquid culture medium was conducted under orbital agitation (120 rpm) and the variables pH, temperature, and carbon source were evaluated (Table 1 and Figure 1). In order to evaluate the effect of the different carbon source concentrations(20 to 40 g L^{-1} of glucose or mannitol, as appropriate) on the growth of the fungi, BAF medium was used, the composition of which in g L^{-1} was: $CaCl_2$ 0.2, KH_2PO_4 0.75, $MgSO_4$ x $7H_2O$ 0.5, $MnSO_4$ 0.005, $ZnSO_4$ 0.001, $FeCl_3$ x $7H_2O$ 0.01, peptone 6.315, yeast extract 0.2, thiamin 0.0005, biotin 0.00001, inositol 0.05 and folic acid 0.0001.The carbon source was glucose for *L. Quieticolor* and mannitol for *R. roseolus*, according to the results of the previous trials. The optimization was carried out in triplicate using Erlenmeyer flasks (100 mL) containing 40 ml of culture medium and two mycelial agar discs (5 mm diameter) as inoculum with a total culture time of 30 days.

Evaluation of the biomass during the optimization stage

At each collection, the mycelia were filtered through filter paper and dried at 60ºC for 48 h, after which the dry weight (g) was determined. The residual volume of each culture medium in each Erlenmeyer flask was also measured. From these data the biomass concentration (g L^{-1}) produced in each trial was calculated using the following formula:

$$X = \frac{B1 - B2}{V}$$

Where, X corresponds to the biomass concentration g L^{-1}, B1 to the mass of the dry sample plus the filter paper (g), B2 to the mass of the filter paper (g) and V to the residual volume of the culture medium (L).

Data analysis

A factor analysis was applied for the response variable biomass. The analyzed factors were culture medium (BAF and MMN) and different carbon sources (glucose, sucrose, and mannitol). The data were subjected to an analysis of variance and the comparison of means was calculated according to the Tukey test ($P<0.05$). For data analysis, the software Statistica v. 6.0 was used. For optimization, the biomass data expressed in g L^{-1} collected from the 15 trials were subjected to a regression analysis using RSM employing the BB experimental design (Box and Behnken, 1960; Ferreira *et al.*, 2007).

Results and Discussion

Mycelial biomass production of *L. quieticolor* and *R. roseolus* in liquid culture medium

L. quieticolor exhibited a greater biomass production in the BAF-glucose medium (47 mg, equivalent to1.2 g L^{-1}), showing significant differences with all the other treatments. For *R. roseolus*, the better culture medium and carbon source was BAF-mannitol (115 mg, equivalent to 3.02 g L^{-1}), presenting significant differences ($P<0.05$) with the other carbon sources (Figure 2).

Growth kinetics of *L. quieticolor* and *R. roseolus* under static and agitated conditions

When comparing the culture conditions (static and agitated) for the two fungal species, it was observed that the agitated condition encouraged mycelial biomass production (Figure 3). Between 15-30 days, *L. quieticolor* increased biomass production under constant agitation with a specific rate (μ) of 0.09 and 7 days of Tdup compared to the 13 days under static condition. At 25 days of culture, *R. roseolus* displayed a considerable increase in biomass production under agitation with a μ of 0.121 and a Tdup of 6 days, whereas under static condition, the duplication was generated at day 8.From the growth kinetics, days under agitation were established for the optimization assays.

Optimization

The growth of *L. quieticolor* and *R. roseolus* was sensitive to slight alterations in the factors such as pH, temperature, and carbon source concentration. The effect of these factors on fungi growth was studied in relation to the total biomass produced expressed ing L^{-1}. *L. quieticolor* presented the maximum biomass production with treatment6 (3.25 g L^{-1}) and *R. roseolus* with treatment 4 (8.60 g L^{-1}) (Table 2). The regression equation obtained after the analysis of variance showed the biomass produced to be a function of the three study factors. Therefore, the predicted response *Y* could be obtained by solving the quadratic equation (2), obtaining:

$$Y_1 = 2.29 + 0.600X_1 - 0.435X_2 + 0.080X_3 - 0.240X_1^2 - 0.375X_2^2 + 0.328X_3^2 - 0.170X_1X_2 - 0.038X_1X_3 - 0.006X_2X_3$$

$$Y_2 = 6.72 + 0.883X_1 + 0.173X_2 + 0.485X_3 - 0.464X_1^2 + 0.517X_2^2 - 0.195X_3^2 + 0.458X_1X_2 - 0.427X_1X_3 - 0.468X_2X_3$$

Where Y_1 and Y_2 were the predicted biomass of *L. quieticolor* and *R. roseolus*, respectively. The coefficients of Equations 3 and 4 are enumerated in Table 3. It can be noted that pH was the variable with the greatest effect on the biomass production by both ectomycorrhizal fungi. The summary of the analysis of variance for the response variable biomass is in Table 4. Based on the results, it may be stated that the model fit is good since the value of R^2=0.85 is close to 1, in particular, 85% of the variability of the variable Y is explained by the adjusted regression model. In addition, the *p* value in the general regression is significant at 5%, thus demonstrating that the model is adequate in the approximation of the RSM of the experimental design.

Analysis of the variables pH and temperature revealed that as the pH increased (from 3.5 to 5.5) and the temperature decreased (from 28 to 20°C), there was an increase in biomass production by *L. quieticolor* (Figure 4A). In contrast, when the temperature decreased (from 24 to 20°C) and the glucose concentration increased (from 30 to 40 g L^{-1}), the mycelial biomass production by the fungus increased (Figure 4B). When analyzing the variables pH and glucose concentration, maximum values of biomass production were observed at the highest pH values and at a concentration of 20 or 40 g L^{-1} glucose; however, when 30 g L^{-1} glucose was used, biomass production dropped (Figure 4C). In the case of *R. roseolus*, as the pH rose (from 4.5 to 5.5) to 28°C, optimal biomass production values were observed(Figure 4D). When the different concentrations of mannitol interacted with the temperature, the biomass increased at a low temperature (20 °C) and at a high concentration of mannitol (40 g L^{-1}). However, when the pH and the different concentrations of mannitol interacted, a directly proportional relation between the increase in the biomass of *R. roseolus*, the pH, and the carbon source concentration was observed (Figure 4F).

The use of statistical models to optimize fungal biomass production and obtain metabolites has increased of late. Examples of this include research being conducted on the entomopathogenic fungus *Cordyceps militaris* and *Metarhizium rileyi* for biomass production (Hsieh *et al.*, 2009; Cui and Yuan, 2011; Song *et al.*, 2017), saprophytic fungi *Lentinus squarrosulus* (Ahmad *et al.*, 2013) for mycelium and exopolysaccharide production, *Ganoderma lucidum* for phenolic compound production and biomass(Zárate *et al.*, 2013; Goh *et al.*, 2016), *Pleurotus* sp. for enzyme production (Saravanakumar *et al.*, 2010) and Filamentous Fungi for Extracellular Lipase Production (Gaurav *et al.*, 2017). Nonetheless, statistical models for research into the optimization of biomass production or metabolites by ectomycorrhizal fungi have scarcely been used (Srinivasan *et al.*, 2000; Liu *et al.*, 2008). For these, several selection criteria are required, particularly when the ultimate goal is the production of large amounts of biomass for the inoculation of plants on a large scale. The selection criteria include the variables pH, concentration and type of carbon source, and culture temperature, which are of great significance in mycelial biomass production.

In this study, the sugar alcohol mannitol, the hexose glucose, and the disaccharide sucrose were used as carbon sources. *L. quieticolor* produced the largest amount of biomass in the BAF-glucose medium, whereas *R. roseolus* presented the greatest biomass production in the same BAF medium, but in the presence of mannitol. Under these conditions, the biomass produced by *R. roseolus* was approximately more than double of what was produced by *L. quieticolor*. Although both species are ectomycorrhizal, they displayed a clear difference in terms of carbon source requirements for optimal biomass production. Studies conducted on the ectomycorrhizal fungi *Infundibulicybe geotropa* (Bull.) Harmaja, *Tricholoma anatolicum* H.H. Doğan & Intini and *Lactarius deliciosus* (L.) Gray, all of epigeous fructification, showed that the greatest mycelial biomass production by *I. geotropa* was obtained using fructose; however, with *T. anatolicum* and *L. deliciosus*, this occurred using glucose (Akatal *et al.,* 2012; Gomez *et al.,* 2016). This is consistent with the results obtained with *L. quieticolor*, which although it produced the greatest amount of biomass in glucose, when it was cultivated in mannitol the amount of biomass was high. Glucose is a carbon source that is easily metabolized by most microorganisms, incorporating it into their metabolism for the rapid production of energy for cell processes (Deacon, 2006; Jonathan and Fasidi, 2001). Ectomycorrhizal fungi transform sugars produced and obtained from plants into mannitol or trehalose to incorporate them into their metabolism (Smith and Read, 2008). Mannitol performs important functions as a form of carbon storage and reducing power, in addition to being stored in vacuoles for the regulation of cell pH (Deacon, 2006). A better use of mannitol by *R. roseolus* could explain its greater production of fungal biomass than *L. quieticolor*.

Culture temperature is another external factor that plays a significant role in the growth of ectomycorrhizal fungi. Most fungi are mesophilic, which commonly grow in a temperature range between10 and 40°C (Jonathan and Fasidi, 2001), and most ectomycorrhizal fungi are in this group. Within the growth temperature range, different degrees of tolerance can be observed, and often the increase in temperature can cause a decrease in fungal growth and sometimes the total cessation (Srinivasan *et al.,* 2000). One exception among the ectomycorrhizal fungi is the case of *P. tinctorius*, with the ability to grow at temperatures between 40 and 42°C, and with a thermal death point of the hyphae at 45°C (Hung and Chein, 1995). For the two species in this study, the optimum temperature for biomass production was found within the normal range of the mesophilic fungi, with the optimum temperature of *R. roseolus* (28°C), being higher than that of *L. quieticolor* (24°C). The optimum temperatures that these two fungal species present make them good candidates for biomass production on a large scale in a bioreactor due to the low energy requirement needed to maintain the temperature control during the culture. Moreover, the pH was the most significant variable in the optimization process *in vitro*. The optimum pH of *R. roseolus* and *L. quieticolor* for biomass production was 5.5, which is related to the fact that the soils where these species are developed are slightly acidic.

When evaluating growth kinetics, it was corroborated that agitation stimulated fungal biomass production. This may be related to a homogenous oxygenation in the flasks and at the same time to a continuous fragmentation of the mycelium, thereby generating new points of active growth and allowing a greater mass transfer of the nutrients towards the fungi (Teoh *et al.,* 2010; Teoh and Don, 2012). The highest specific growth rate (μ) for *L. quieticolor* and *R. roseolus* was recorded in agitation (0.09 and 0.121 day^{-1}, respectively), with these rates being higher than those displayed under static conditions. The cell duplication time (Tdup) is the time that mediates between two successive duplications, and in this case, it depended on the culture and the fungal species. For *L. quieticolor* and *R. roseolus*, the maximum cell duplication time was 7 and 6 days, respectively, with agitation, much lower than the 13 and 8 days under static conditions.

RSM made it possible to determine the optimal conditions in the experimental area, obtaining an increase in biomass production of 1.2 for 3.25 g L^{-1} in the case of *L. quieticolor* at pH 5.5, 24ºC culture temperature, and 20 g L^{-1} of glucose concentration. For *R. roseolus*, the increase in biomass was 3.02for 8.6 g L^{-1} at pH 5.5, 28°C culture temperature, and 30 g L^{-1} mannitol concentration. This is a similar increase of 2.71 and 2.85 times in biomass production for *L. quieticolor* and *R. roseolus*, respectively, under optimized conditions. For the ectomycorrhizal fungus *P. tinctorius* (Srinivasan *et al.,* 2000), an optimum growth range was found at pH 5.8-6 and a temperature of 29-30°C; when cultivated between 40 and 45 days in these conditions, the maximum biomass production was 1.13 g L^{-1}, far below what was reported for *L. quieticolor* and *R. roseolus* and mainly due to the slow growth of *P. tinctorius* in artificial culture media. Other biomass optimization processes have found a 1.4-fold increase in

the biomass production of the saprophytic fungus *C. versicolor* (Wang *et al.*, 2012) and 1.9-fold in the entomopathogenic fungus *C. militaris* (Ahmad *et al.*, 2013). This underscores the importance of applying statistical designs to produce fungal biomass.

Table.1 Three-factor Box-Behnken experimental designs

Trial N°	pH	Temperature (ºC)	Glucose/Mannitol (g L^{-1})
1	3.5 (-1)	20 (-1)	30 (0)
2	5.5 (1)	20 (-1)	30 (0)
3	3.5 (-1)	28 (1)	30 (0)
4	5.5 (1)	28 (1)	30 (0)
5	3.5 (-1)	24 (0)	20 (-1)
6	5.5 (1)	24 (0)	20 (-1)
7	3.5 (-1)	24 (0)	40 (1)
8	5.5 (1)	24 (0)	40 (1)
9	4.5 (0)	20 (-1)	20 (-1)
10	4.5 (0)	28 (1)	20 (-1)
11	4.5 (0)	20 (-1)	40 (1)
12	4.5 (0)	28 (1)	40 (1)
13	4.5 (0)	24 (0)	30 (0)
14	4.5 (0)	24 (0)	30 (0)
15	4.5 (0)	24 (0)	30 (0)

Table.2 Experimental and theoretical predicted values for biomass expressed in g L^{-1}

Trial n°	*Lactarius quieticolor*		*Rhizopogon roseolus*	
	Actual	Predicted	Actual	Predicted
1	1.21	1.34	5.86	6.18
2	2.79	2.88	6.66	7.03
3	0.90	0.81	5.97	5.61
4	1.80	1.67	8.60	8.29
5	2.01	1.66	4.41	4.27
6	3.25	2.94	7.08	6.89
7	1.58	1.90	5.90	6.09
8	2.67	3.02	6.86	7.00
9	2.37	2.59	6.08	5.92
10	1.30	1.73	6.69	7.20
11	3.20	2.76	8.33	7.82
12	2.10	1.88	7.07	7.23
13	2.27	2.29	6.65	6.72
14	2.27	2.29	6.60	6.72
15	2.32	2.29	6.92	6.72

Table.3 Model coefficients

	Biomass	
Variables	*L. quieticolor*	*R. roseolus*
B_0	2.290	6.720
B_1	0.600	0.883
B_2	-0.435	0.173
B_3	0.080	0.485
B_{11}	-0.240	-0.464
B_{22}	-0.375	0.517
B_{33}	0.328	-0.195
B_{12}	-0.170	0.458
B_{13}	-0.038	-0.427
B_{23}	-0.006	-0.468
$\boldsymbol{Q^2}$	0.73	0.74
$\boldsymbol{R^2}$	0.85	0.85

Table.4 Regression analysis for the amount of biomass (quadratic response surface model fitting) A) *L. quieticolor* and B) *R. roseolus*

(A)					(B)			
Source	Sum of squares	Degree of freedom	Mean squares	F value ($P<$ 0.001)	Sum of squares	Degree of freedom	Mean squares	F value ($P<$ 0.001)
Model	17.3	9	1.920	21.2	38.62	9	4.291	21.8
Residual	3.170	35	0.090		6.867	35	0.196	
Lack of fit	2.88	3	0.963	10.76	3.44	3	1.14	10.69
Pure error	0.28	32	0.008		3.43	32	0.107	
Correlation total	20.276	44			45.48	44		

Fig.1 The low, middle, and high levels of the three variables under optimization (temperature, pH, and carbon source concentration)

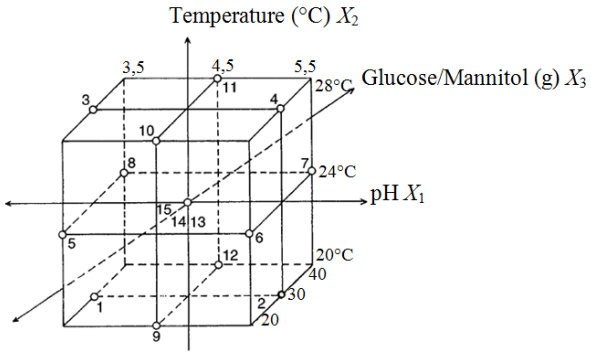


Fig.2 Production of mycelium biomass by (A) *L. quieticolor* and (B) *R. roseolus* cultivated in liquid medium BAF and MMN, using mannitol, glucose, and sucrose as carbon sources (10 gL^{-1}). Incubation time: 30 days. WCS: without carbon source

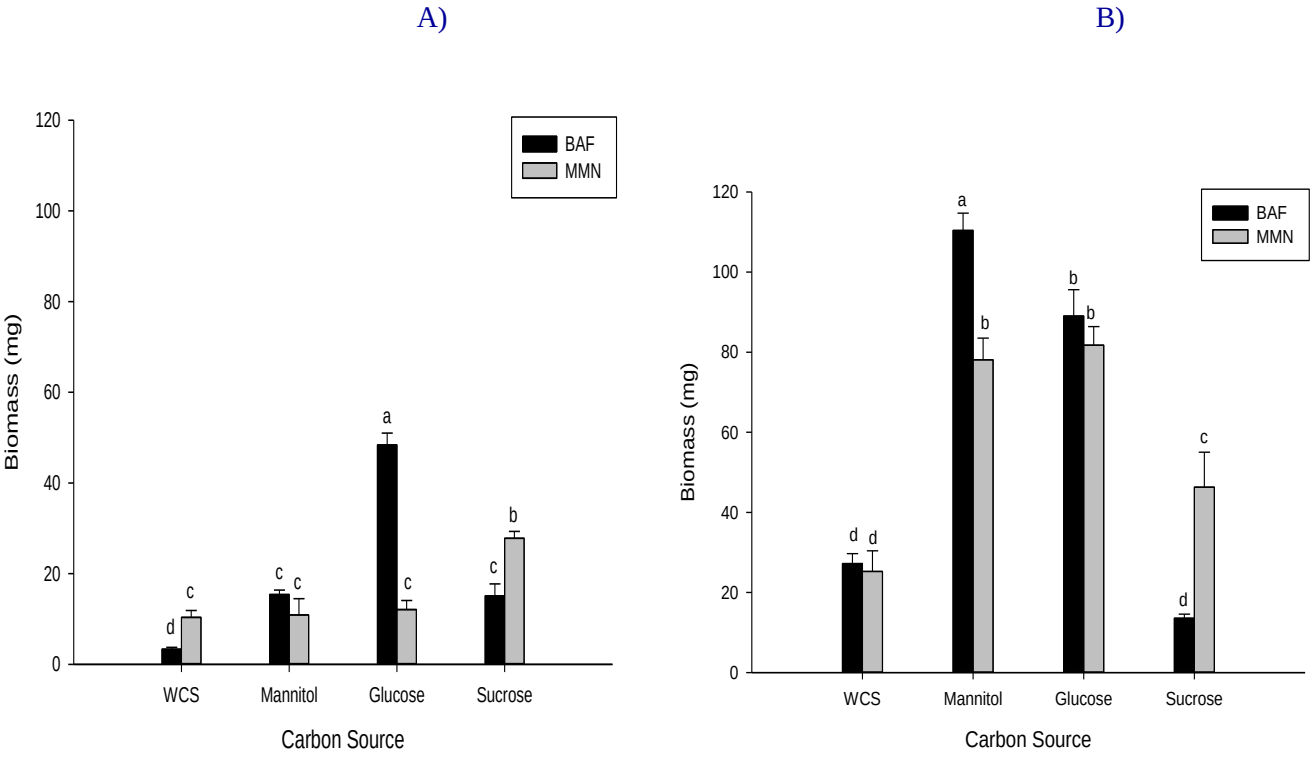


Fig.3 Growth kinetics at pH 5.5 for L. quieticolor (A) and *R. roseolus* (B) in agitated and static conditions. µ: specific growth rate. Tdup: time of cell duplication

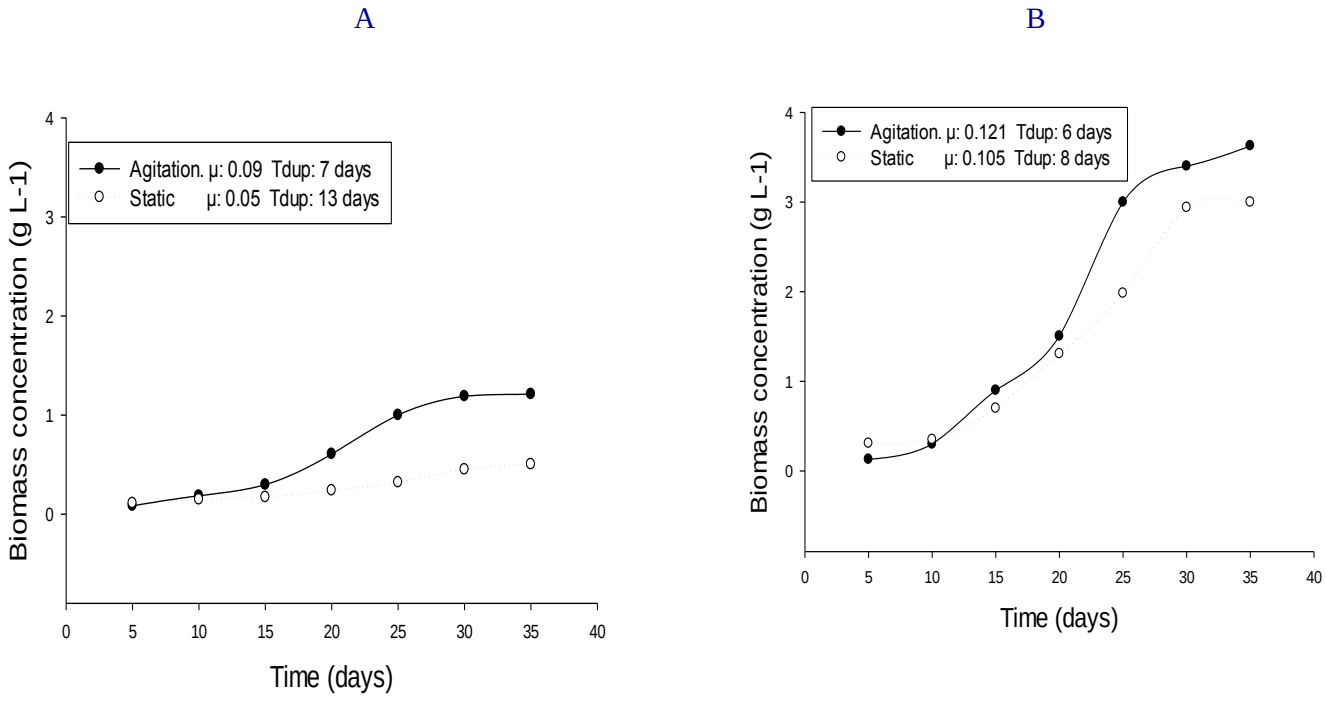
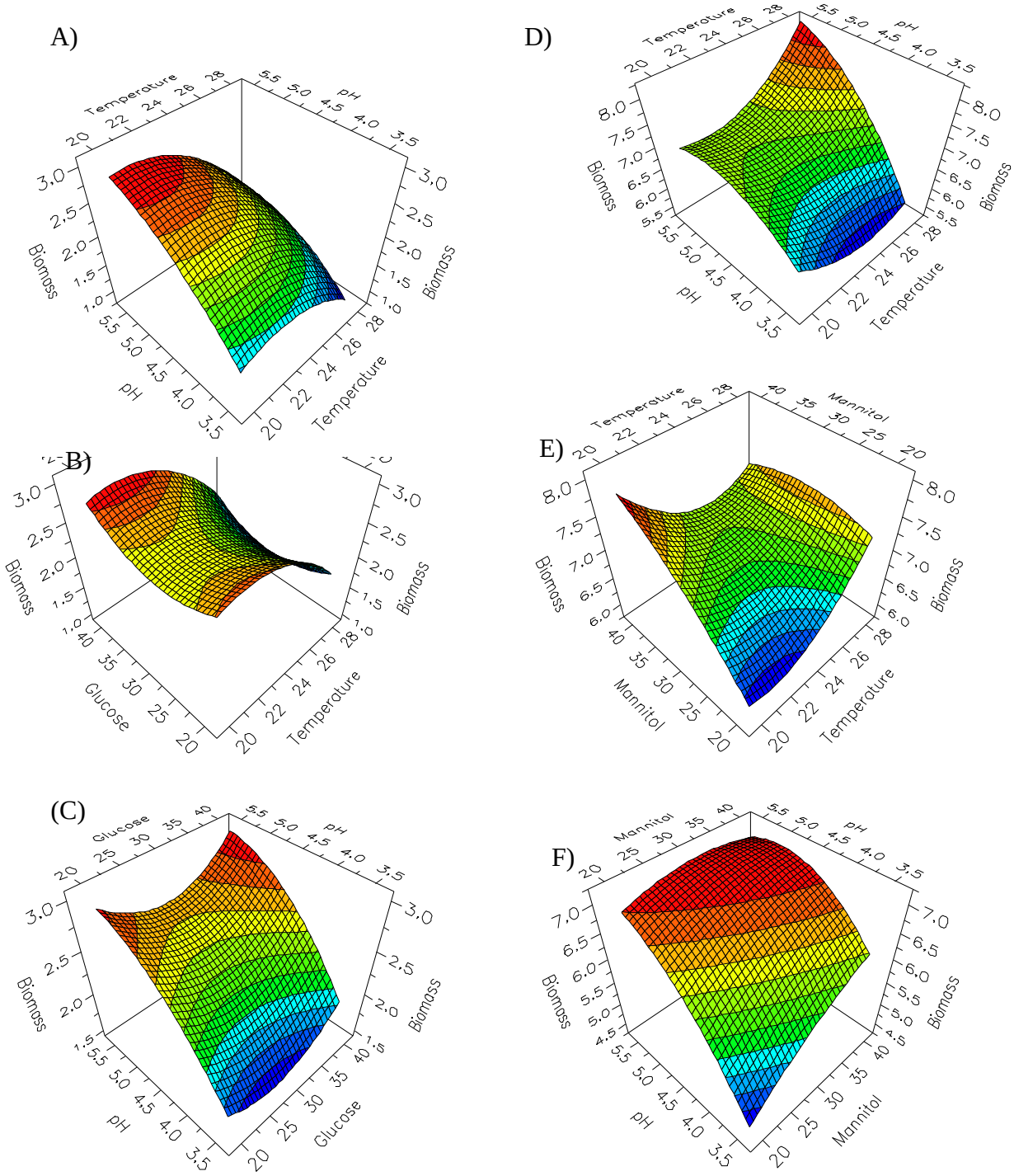


Fig.4 Response surface curve (3D plot) of mycelium biomass from *L. quieticolor* (A-C) and *R. roseolus* (D-F), showing the interaction between (A-D) pH and temperature, (B-E) carbon source and temperature and (C-F) pH and carbon source



Although the statistics, R^2 correlation coefficient, and Q^2predictivity (Table 3) represent the explanation rate of the total variability found in the response and the prediction rate that the model presents, respectively, these do not provide any information about the reliability of the model. For this, the regression F (model) must be greater than the lack of fit F. Furthermore, the *P* value of the regression must be less than 0.05 (Montgomery, 2002). Under these assumptions, the ANOVA (Table 4 A and B) for the biomass production (g L^{-1}) indicated that

the model for both fungal species was satisfactory. Therefore, the response surface for the biomass in the optimization stage was statistically reliable. The response surface image helped to evaluate the effect of any combination of two factors on the fungi growth; therefore, for the effects of the interactions pH-temperature, carbon source-temperature, and carbon source-pH, a favorable response could be achieved.

Conclusion

Through the optimization process using RSM, it was possible to establish the conditions of carbon source concentration, culture medium, pH, and culture temperature for the maximum production of biomass in liquid medium under agitated conditions with *R. roseolus* and *L. quieticolor*, prior to determination of the best culture medium and carbon source. In the stage following this study, biomass production will be scaled to bioreactors, under the already optimized conditions, with the aim of obtaining large amounts of *R. roseolus* and *L. quieticolor* biomass for application in inoculation tests of *P. radiata* nursery plants.

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Disclosure statement

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