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TECHNICAL NOTE

IN VITRO GROWTH OF NINE EDIBLE ECTOMYCORRHIZAL FUNGI UNDER A RANGE OF pH CONDITIONS

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ABSTRACT

Ectomycorrhizal fungi are considered to play an essential role in the development of forest ecosystems and can protect plant against pathogenic infections. Among other factors, soil pH may affect the successful inoculation of forest seedlings in nurseries. The effect of pH on the growth rate of strains of nine species of edible ectomycorrhizal (ECM) fungi was evaluated *in vitro*. In the experiments, *Boletus edulis*, *B. aereus*, *B. pinophilus*, *B. fragrans*, *Amanita rubescens*, *Xerocomus ferrugineus*, *Lactarius deliciosus*, *Lactarius sanguifluus* and *Suillus luteus* were grown in Petri dishes containing modified Melin Norkrans medium and adjusted at seven different pH levels. Colony area was measured at 7-day intervals for 8 weeks. Final fungal biomass and residual pH of the medium at 8th week were also measured. The optimum pH levels and pH tolerance ranges for the tested ECM fungal species are presented and discussed in the text. The results showed that the greatest growth *in vitro* was produced by *A. rubescens* and *S. luteus* at high pH levels (between 6.5-8.5), and by *X. ferrugineus* at low pH (3.5-6.5). Almost all the strains acidified the medium where they were grown after eight incubation weeks.

Additional keywords: Forest ecosystems, forest seedlings, fungus biomass, incubation, nurseries

RESUMEN

Crecimiento in vitro de nueve especies de hongos ectomicorrízicos comestibles bajo diferentes condiciones de pH

Se considera que los hongos ectomicorrízicos desempeñan un papel esencial en el desarrollo de los ecosistemas forestales y pueden proteger a las plantas contra infecciones patógenas. Entre otros factores, el pH del suelo puede afectar la inoculación exitosa de plántulas forestales en viveros. Se evaluó *in vitro* el efecto del pH sobre la tasa de crecimiento de cepas de nueve especies de hongos ectomicorrízicos comestibles (EMC). En los experimentos, se cultivaron *Boletus edulis, B. aereus, B. pinophilus, B. fragrans, Amanita rubescens, Xerocomus ferrugineus, Lactarius deliciosus, Lactarius sanguifluus* y *Suillus luteus* en placas de Petri que contenían medio Melin Norkrans modificado, y se ajustaron a siete niveles de pH diferentes. El área de la colonia se midió a intervalos de 7 días durante 8 semanas. También se midieron la biomasa fúngica final y el pH residual del medio a la octava semana. Los niveles de pH óptimos y los rangos de tolerancia de pH para las especies de hongos EMC probadas se presentan y analizan en el texto. Los resultados mostraron que el mayor crecimiento *in vitro* lo produjeron *A. rubescens* y *S. luteus* a pH alto (entre 6,5-8,5), y *X. ferrugineus* a pH bajo (3,5-6,5). Casi todas las cepas acidificaron el medio donde crecieron después de ocho semanas de incubación.

Palabras clave adicionales: Biomasa fúngica, ecosistemas forestales, incubación, viveros forestales

INTRODUCTION

The association between trees and ectomycorrhizal (ECM) fungi is very well-known and established (Boeraeve et al., 2018; Liu et al., 2020; Milton et al., 2021). Ectomycorrhizas, which are considered to play an essential role in the development of forest ecosystems (Domínguez and Albanesi, 2019), improve vigour and growth in forest plants (Sebastiana et al., 2013; Sultana et al., 2018; Liu et al., 2020). In addition to that, they have been shown to protect plant against pathogenic infections (Mohan et al., 2015; Milton et al., 2021; Kebert et al., 2022).

Inoculating artificially forest seedlings with specific ECM fungus is a recognized tool to improve seedlings survival and early growth in forest plantation programs as stated previously (Turjaman et al., 2006). The use of edible ECM fungi in forest plants inoculations could increase a multiple use and economic value of forest ecosystems, mainly in those of Mediterranean areas where timber production is usually very low. It has been stated that in many Spanish forests the economic value of the mushroom production has been at least similar than that of the wood (Díaz et al., 2003).

Optimum pH and pH tolerance are two of the most important criteria to select ECM fungi for seedlings inoculation programs in nurseries, and it is known that soil pH can strongly affect both ECM formation, with regard to the symbiotic

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species involved and their infective ability (Carrino et al., 2016; Glassman et al., 2017; Ge et al., 2017; Burke et al., 2021). Therefore, an ECM species, to be inoculated in plants used in afforestation programs, must be able to spread in a wide range of soil conditions.

In vitro growth in pure culture has been shown to be a very useful variable to test the ability of how ECM fungi develop under different pH levels (Daza et al., 2006; Sánchez et al., 2001). A great variability in the optimum pH level has also been stated both among species as well as among isolates (Matsuoka et al., 2016; Boeraeve et al., 2018). In a multi-year regional-scale survey, it was found that soil pH has the strongest effect on the diversity of fungi, and in conifer species richness has a positive effect on overall fungal diversity (Tedersoo et al., 2020). Therefore, the evaluation of several isolates of the same species is essential to test the suitability of ECM fungi at different pH levels, being aware that these *in vitro* conditions are just an indicator of what may happens in more realistic conditions including bioassays under field conditions, due to the more complex factors involved.

The aim of the present study was to evaluate *in vitro* the ability of nine strains of edible ECM fungi to grow under different pH levels with the goal of determining the optimum pH value for each strain. The incubation time, just before ECM growth decrease, was also determined in order to maximize the ECM inoculum production, which is an important limitation in the inoculation programs.

MATERIALS AND METHODS

Nine ECM fungi, which are described in Table 1, were collected in different forest stands of Spain, where a soil sample was extracted from the first 20 cm to determine its pH. Strains were isolated from basidiocarps. Strains were maintained in Petri dishes containing solid modified Melin Norkrans (MMN) medium at 22 ºC in the dark. Colonies were subcultured in fresh medium at intervals of three months to avoid degeneration of mycelium. Forty-five days before testing, all the strains were subcultured as explained above in order to obtain an aerial mycelium suitable for experiments. The identification of the species was conducted by macro and micromorphological characters.

Table 1. Edible ECM strains and site characteristics where fungi were isolated

Species	Code	Origin (Province)	Dominant vegetal species	Altitude (m)	Mean anual precipitation (mm)*	Soil pH
Amanita rubescens Pers.	Ar	Perales (Palencia)	*Quercus ilex y Quercus faginea*	810	410	6.1
Boletus aereus Bull.	Ba	Perales (Palencia)	*Quercus ilex y Quercus faginea*	810	410	6.3
Boletus edulis Bull.	Be	Torla (Huesca)	*Pinus uncinata*	1090	1504	5.1
Boletus fragrans Vitt.	Bf	Perales (Palencia)	*Quercus ilex y Quercus faginea*	810	410	6.4
Boletus pinophilus Pil. & Derm	Bp	Rionegro del Puente (Zamora)	*Castanea sativa*	960	990	5.2
Lactarius deliciosus (L.: Fr.) S.F. Gray	Ld	Osorno (Palencia)	*Pinus pinaster*	800	525	7.2
Lactarius sanguifluus (Paulet) Fries	Ls	Osorno (Palencia)	*Pinus pinaster*	800	525	7.2
Suillus luteus (L.: Fries) Roussel	Sl	Celadilla (Palencia)	*Pinus sylvestris*	980	630	5.7
Xerocomus ferrugineus (Schaeffer) Bon.	Xf	Perales (Palencia)	*Quercus ilex y Quercus faginea*	810	410	6.4

*Data from INM (Meteorology Nacional Institute) from Spain

A young, actively growing plug of mycelium (Ø = 5 mm) of each strain was transferred on to the surface of Petri dishes containing 20 mL of solid MMN medium adjusted at seven pH levels

ranging between 2.5 to 8.5 at one-unit intervals. The pH was adjusted using 1N HCl and 1N NaOH as required. Five replicates of each treatment were incubated at 22 ºC in the dark.

The evaluation of the fungal development at the different pH levels was assessed by the colony area and dry fungal biomass. Diameters of the colony were measured with a ruler to an accuracy of 1 mm at intervals of 7 days for 8 weeks, and the colony area was calculated with the mathematical formula $A = \frac{\pi}{4} \cdot (r_1 + r_2) \cdot (r_3 + r_4)$ where r_1, r_2, r_3 and r_4 are the four perpendicular colony radial measures. At the other hand, mycelium was harvested on a Whatman No.1 filter and oven dried at 80 ºC for 48 h (Srinivasan et al., 2000), after which dry biomass was weighed. After mycelium harvesting, final pH level of the culture medium was measured by means of a pH electrode meter.

Colony area and dry biomass, along with weekly average increases (WAI) of the colony area, after 8 weeks of incubation, were subjected to analysis of variance (ANOVA) and Bonferroni test for comparisons. Sixty-three treatments (nine fungi and seven pH values), with five replicates each, where established in the study. Assumptions of normality and homoscedasticity were assured by a Kolmogorov-Smirnov test and Levene´s test, respectively. In order to evaluate the association between the colony area and the dry biomass, a correlation analysis was performed. All the tests were conducted using the 99 ed. Statistica 5.5 software.

RESULTS

When the strains were compared with each other, *Amanita rubescens* (*Ar*) produced the greatest WAI of the colony (4.278±0.38) and *Boletus aereus* (*Ba*) the lowest (0.384±0.04). The strains Ar and *Suillus luteus* (Sl) showed their main response at high pH levels (between 6.5 and 8.5), and *Lactarius sanguifluus* (*Ls*) and *Lactarius deliciosus* (*Ld*) only at pH 6.5 and 8.5, respectively. At pH 2.5 no strains had good performance, although *Boletus pinophilus* (*Bp*) behaved well at low pH (between 3.5 and 6.5). The strains *Boletus fragrans* (*Bf*) and *Xerocomus ferrugineus* (*Xf*) showed a good WAI through the whole range of pH above 2.5. On the other hand, in the strain *Ba*, a clear optimum pH value was not observed, while it produced no growth at the highest pH (Table 2).

The WAI of the colony area was strongly affected by pH as well as the strain and their interactions. The variable ranged between 0 at pH 2.5 in *Ls* and 7.093 cm^2 at pH 6.5 in *Ar*. The strains showed similar pH preferences when colony area and dry biomass after 8 weeks were used as response variables (Table 3).

The trend of colony growth suggests interactions of pH with time (Figure 1). In this sense, *Ar* colonized the Petri dish completely at pH 6.5 in the 6^{th} week, so no more differences were found afterwards. Also, in the 8^{th} week, *Ld* showed the greatest growth at pH 8.5; however, until the 4^{th} week the greatest growth of this species occurred significantly at pH 6.5 and 7.5, and almost no response occurred from pH 2.5 through 5.5.

The correlation analysis carried out between the final colony area (FCA) and dry weight (DW), showed variable level of association with regard to the strain considered: in *Ld*, *Bp*, *Ar*, and *Ba*, the highest coefficient (r) was observed, whilst *Xf*, *Bf*, and *Sl* exhibited the lowest ones (Table 3).

When pH was measured at the end of the experiment (8 weeks later), it was observed a strong variation regarding the initial pH. In almost all the cases, a decrease at the pH level was observed, and the most important pH decreases occurred when the initial pH was very high. Only at low initial pH (2.5-3.5), some strains showed slight increases in the final pH, like *Bp* and both *Lactarius* species (Table 3).

DISCUSSION

Culture medium pH strongly affected the *in vitro* growth of all tested strains of ECM fungi, as it has been previously described for several ECM isolates (Sarker et al., 2007). It is clear that a vigorous growth is a very important aspect to select ECM fungi for seedlings inoculation programs, and the results presented in this paper showed *Ar*, *Xf* and *Ld* to produce the greatest colony area. However, optimum pH and pH tolerance should also be considered in that selection. The strains evaluated here showed significant differences as in their optimum pH preference and their tolerance to grow at different pH values. The strain *Sl* produced the greatest growth at pH 8.5 but also presented good growth

at pH 6.5 and 7.5, showing their basophilic preference such as it has been recorded in previous works (Sánchez et al., 2001, Khan et al., 2013). Additionally, this species has grown well in pH from 4 to 7 (Zhu et al., 2008), showing a wide tolerance range. Other wide ranges were observed in the strains of *Xf* which spread well at pH 3.5-7.5, and *Ar* and *Sl* at 6.5-8.5. On the other hand, *Bp* grew well at pH 4.5-6.5, showing a clear acidophilic preference. Vázquez et al. (2002) found that among seven species of ECM studied, most of them showed better growth at 4.0 to 7.0 pH, and in contrast, the strain of *Terfezia olbiensis* was the only one that grew better at pH of 8.0.

Table 2. Weekly average increases (WAI) of the colony area (cm^2) after 8-week incubation in nine ECM strains at different pH's

	pH							
Strain code (from Table 1)	**2,5**	**3,5**	**4,5**	**5,5**	**6,5**	**7,5**	**8,5**	**Mean***
Ar	0.515 d	4.244 bc	2.826 c	3.323 c	7.9093 a	5.545 ab	6.398 a	4.278 ± 0.38
Ba	0.303 bc	0.668 a	0.309 b	0.627 a	0.603 a	0.124 bc	0.055 c	0.384 ± 0.04
Be	0.280 c	1.078 b	1.290 ab	1.164 b	1.655 a	1.403 ab	0.000 c	0.981 ± 0.10
Bf	1.045 c	1.517 ab	1.796 ab	1.715 ab	1.364 bc	1.621 ab	1.945 a	1.572 ± 0.06
Bp	0.060 c	0.410 b	0.627 ab	0.730 a	0.895 a	0.065 c	0.043 c	0.404 ± 0.06
Ld	0.084 e	0.720 de	1.094 d	1.312 d	2.659 c	4.433 b	5.530 a	2.166 ± 0.32
Ls	0.000 e	0.319 d	0.548 d	0.813 c	1.674 a	1.190 b	1.005 cb	0.792 ± 0.09
Sl	0.374 c	1.067 bc	1.331 b	1.569 b	2.446 a	2.969 a	3.120 a	1.839 ± 0.17
Xf	1.193 c	3.502 a	3.334 ab	3.597 a	4.192 a	2.865 abc	1.645 bc	2.904 ± 0.22

Mean values in the same row with distinct letters are significantly different ($P \leq 0.05$) according to Bonferroni test.
*Average increments when combining all pH together.

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Although these results may suggest the ecological preference of the tested ECM fungi, important aspect to have into account in inoculation programs; they must, however, be regarded with caution, since it might be a lack of consistency between results obtained on agar and those on field conditions. Thus, further studies, including effects under *in vivo* conditions, would be required to confirm those preferences. Nevertheless, literature shows that several ECM species, like *Bp*, *Ld* and *Sl* isolates have similar pH preference on *in vitro* culture than that observed in the field where they were collected, and that many ECM fungi can grow better under acidophilic conditions (Yamanaka, 2003; Zhu et al., 2008). Results presented here, and those obtained by Sánchez et al. (2001), do not agree well with that statement, indicating a greater variation than that usually thought. These differences between *in vitro* and *in vivo* behaviour could be related with the more diverse composition of the substrates in nature. So, other organisms as mycorrhizal helper bacteria (bacteria that promotes the establishment of the root-fungus symbiosis–MHB) use to be present in the forest soils causing modifications on it (Rigamonte et al., 2010). Bacteria of the genus *Pseudomonas* is included in this groups of microorganisms in association with fungi and plants that can act as MHB. On the other hand, this study has been carried out with only one culture medium (MMN), and it is well known that the growth and conditions of fungi can change when they are cultivated on different growth media (Barros et al., 2006). That is why these results must be taken with caution, before being applied.

Regarding the minimum incubation time required to maximize the mycelial production, the results showed two important aspects to have into account. First of all, most of the strains did not decrease their growth rate along the eight weeks the experiment remained. The second aspect was that, in the cases where strains decreased their growth time before the experiment ended, this time depended on the pH level of the medium where it was grown. For instance, *Ld* growth at pH 5.5 and 6.5 decreased from the fourth week, but at all the other pH levels that decrease was not so evident.

The correlation between the final colony area and dry weight (biomass production) was high (r>0.8) in most of the cases (Table 3). As mentioned before, it was only low (r<0.5) in *Xf* and *Bf*. The lowest correlation values corresponded to those species which produce in their growth a large quantity of aerial mycelia made up of lax hyphae. Therefore, in such species, colony area would not be a good indicator of their growth, and the use of dry biomass, as response variable to estimate growth, would be better.

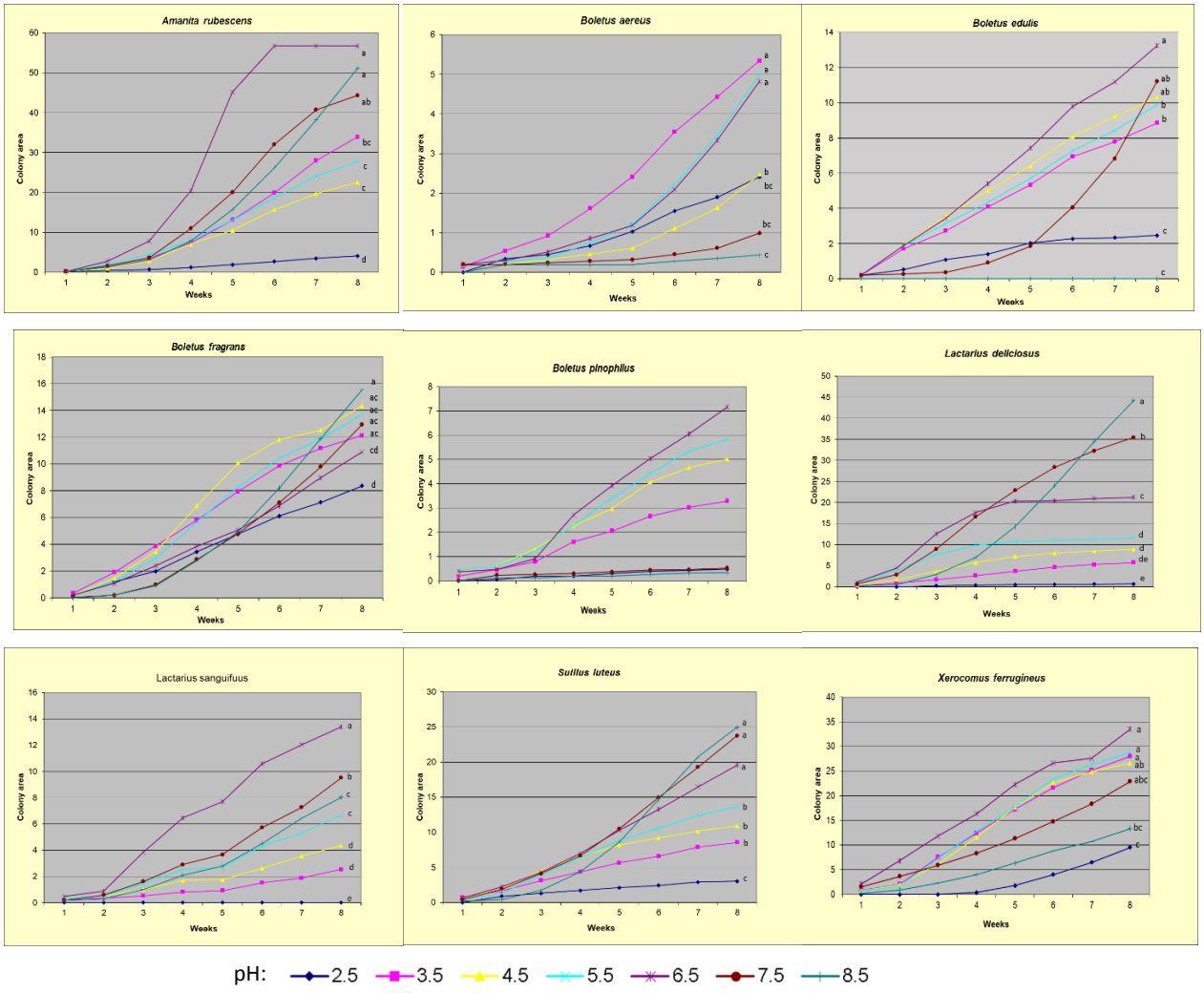

Figure 1. Average growth (cm^2) of the colony of nine species of ECM strains during eight weeks at the tested pH values. The lengths of Y-axis are different among the species

The pH preference of the ECM fungi was changing with time. This aspect must be considered in order to rank species in terms of pH preference since it could be different regarding the incubation time. This fact could be explained by the changes in the medium pH derived from the fungal activity. During the *in vitro* development of mycelia some ions are taken up, which should lead to a reduction in pH (Tang and Rengel, 2003). That explanation would be corroborated by the results obtained in the present work, which showed a decrease in the medium pH in almost all the strains at the end of the experiment, as it was expected in unbuffered conventional culture media (Yamanaka, 2003). Nevertheless, a slight pH increase of the culture medium was also observed in the lowest initial pH levels (2.5-3.5) in the strains of *Bp*, *Ls* and *Ld*, which were indeed collected from basic soil.

Table 3. Dry weight (DW, mg), final medium pH (FpH), and Pearson coefficient (r) of the correlation between the final colony area (FCA) and DW at the 8th week

	Amanita rubescens		*Boletus aereus*		*Boletus edulis*	
pH	**DW**	**FpH**	**DW**	**FpH**	**DW**	**FpH**
2.5	31.0 e	2.318 g	16.2	2.34	31.6 c	2.458
3.5	63.6 cd	2.600 f	30.2 a	2.50	54.8 b	2.456
4.5	47.6 ed	2.696 e	9.0 cd	3.93	52.0 bd	2.636
5.5	48.2 ed	2.806 d	23.8	3.13	57.4 b	2.618
6.5	124.4 a	3.098 c	27.6 a	3.87	70.0 b	2.866
7.5	82.4 bc	4.218 b	7.0 d	5.94	107.2 a	4.096
8.5	90.2 b	4.824 a	4.2 d	5.88	1.8 d	6.200
r	FCA-DW = 0.917		FCA-DW = 0.907		FCA-DW = 0.844	

	Boletus fragrans		*Boletus pinophilus*		*Xerocomus ferrugineus*	
pH	**DW**	**FpH**	**DW**	**FpH**	**DW**	**FpH**
2.5	41.2 c	2.266 e	1.0 c	2.78	14.4 d	2.464
3.5	63.4 bc	2.444	23.2 b	3.00	45.4 c	2.424
4.5	60.4 bc	2.592 d	36.6 a	2.76	56.6 bc	2.502
5.5	57.2 bc	2.636	41.6 a	2.85	56.8 bc	2.52 d
6.5	59.2 bc	2.868 c	48.4 a	3.24	77.4 ab	2.756
7.5	94.2 a	3.974 b	9.6 c	6.40	95.8 a	4.034
8.5	76.0 b	4.608 a	1.0 c	6.43	74.4 ab	4.846
r	FCA-DW = 0.496		FCA-DW = 0.941		FCA-DW = 0.448	

	Lactarius deliciosus		*Lactarius sanguifluus*		*Suillus luteus*	
pH	**DW**	**FpH**	**DW**	**FpH**	**DW**	**FpH**
2.5	11.6 c	2.722 c	6.0 f	2.61	49.8 c	2.354 f
3.5	38.4 bc	2.774 c	10.8	3.64	53.0 c	2.56 ef
4.5	39.8 bc	2.810 c	18.0	3.93	47.0 c	2.668
5.5	53.4 bc	2.724 c	38.8	4.05	59.0 bc	2.806
6.5	83.8 ab	2.938 c	43.4 a	4.03	63.6 ab	3.242
7.5	121.0 a	4.120 b	30.2	5.50	82.2 a	4.344
8.5	108.2 a	5.426 a	27.2	5.94	71.4 ab	4.928
r	FCA-DW = 0.967		FCA-DW: 0.862		FCA-DW = 0.649	

Values in the same row with distinct letters are significantly different according to Bonferroni test ($P \leq 0.05$)

CONCLUSION

This paper presents the optimum pH levels and pH tolerance ranges *in vitro* for the tested ECM fungal species. The greatest growth at high pH levels (between 6.5-8.5) was produced by *Amanita rubescens*, followed by *Lactarius deliciosus* and *Suillus luteus*. In general, the growth decreased with the pH, and no strain had good behaviour at pH 2.5, although *Boletus fragrans* and *Xerocomus ferrugineus* showed a good performance through the whole range of pH above 3.5. The growth increase rate of the colony area was strongly affected by pH as well as the strain. Almost all the strains acidified the medium where they were grown after eight incubation weeks. The

production of edible mushrooms *in vitro* is of great interest due to its possibilities in reforestation, and even for its industrial production, where its demand only grows. This work is a contribution to the cultivation of edible species of interest, and will undoubtedly help increase the possibilities of future commercialization. However, future *in vivo* tests are needed to establish the real possibilities of micorrhization with these species in the forest.

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