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*Full Length Research Paper*

# Mycelial growth requirements of *Lactarius pyrogalus* and *Lactarius controversus*

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**The objective of this study was to determine the requirements including culture medium, temperature, pH, carbon and nitrogen sources for the best mycelium growth of *Lactarius pyrogalus* and *Lactarius controversus*. Different inoculum media containing peat and peat : vermiculite mixtures (1:4, 1:6, 1:8 and 1:10 v:v) were also investigated for vegetative inoculum production. In terms of mycelium growth rate and mycelial characteristics, the most suitable culture media were potato dextrose agar and biotin aneurin folic acid agar for *L. pyrogalus* and *L. controversus*. Although *L. pyrogalus* had higher mycelium growth rate than *L. controversus,* they showed similar trend at different temperature. 25°C and 4.5 to 6.0 pH values gave the best results for mycelial growth of these mushrooms. Mannitol, glucose, dextrose and maltose were the most suitable carbon sources for *L. pyrogalus*, while mannitol, and lactose were the best carbon sources for *L. controversus* and significantly enhanced mycelial growth. Malt extract, peptone, yeast extract and $Ca(NO_3)_2$ were found to be the most suitable nitrogen sources for both *Lactarius* species, except for peptone in *L. controversus*. The lowest mycelial growth was determined in xylose among carbon sources, and $NH_4NO_3$ and $(NH_4)_2HPO_4$ among nitrogen sources for both *Lactarius* species. Peat : vermiculite mixtures in the rate of 1:4 and peat were the most suitable vegetative inoculum medium for *L. pyrogalus* and *L. controversus*, respectively, when the mycelium growth rate and days to complete mycelium running took into consideration.**

**Key words:** *Lactarius pyrogalus, Lactarius controversus,* growth condition, mycelial growth.

## INTRODUCTION

*Lactarius pyrogalus* (Bull.) Fr. and *L. controversus* (Pers.) are edible ectomycorrhizal fungi, which belong to the class basidiomycetes, the order *Russulales* and the family *Russulaceae* (Heilmann-Clausen et al., 1998). These two fungi species are widely distributed in the Black Sea region of Turkey (Peksen and Karaca, 2000).

*L. pyrogalus* is a delicious edible mushroom and it is known as the hazel milk-cap in Turkey. It grows naturally in spring and autumn seasons under hazel trees (Peksen and Karaca, 2003) and forms ectomycorrhizal association with *Corylus avellana. L. pyrogalus* is an excellent source of protein, dry matter, ash and mineral elements such as potassium, magnesium, phosphorus, ferrous and mangan (Peksen et al., 2008).

*L. controversus,* namely willow or acrid mushroom, is another popular edible mushroom existed in macrofungi flora of Turkey and it associates with species of *Populus* and *Salix* (Peksen and Karaca, 2000). Although most of people in many parts of the world doesn't prefer *L. controversus* due to its very acrid taste, people in the Black Sea Region of the Turkey commonly consume it. It was also reported that *L. controversus* is rich in protein and mineral elements (Peksen et al., 2007).

During rainy periods in both spring and autumn seasons, large quantities of these mushrooms are collected from hazelnut orchard and forest, respectively. *L. pyrogalus* and *L. controversus* are preferred by the consumers because of its delicious flavor and nutritional value. They are sold in local or public markets or directly

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**Table 1.** Chemical composition of the culture media used in the study.

| Medium | Chemical composition |
|---|---|
| PDA | 200 g potato, 20 g dextrose, 20 g agar, 1 l distilled water |
| BAF | 30.0 g glucose, 2.0 g peptone, 0.2 g yeast extract, 0.5 g $KH_2PO_4$, 0.5 g $MgSO_4.7\ H_2O$, 10.0 mg $FeCl_3.6\ H_2O$, 1.0 mg $ZnSO_4.7\ H_2O$, 5.0 mg $MnSO_4$, 100.0 mg $CaCl_2.2\ H_2O$, 50.0 µg thiamine HCl, 1.0 µg biotin, 100.0 µg folic acid, 50.0 µg inositol, 15 g agar, 1 l distilled water |
| ME | 20 g malt extract, 15 g agar, 1 l distilled water |
| MMN | 10 g glucose, 3 g malt extract, 0.25 g $(NH_4)_2HPO_4$, 0.025 g NaCl, 0.5 g $KH_2PO_4$, 0.05 g $CaCl_2$, 0.15 g $MgSO_4.7\ H_2O$, 0.012 g $FeCl_3$. 6 $H_2O$, 0.003 g thiamine, 15 g agar, 1 l distilled water |
| M40 | 10 g glucose, 5 g malt extract, 0.25 g $(NH_4)_2\ HPO_4$, 66.8 mg $CaCl_2$. 2 $H_2O$, 500 mg $KH_2PO_4$, 25 mg NaCl, 150 mg MgSO4.7 $H_2O$, 10 mg thiamine, 0.5 ml %1'lik FeCl, 15 g agar, 1 l distilled water |

PDA: potato dextrose agar, BAF: biotin aneurin folic acid agar, ME: malt extract agar, MMN: modified Melin-Norkrans agar, M40: modified M40 medium.

consumed by the family members of the collectors. These mushrooms have high economic importance because of source of income and also valuable human food for rural public. Recently, remarkable decrease was occurred in quantity of these mushrooms collected from the nature in Turkey. The researches conducted to understand the symbiotic growth and development of ectomycorrhizal mushrooms are not only on the improvement and practice of symbiotic field cultures but also on the development of pure cultures. Maintenance of fungi in pure cultures, preparation of fungal inoculum, and inoculation of seedlings are several steps of artificial mycorrization. Preparation of vegetative inoculum is an important process to supply inoculum required for inoculation and to guarantee ectomycorrhizal formation on seedling roots. Calam (1971) reported that the mycelial growth depends on some factors such as culture media, pH, temperature and nutrient elements. These factors greatly affect the formation and growth of ectomycorrhizal fungi both in the laboratory condition and field (Lilleskov et al., 2002). Many studies have determined the optimal growth conditions and nutritional requirements for the mycelial growth and the cultivation of ectomycorrhizal mushroom species (Baar et al., 1997; De Araujo et al., 1998; Guerin-Laguette et al., 1998; Guerin-Laguette et al., 2000; Sanchez et al., 2001; Yamada et al., 2001; Parlade et al., 2004; Flores et al., 2005; Xu et al., 2008; Guler and Ozkaya, 2009). However, there isn't any report on the growth conditions and the nutritional requirements to improve mycelial growth of *L. pyrogalus* and *L. controversus*. The objectives of this study were to determine the suitable culture medium and growth conditions such as temperature, pH, carbon and nitrogen sources for the best mycelial growth of *L. pyrogalus* and *L. controversus* and to reveal response of mushroom species to those factors. The study was also aimed to determine the most suitable medium for vegetative inoculum production.

## MATERIALS AND METHODS

### Sporocarp isolation

*L. pyrogalus* and *L. controversus* mushrooms samples were collected from the Samsun province of Turkey during autumn season and identified according to conventional description method by Prof. Dr. Annemieke Verbeken from the University of Gent, Belgium (Heilmann-Clausen et al., 1998). The pure mycelial culture was maintained by propagating pieces of the fruit-body caps on Modified Melin Norkrans medium (Jonathan and Fasidi, 2003) the cultures were stored at 4°C, and subcultured every 3 months (Brundrett et al., 1996).

### Effect of nutrient media on mycelial growth

To determine the effect of different culture media on the mycelial growth of *L. pyrogalus* and *L. controversus,* the following nutrient media were used: (1) potato dextrose agar (PDA); (2) biotin aneurin folic acid agar (BAF) (3) malt extract agar (ME); (4) modified Melin-Norkrans agar (MMN); (5) modified M40 medium. Chemical compositions of the nutrient media are given in Table 1. Prepared media were autoclaved at 121°C and 15 psi pressure for 20 min and each medium was poured into 9 cm Petri dishes. The plates were inoculated with an agar-mycelium disc (5 mm in

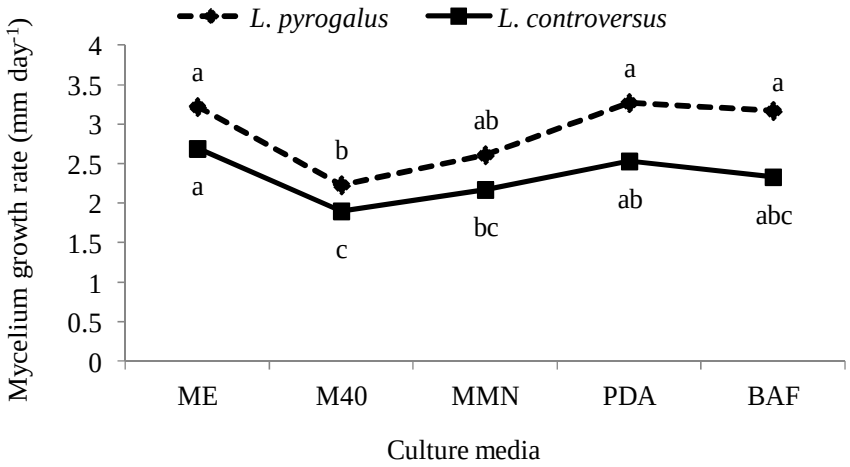


**Figure 1.** Effect of culture media on mycelium growth rate (mm $day^{-1}$) of *L. pyrogalus* (upper line) and *L. controversus* (lower line). Means followed by the same letters on the same line are not statistically different by Duncan's multiple range test *(P* < 0.01).

diameter), sealed with parafilm and incubated at 25°C in the dark. The experimental design was a completely randomized design (CRD) with 6 replications.

**Effect of initial pH and temperature on mycelial growth**

The effects of different temperatures and pH on mycelial growth of *L. pyrogalus* and *L. controversus* were investigated on PDA and BAF. The pH values of the media were adjusted to 4.0, 4.5, 5.0, 5.5 and 6.0 by adding of NaOH or (HC1), and media were autoclaved at 121°C for 20 min PDA and BAF media were poured into the 9 cm diameter Petri dishes and inoculated with a 5 mm in diameter mycelial discs of *L. pyrogalus* and *L. controversus* and incubated at 15, 20, 25 and 30°C in the dark with 5 replications.

**Effect of carbon and nitrogen sources on mycelial growth**

Seven carbon sources including glucose, lactose, maltose, dextrose, mannitol, xylose and sucrose were tested to determine their effects on mycelial growth. Each of them was added to the MMN medium separately as a carbon source (10 g $1^{-1}$). The MMN medium without carbon was used as C-free control medium. Malt extract, yeast extract, peptone, $(NH_4)_2HPO_4$, $NH_4NO_3$ and $Ca(NO_3)_2$ were nitrogen sources. Nitrogen sources were separately added into the MMN medium at a concentration of 3.25 g $1^{-1}$ instead of 3 g malt extract and 0.25 g $(NH_4)_2HPO_4$ which are presented in the MMN medium. The MMN medium without nitrogen was used as N-free control medium. The media were autoclaved at 121°C for 20 min and each medium was poured into 9 cm Petri dishes. The plates were inoculated with an agar-mycelium disc (5 mm in diameter) of *L. pyrogalus* and *L. controversus* and incubated at 25°C in the dark with 8 replications.

Mycelium growth rate (mm $day^{-1}$) was determined by daily measurements from the two different sections of the colony diameter using a digital caliper, and was calculated to obtain a final value. Morphological characteristics of the mycelia such as colony texture, color and mycelial density were noted. Mycelial density was also observed visually as 0: No growth, 1: Very sparse, 2: Sparse, 3: Moderate, 4: Dense, 5: Very dense.

**Vegetative inoculum preparation**

Five different vegetative inoculum media, containing peat (P) and peat:vermiculite (P:V) mixtures (1:4, 1:6, 1:8 and 1:10 v:v), were tested in this study. Vegetative inoculum media, in 250 ml small culture bottles containing 230 ml of peat or different peat:vermiculite mixtures, were autoclaved at 121°C for 1.5 h. After 24 h, 80 ml BAF liquid medium were added into vegetative inoculum media and autoclaved again at 121°C for 30 min. The moisture c ontent (Kacar, 1994) and pH (Rowell, 1996) of vegetative inoculum media were determined after sterilization. The small culture bottles were separately inoculated with 3 mycelial discs of 5 mm diameter of *L. pyrogalus* and *L. controversus*. The inoculated bottles were incubated at 25°C in the dark with 5 replications. My celium growth rate (cm $day^{-1}$) was determined by daily measurements from the two different sections of the culture bottles. The number of days from inoculation to time that bottle completely covered by mycelium was recorded as days to complete mycelium running (days).

**Statistical analysis**

The data obtained from the experiments were subjected to analysis of variance using the SPSS statistical programme and means showing statistical significance were compared by Duncan's multiple range test.

## RESULTS AND DISCUSSION

The best mycelium growth was obtained from PDA, ME, BAF and MMN media and the lowest mycelial growth was determined on M40 *(P < 0.01*) in *L. pyrogalus.* ME, PDA and BAF media gave the highest mycelium growth rate while the lowest mycelial growth rate was recorded on MMN and M40 *(P < 0.01*) in *L. controversus.* Mycelium growth rate of *L. pyrogalus* was found to be higher than that in *L. controversus* (Figure 1). Mycelia on all culture media were white and cottonish according to visual observations, and both *Lactarius* species had high

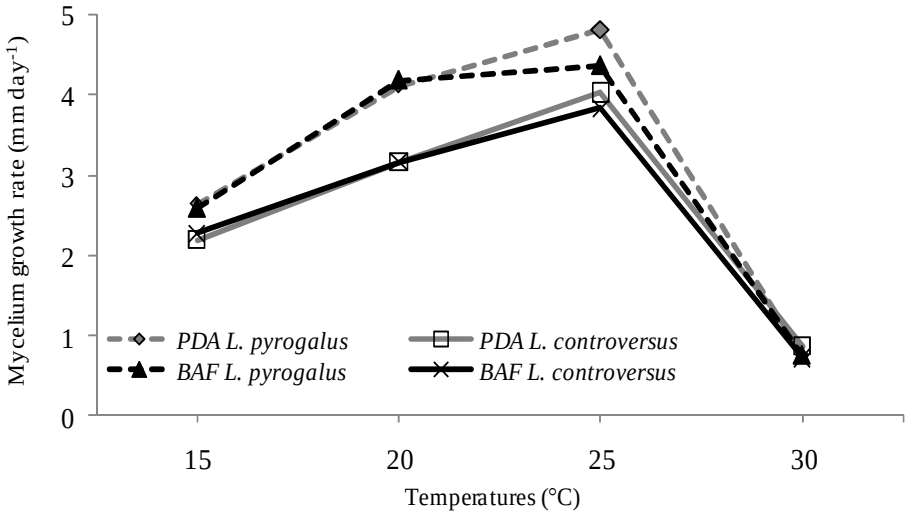


**Figure 2.** Effect of temperatures on mycelium growth rate (mm $day^{-1}$) of *L. pyrogalus* and *L. controversus* growth on PDA and BAF.

mycelia density on PDA and BAF media as compared to the other media.

MMN has been referred as the most commonly used medium and it is usually offered for the best mycelial growth in studies on ectomycorrhizal fungi (Marx and Kenney, 1982; Kumar and Satyanarayana, 2002). However, effect of culture media on the mycelial growth varies with mushroom species. Our results were in agreement with the results of De Araujo et al. (1998) stated that PDA is favorable for the mycelium growth of *Pisolithus tinctorius, Suilllus collinitus, Lactarius deliciosus* and *L. sanguiflius* strains. In another study, the highest growth rate and colony diameter for *Laccaria bicolor* were obtained from ME and BAF media, whereas the highest biomass production was observed in BAF and SAB media. The MNM, ING and HG media gave the lowest values for diameters and biomass production of the colonies (Santiago-Martinez et al., 2003).

PDA medium was better than BAF medium for mycelia growth in both *L. pyrogalus* and *L. controversus*. Althought mycelium growth rate of *L. pyrogalus* on all media was higher than that in *L. controversus,* the mycelium growth of *L. pyrogalus* and *L. controversus* on both PDA and BAF culture media showed similar trend depending on the temperature. Statistical analysis of the data on the mycelium growth rates revealed that 25℃ were the best temperature for mycelial growth of *L. pyrogalus* and *L. controversus* in both PDA and BAF media, followed by 20℃. The lowest mycelium growth was determined at 30℃ for both *Lactarius* species (Figure 2). The optimal growth temperature of *Amanita caesarea* was 24 to 28℃ (Daza et al., 2006). Xu et al. (2008) reported that the optimum temperature for three pure strains including *Boletus edulis, Lactarius deliciosus* and *L. insulsus* ectomycorrhizal fungi was 25 to 28℃ and high temperatures induced the fungi death. Kalyoncu et al. (2009) found that optimal temperatures for six *Morchella* species were 25 and 20℃.

The mycelium growth was low at 15℃ and drastically decreased at 30℃ (Figure 2). The mycelial growth reduction at 15℃ and above 25℃ might be sourced f rom the drastically reducing metabolic activities of the fungus that allowing the absorption of essential nutrients needed for growth (Garraway and Evans, 1984). In the present study, a weak mycelial growth at 30℃ could be attr ibuted to the denaturation of important enzymes which catalyze fungal metabolic processes (Jonathan et al., 2004). The mycelium of *L. pyrogalus and L. controversus* did not show any change in their color on both PDA and BAF culture media at all temperatures. However, the mycelia growth was found to be slender and the pellet of mycelia was compact at 30℃.

For the mycelium growth rate, the response of *L. pyrogalus* and *L. controversus,* growth on PDA and BAF, to different pH level of the media varied between 4.0 and 6.0 is shown in Figure 3. While the mycelium growth of *L. controversus* on PDA at pH 4.0 decreased, the mycelium growth of *L. pyrogalus* on PDA at investigated pH values was found to be non significant. The mycelial growth evidently decreased on BAF medium at pH 4.0 and 4.5 in *L. pyrogalus* and *L. controversus,* respectively. It was clearly shown that the effects of the initial pH of medium on mycelium growth varied with culture media and the mushroom species. The best suitable pH range for mycelium growth rate was found between 4.5 and 6.0 in both *Lactarius* species and also PDA and BAF culture media. According to visual observations, pH level of the PDA and BAF media had no significant effect on the morphological characteristics of the mycelia. The mycelia were white and cottony. Han et al. (1993) reported that

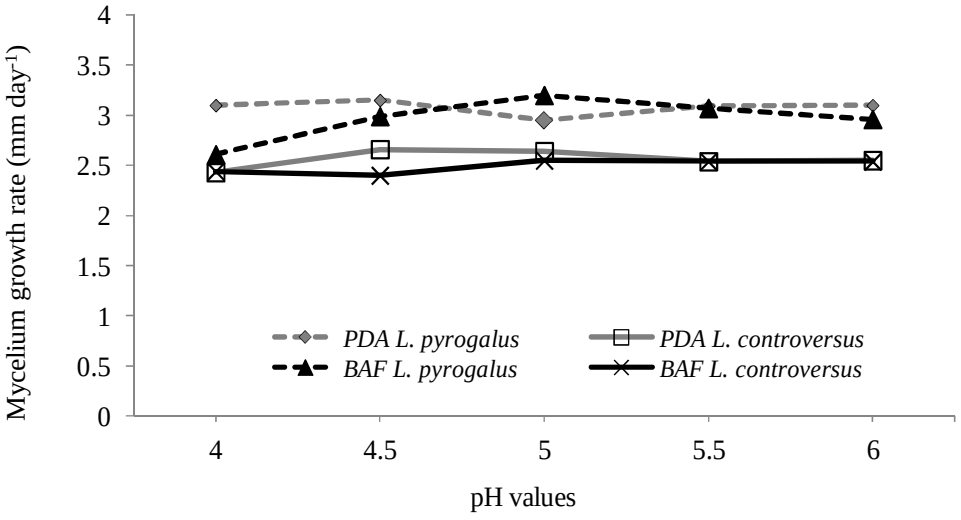


**Figure 3.** Effect of pH level on mycelium growth rate (mm $day^{-1}$) of *L. pyrogalus* and *L. controversus* growth on PDA and BAF.

acidic growth conditions are favorable for mycorrhizal fungi. Xu et al. (2008) stated that pH had a slight effect on the growth of the three ectomycorrhizal fungi, and the optimum pH values were 6.0 for *L. deliciosus,* 5.0 for *B. edulis,* 6.0-7.0 for *L. insulsus,* respectively.

Temperature and pH are the important environmental factors that promoting the growth of fungi (Garraway and Evans, 1984). There was a significant *(P < 0.01)* interaction between temperature and pH in terms of mycelial growth rate in both *Lactarius* species, and the best mycelial growth was determined in pH between 4.5 and 6.0 at 25℃ (Figure 4).

Carbon sources had significant effect on mycelia growth rate in both *Lactarius* species. Mannitol, glucose, C-free, dextrose and maltose gave the highest mycelial growth rate in *L. pyrogalus* while mannitol, C-free and lactose were the highest for *L. controversus* (*P* < 0.01) (Figure 5). However, mycelial growth in the C-free medium was weak and loosely woven in both *Lactarius* species. The lowest mycelial growth rate was determined in xylose in both *Lactarius* species. Our results were in agreement with Daza et al. (2006) stated that mannitol produced the largest radial growth, and mannitol and glucose yielded the highest mycelium dry weights, but xylose inhibited the growth of all the strains of *A. caesarea* and also by Fasidi and Akwakawa (1996) and Jonathan and Fasidi (2003) who reported mannitol as the most suitable sugar alcohol for the growth of mushrooms. The best vegetative mycelium development of *M. conica* was obtained from malt extract agar (ME), wheat agar (WA), potato dextrose agar (PDA) and complete medium yeast extract agar (CYM), however, moderate development occurred in defined media containing glucose, sucrose, maltose and starch (Guler and Ozkaya, 2008).

Among nitrogen sources, the highest mycelium growth rate (*P* < 0.01) for *L. pyrogalus* were recorded in the media containing of malt extract, yeast extract, peptone and $Ca(NO_3)_2$. Yeast extract, malt extract and $Ca(NO_3)_2$ were determined to be the most suitable nitrogen source for the mycelial growth of *L. controversus* (Figure 6). However, N-free media in the both *Lactarius* species have low mycelial density. Their mycelial growth was weak and loosely woven when compared to the others. On the other hand, mycelial density in the medium contained peptone, malt extract and yeast extract was very good. Oort (1981) had been reported that inclusion of yeast extract is essential for growth in genera *Lactarius.* The presence of nitrate ion has a negative effect on the development of some ECM fungi (Griftin, 1994). Ammonium is generally recognized as the most readily utilable source of N for the most of ECM fungi (Smith and Read, 1997; Rangel-Castro et al., 2002; Sangtiean and Schmidt, 2002), but the results from the present study of *L. pyrogalus* and *L. controversus* do not support this assertion. Our results is contrary to that of Daza et al. (2006) who reported that the greatest mycelium dry weight yields of *A. caesarea* were obtained with ammonium. Guler and Arkan (2000) stated that *M. esculenta* mycelia have a very slow growth rate in agar media to which sodium nitrate has been added. It has been observed that the colonization characteristics of vegetative mycelium developed in solid nutritional environ ments showed differences for mycelium quality. The effect of nitrogen source on the mycelia growth depended on species, culture media and growth conditions (Lin and Yang, 2006). The lowest mycelial growth was found in $NH_4NO_3$ and $(NH_4)_2HPO_4$ for *L. pyrogalus* and *L. controversus*. Mycelial density in the both control media (C-free and N-free media) was low due to limited nutrition in the growth media.

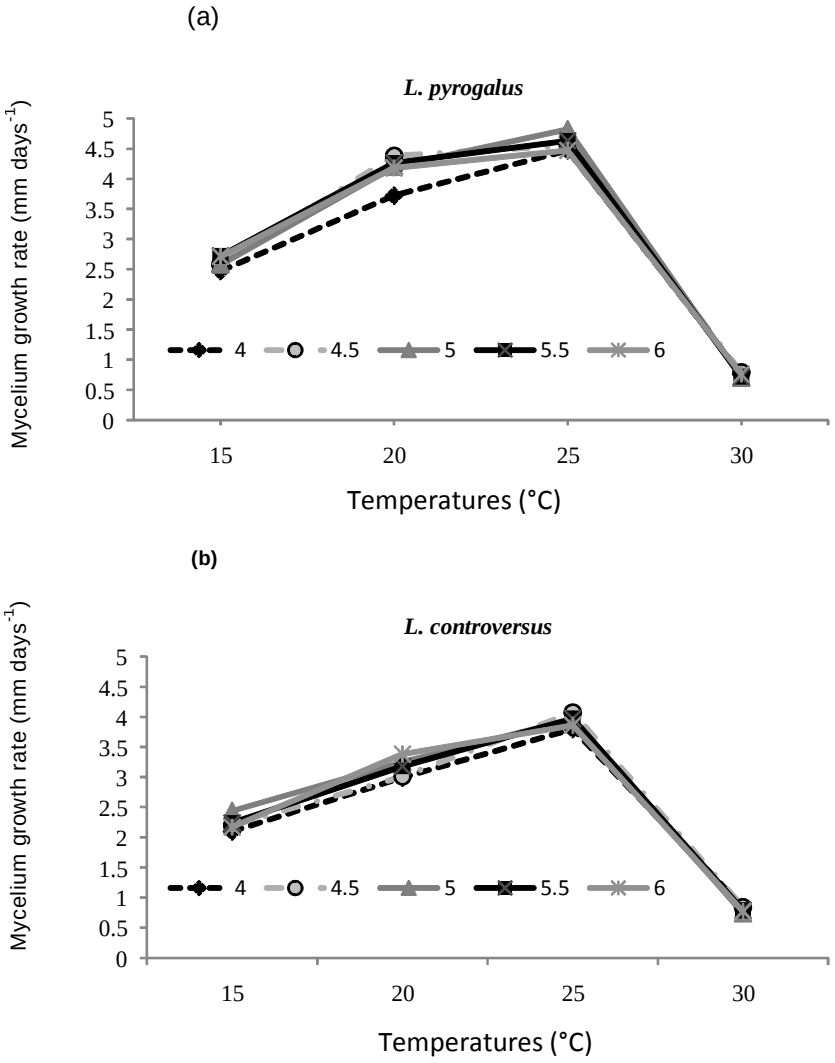


**Figure 4.** Effects of pH levels of the growth media on mycelium growth rate of *L. pyrogalus* (a) and *L. controversus* (b) at different temperatures.

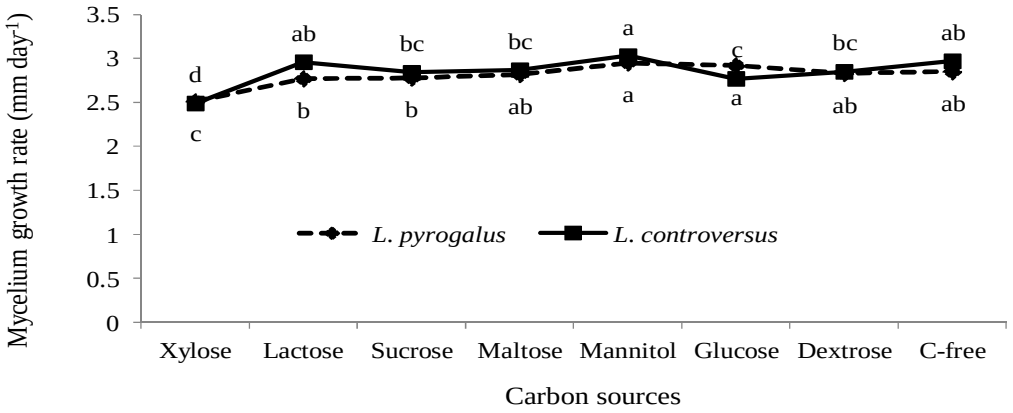


**Figure 5.** Effect of carbon sources on mycelium growth rate (mm day$^{-1}$) of *L. pyrogalus* (lower line) and *L. controversus* (upper line). Means followed by the same letters on the same line are not statistically different by Duncan's multiple range test *(P* < 0.01).

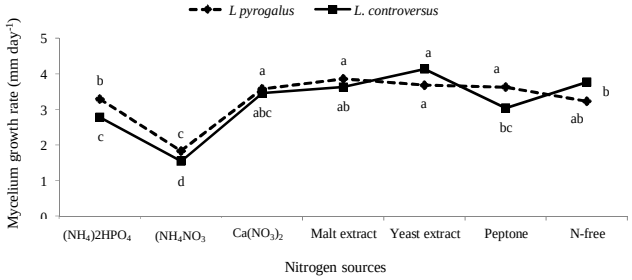


**Figure 6.** Effect of nitrogen sources on mycelium growth rate (mm day$^{-1}$) of *L. pyrogalus* (upper line) and *L. controversus* (lower line). Means followed by the same letters on the same line are not statistically different by Duncan's multiple range test *(P* < 0.01).

**Table 2.** Moisture contents and pH values of the vegetative inoculum media and their effects on mycelial growth.

| Media | Moisture (%) | pH after sterilization | *L. pyrogalus* | | *L. controversus* | |
|---|---|---|---|---|---|---|
| | | | Mycelium growth rate (cm day$^{-1}$) | Days to complete mycelium running | Mycelium growth rate (cm day$^{-1}$) | Days to complete mycelium running |
| | 51.8 | 5.25 | 0.64b* | 17.00a** | 0.56a** | 19.60c** |
| P:V (1:4) | 48.2 | 5.65 | 0.76a | 13.40d | 0.27b | 33.20b |
| P:V (1:6) | 47.5 | 5.80 | 0.72a | 14.00cd | 0.25b | 33.40b |
| P:V (1:8) | 47.1 | 5.95 | 0.70ab | 16.00ab | 0.24b | 36.60a |
| P:V (1:10) | 42.9 | 6.00 | 0.72a | 15.40bc | 0.24b | 37.40a |

P (Peat), P:V (Peat:Vermiculite), *significant at the 0.05 probability levels, **significant at the 0.01 probability levels (Means followed by the same letters in the columns are not statistically different by Duncan's multiple range test).

Moisture contents and pH values of the vegetative inoculum media were found between 42.9 and 51.8%, and 5.25 and 6.00, respectively. Responses of *Lactarius* species to vegetative inoculum media were different. In *L. pyrogalus*, the fastest growth and the shortest time were determined on peat:vermiculite mixture in the rate of 1:4. Mycelia showed the fastest growth and also completed its growth in the shortest time on peat in *L. controversus* (Table 2).

PDA and BAF were the most suitable culture media for *L. pyrogalus* and *L. controversus* when the mycelium growth rate and mycelial characteristics took into consideration. 25$^{o}$C was the peak point for mycelium growth of *Lactarius* species, than mycelium growth drastically decreased at 30$^{o}$C in PDA and BAF. pH levels between 4.5 and 6.0 were found to be suitable for mycelium growth of *Lactarius* species. The best mycelial growth for *L. pyrogalus* was determined in the presence of dextrose, mannitol, glucose and maltose as carbon sources, and malt extract, peptone, yeast extract and $Ca(NO_3)_2$ as nitrogen sources. Mannitol and lactose as carbon sources and yeast extract, malt extract and $Ca(NO_3)_2$ as nitrogen sources were the most suitable for mycelial growth of *L. controversus.* The lowest mycelial growth was determined in $NH_4NO_3$ and $(NH_4)_2HPO_4$ among N sources for *Lactarius* species. In recent years, productivity of *L. pyrogalus* which is an ectomycorrhizal mushroom is reduced in hazelnut orchard in Black Sea region of Turkey. Ammonium nitrate is commonly used in hazelnut orchards as a main nitrogenous fertilizer. This fertilizer can have a negative effect on *L. pyrogalus* productivity. Therefore, the effect of different fertilizers on both productivity of *L. pyrogalus* and host plant development should be investigated in more detail. Thus the preliminary study on nitrogen fertilizers of *L. pyrogalus* would be able to help in understanding the process of mycorrhization in hazelnut orchards. The best vegetative inoculum medium was peat:vermiculite mixtures in the rate of 1:4 for *L. pyrogalus* and peat for *L. controversus*.

As a result, *L. pyrogalus* and *L. controversus* are important local edible mushroom species due to their common consumption in the rural population and economic value in the markets. Thus, obtaining of new

knowledges on growth conditions and nutritional requirements of *L pyrogalus* and *L. controversus* would be useful for improving cultivation technology of these mushroom species.

## ACKNOWLEDGEMENTS

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