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Pure culture isolation and optimal conditions for the mycelia growth of *Lactarius sanguifluus*: An edible ectomycorrhizal mushroom

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Abstract
The aim of the study was to evaluate different solid media for the best mycelial growth of *Lactarius sanguifluus*. Ten solid media tested for best mycelial growth of *L. sanguifluus* and out ten solid media best mycelial growth was observed in Potato Dextrose Agar Medium. Temperature of 25°C was observed as optimum temperature of maximum growth of mycelium of this mushroom. PH 5.0 was observed as best pH for mycelial growth whereas the mycelium was found to grow better under the dark conditions in comparison to light.

Keywords: *Lactarius sanguifluus*, ectomycorrhizal mushroom, mycelial growth

Introduction
Lactarius sanguifluus, commonly known as the bloody milk cap, is belonging in the family russulaceae. *L. sanguifluus* is an edible and ectomycorrhizal fungi fruiting bodies grow scattered or in groups on the ground under conifers forest. When bruised or cut, the fruit bodies ooze a blood-red to purple latex that slowly turns greenish upon exposure to air. The pileus is orangish to reddish-brown, and become funnel-shaped with age. The gills are pinkish to purplish in colour. In forest soils, ectomycorrhizal fungi can contribute upto one-third of microbial biomass (Hogberg and Hogberg, 2002) [5]. They are associated with almost all feeder roots of woody plants in boreal, temperate and some subtropical forests (Smith and Read, 1997) [15] and more than 95% of the short roots of boreal forests trees are colonised by ectomycorrhizal fungi (Taylor *et al*., 2000) [16]. These fungi play a crucial role in the growth and survival of forest trees by enhancing nutrient acquisition (Landeweert *et al*., 2001) [9], drought tolerance (Morte *et al*., 2000) [10] and pathogen resistance of their hosts (Branzanti *et al*. 1999) [1]. In return, the autotrophic hosts provide carbohydrates to their heterotrophic fungal partners. The pure culture of mycorrhizal fungi can be utilized for *in vitro* mycorrhiza synthesis and mass multiplication the mycelium for nursery inoculation which can help for the successful establishment of seedling in afforestation practices of conifer trees.

Materials and Methods
In vitro isolation
In vitro cultures of *Lactarius sanguifluus* were raised from the pileus and stipe portion of the healthy and fresh fruiting bodies. The specimens were first wash with distilled water and then the tissues from pileus and stipe portion were cut with the help of sterilized blade. The bits of tissue (2-3 mm) were taken by sterilized forceps and dipped in 0.1% Mercuric Chloride solution for 5-10 seconds and then washed with sterilized distilled water. Now the tissue was placed on sterilized filter paper to remove the excess moisture. These bits of tissue were then transferred aseptically into Petri plates containing nutrient medium with the help of sterilized forceps. Petri plates were then incubated at ambient temperature for at least 7-9 days and observed regularly for the appearance of culture. The actively growing mycelial colonies were subcultured to obtain pure cultures. Ten solid media have been tried during the present studies. All the media were prepared following Tuite (1969) [18].

Preparation of inoculum
Inoculum used in this study was obtained from the periphery of actively growing mycelial colonies. Mycelial discs of 5 mm diameter were taken out with a presterilized borer under aseptic conditions, to be used as inoculum in different solid media.

Recording of vegetative growth in solid media
Vegetative growth of mycelium in the solid media was measured by taking the diameter of colony in two directions at right angles. Five replicates of each medium were used and average values were taken for comparison of growth in different media. The medium with best vegetative growth was used in further studies i.e. for studying the effect of temperature, pH and light and darkness.

Effect of Temperature
For the study of temperature requirement of the fungus, inoculated Petri plates and flasks were incubated at the following temperatures viz. 5, 10, 15, 20, 25, 30, 35 and 40°C in separate incubators on the best suited solid medium.

Effect of Hydrogen Ion Concentration (pH)
To record the effect of different pH on the growth of this fungus the best solid media was adjusted at different pH levels, viz. 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5. The pH was adjusted with the help of N/10 NaOH or N/10 HCl. The pH was checked with the help of digital type Phillips pH meter. The inoculated Petri plates and flasks were incubated at best suited temperature and after that the growth was measured.

Effect of Light and Darkness

Best selected solid medium with optimum pH was inoculated and was given light and dark treatment at optimum temperature. Growth was observed after incubation period.

Statistical analysis of the data

The data obtained for mycelial growth under different conditions were from five replicates. All data obtained was statistically analyzed. To find out the significance of difference between the mean values, one way analysis of variance (ANOVA) test and student's t-test was applied. Tukey's multiple compression test was used to determine honestly significant difference (HSD) values for significance among means.

Results and Discussion

Mycelial characteristics

Pure culture of *Lactarius sanguifluus* was isolated on Modified Melin-Norkans Medium (MMN). The growth pattern of *L. sanguifluus* was recorded when incubated at ambient temperature (25°C) in Petri plates. Mycelium in the colony form concentric zones during its growth. The margin of the colony was irregular (Fig. 1A) and maximum growth in Petri plates was achieved after 7 days. Therefore, in subsequent experiments the final data relating to growth of mycelium were recorded only after 7 days only.

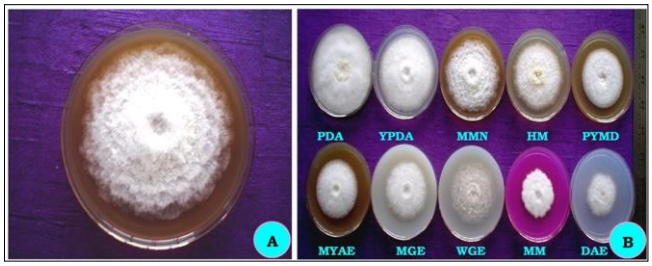

Fig 1: (A) Petri plate containing pure culture of *Lactarius sanguifluus* (B) Petri plates showing cultural characteristics of *Lactarius sanguifluus* on different solid media

Growth of mycelium on different solid media

Out of ten solid media tested for mycelial growth of *L. sanguifluus*, the maximum mycelial growth was recorded in Potato Dextrose Agar (8.02±0.23 cm) whereas minimum growth was recorded in Dimmick Agar Extract (4.00±0.23 cm). Thus, Potato Dextrose Agar was used as best solid medium for the mycelial growth of *L. sanguifluus*. Hence it has been now used as basal solid medium in subsequent studies. Table-1 and Fig. 1B.

Table 1: Effect of different solid media on mycelial growth of *Lactarius sanguifluus*. Mean ± S.D. followed by the same letters are not significantly different by One Way ANOVA with Tukey's Multiple Comparison Test ($p \leq 0.05$).

Sr. No.	Name of Medium	Colony Diameter (cm) (Mean ± S.D.)
1.	Potato Dextrose Agar (PDA)	8.02 ± 0.23^{a}
2.	Yeastal Potato Dextrose Agar (YPDA)	7.40 ± 0.24^{b}
3.	Modified Melin Norkran's Medium (MMN)	7.24 ± 0.24^{b}
4.	Hagem's Agar (HM)	6.88 ± 0.17^{b}
5.	Pridham Yeast Malt Dextrose Medium (PYMD)	6.00 ± 0.28^{c}
6.	Malt Yeast Agar Extract (MYAE)	5.70 ± 0.14^{c}
7.	Maize Grain Extract (MGE)	5.62 ± 0.12^{c}
8.	Wheat Grain Extract (WGE)	5.10 ± 0.24^{d}
9.	Martin's Medium (MM)	4.60 ± 0.23^{e}
10.	Dimmick Agar Extract (DAE)	4.00 ± 0.23^{f}

In a similar study Hung and Chien (1978) [6] recorded the physiological parameters of two ectomycorrhizal fungi (*P. tinctorius* and *S. bovines*) in five media. Out of five media tested *P. tinctorius* grew well on Modified Melin-Norkan's Medium (MMN) while *S. bovinus* grew equally on all the media tested. France and Reid (1984) [4] tested four ectomycorrhizal fungi for their ability to grow on nutrient media either supplemented with ammonium-nitrogen or nitrate-nitrogen or in the absence of an inorganic source. *Pisolithus tinctorius, Cenococcum geophilum* and *Thelephora terrestris* exhibited greater growth on ammonium-nitrogen. *Suillus granulatus* grew better on the nitrate-nitrogen nutrient media. Sharma and Mishra (1988) [13] recorded maximum growth of *L. laccata* on Modified Melin-Norkran's Medium.

Effect of different temperature on mycelial growth

To record the effect of temperature on mycelial growth, the fungus was inoculated on the basal solid medium i.e. Potato Dextrose Medium in Petri plates which were incubated at temperatures ranging from 5-40°C in different incubators. It is clear from the Table-2 and Fig. 1B that growth of mycelium was best at 25°C (8.10±0.14 cm) followed by 30°C (7.48±0.17 cm) and minimum growth was observed at 10°C (2.78±0.17 cm) whereas growth was completely ceased at 5°C and 40°C.

Table 2: Effect of different temperature on mycelial growth of *Lactarius sanguifluus*. Mean ± S.D. followed by the same letters are not significantly different by One Way ANOVA with Tukey's Multiple Comparison Test ($p \leq 0.05$).

Sr. No.	Temperature (^{O}C)	Colony Diameter (cm) (Mean ± S.D.)
1.	5	0.00 ± 0.00^{e}
2.	10	2.78 ± 0.17^{d}
3.	15	4.50 ± 0.14^{c}
4.	20	7.24 ± 0.27^{b}
5.	25	8.10 ± 0.14^{a}
6.	30	7.48 ± 0.17^{b}
7.	35	4.78 ± 0.17^{c}
8.	40	0.00 ± 0.00^{e}

Thus 25°C was considered as the optimum temperature for mycelial growth of *L. sanguifluus*. Hence this temperature was used in further studies Table-2 and Fig. 1B.

Chang and Chien (1988) [2] observed that the optimum temperature for most of the ectomycorrhizal fungi ranges from 22°C to 27°C with maximum growth at 25°C while in most of the cases growth stopped below 10°C and above

35°C. However Singh and Lakhanpal (1988) [14] observed 30°C as the optimum temperature for the mycelial growth of *Trappeinda himalayansis* (*Octaviania densa*). The excess heat may reduce fungal growth and mycorrhizal colonization. Cline *et al*. (1987) [3] showed that optimal growth of *Pisolithus tinctorius* isolates varied between 21°C and 32°C. All isolates studied had reduced growth at 38°C and suggest that the climate of the place of origin of an isolate may impact its ability to grow and form ectomycorrhiza at varying temperatures. Thus the results obtained in the present study are in agreement with the results of most of the earlier reports.

Effect of Hydrogen Ion Concentration (pH)

To study the effect of pH on the growth of mycelium, the pH of the basal solid medium (Potato Dextrose Agar Medium) was adjusted at different pH levels ranging from 4.0-8.5. For each pH level the fungus was inoculated and incubated at optimum temperature of 25°C in different incubators and it is It is clear from Table-3 and Fig. 1B that the maximum growth of mycelium was supported at pH 5.0 (8.38±0.13 cm) whereas minimum mycelial growth was recorded in pH 8.5 (3.66±0.14 cm). Thus pH 5.0 was recorded as ambient pH for mycelial growth of *L. sanguifluus*, hence to see the effect of light and darkness on the mycelial growth of this mushroom was carried out at this pH only.

However, Peng and Chien (1988) [11] reported that *Boletus griseus* grew profusely at acidic and neutral pH while *Suillus bovines* at neutral and low basic pH. Thapar (1988) [17] reported that the pH requirements varied among different isolates of *Cenococcum graniforme*. *Laccaria laccata* showed maximum colony growth at pH 5.0 while its dry weight was maximum at pH 7.0 (Jha *et al*., 1990) [8]. These authors also observed better colony diameter of *P. tinctorius* at pH 7.0 and dry weight at pH 6.0. The selective ion uptake and production of organic acids by the mycelium may account to their variability in growth at different pH (Hung and Trappe, 1987) [7]. The organic acids of ECM fungi may help in increasing the uptake of phosphorus either through chelating the metals or increasing the phosphatase activity.

Table 3: Effect of different pH on mycelial growth of *Lactarius sanguifluus*. Mean ± S.D. followed by the same letters are not significantly different by One Way ANOVA with Tukey's Multiple Comparison Test ($p \leq 0.05$).

Sr. No.	pH	Colony Diameter (cm) (Mean ± S.D.)
1.	4.0	4.98 ± 0.19^{g}
2.	4.5	7.04 ± 0.19^{d}
3.	5.0	8.38 ± 0.13^{a}
4.	5.5	8.00 ± 0.14^{b}
5.	6.0	7.60 ± 0.14^{c}
6.	6.5	6.92 ± 0.17^{d}
7.	7.0	6.20 ± 0.14^{e}
8.	7.5	5.70 ± 0.14^{f}
9.	8.0	4.48 ± 0.17^{h}
10.	8.5	3.66 ± 0.14^{i}

Effect of Light and Darkness on mycelial growth

To record the effect of light and darkness on the growth of *L. sanguifluus* mycelium, petriplates containing basal solid medium (Modified Melin Norkran's Medium) adjusted at pH 5.0 were inoculated and incubated at 25°C in light and darkness. It is clear from the results that the growth of mycelium was better in dark (8.32±0.19 cm) than in light (7.90±0.16 cm) Table-4 and Fig. 1B.

Table 4: Effect of light and darkness on mycelial growth of *Lactarius sanguifluus*. Mean ± S.D. followed by the same letters are not significantly different by student's t-test Comparison Test ($p \leq 0.05$).

Sr. No.	Treatments	Colony Diameter (cm) (Mean ± S.D.)
1.	Light	7.90 ± 0.16^{b}
2.	Dark	8.32 ± 0.19^{a}

Our results are also in agreement with the results of Hung and Chien (1978) [6] who also reported the inhibition in the growth of *Pisolithus tinctorius* and *Suillus bovines* under light conditions. Inhibitory effect of light on the growth of *Laccaria laccata* and *Amanita muscaria* was also reported by Raman and Thiagarajan (1988) [12].

Conclusions

During present studies ten solid media were tested for the mycelial growth of *L. sanguifluus*, and Potato Dextrose Agar Medium showed maximum mycelial growth. The data on the effect of different temperatures on the growth of this mushroom clearly indicate that maximum growth was recorded at 25°C whereas minimum growth was observed at 10°C. Maximum mycelial growth was achieved at pH 5.0 whereas the mycelium was found to grow better under the dark conditions in comparison to light.

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