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Research Article

In vitro Isolation and Optimization of Favourable Culture Conditions for the Mycelial Growth of *Amanita ceciliae* – A Mycorrhizal Mushroom

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ABSTRACT

In the present study, different solid media were evaluated for the best mycelial growth of Amanita ceciliae, out of ten solid media tested, A. ceciliae showed maximum mycelial growth on Potato Dextrose Agar Medium. Various factors such as temperature, pH, light and darkness were also investigated. The best mycelial growth was observed at 20°C. To determine the optimum pH, the mycelium was grown in best suited solid media at optimum temperature and at different pH levels. 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.

***Key words*: *Amanita ceciliae*, *Vitro isolation*, *Cultural conditions*, *Mycelial growth*.**

INTRODUCTION

Amanita ceciliae, is a ectomycorrhizal basidiomycetous fungus. It grows Solitary to scattered on soil in mixed forests, mostly under conifers and fruits from July to August. *A. ceciliae* form ectomycorrhiza with many hardwoods and conifers. Fungi and plants have a long history of intimate association and are adapted to penetrate and live within their nutritional substrate and one of the most common fungal substrates is the tissue of plants, either living or dead. The colonization of land by plants was probably facilitated by fungal mutualists (Pirozynski & Malloch, 1975) but fungal parasites, no doubt accompanied them as well. These fungi play a crucial role in the growth and survival of forest trees by enhancing nutrient acquisition (Landeweert et al., 2001), drought tolerance (Morte et al., 2000) and pathogen resistance of their hosts (Branzanti et al., 1999). 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.

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MATERIALS AND METHODS

In vitro isolation:

In vitro cultures of *Amanita ceciliae* 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 8-10 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).

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 40ºC 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 *A. ceciliae* was isolated on Potato Dextrose Agar Medium (PDA). The growth pattern of *A. ceciliae* was recorded when incubated at ambient temperature (25°C) in Petri plates. The colour of the colony was white and dense mat of mycelium was formed on the medium. Maximum growth in Petri plates was achieved after 8 days. Therefore, in subsequent experiments the final data relating to growth of mycelium were recorded only after 8 days. (Fig. 1A).

Growth of mycelium on different solid media:

It is evident from Table-1 and Fig. 1B that out of ten solid media were tested for mycelial growth of *A. ceciliae*. The maximum mycelial growth was observed on Potato Dextrose Agar Medium (8.80±0.16 cm), followed by Hagem's Medium (8.60±0.23 cm). The minimum growth was recorded in Dimmick

Agar Extract (1.20±0.14 cm). Thus, Potato Dextrose Agar was selected as best solid medium for the mycelial growth of *A. ceciliae*. Hence it was used as basal solid medium in subsequent studies and for the maintenance of cultures.

In similar studies Hacskaylo et al. (1965) used Hagem's agar for isolating six ECM fungi (*Amanita rubescens*, *Suillus luteus*, *S. punctipes*, *Russula emitica*, *Cenococcum graniforme* and *Rhizopogon roseolus*) which form ectomycorrhiza with *Pinus virginiana*. Cripps (2001) collected 54 species of ectomycorrhizal fungi. Out of these about half of the fungi were isolated in culture on MMN medium. Cotter (1987) described cultural characteristics of eight species of *Suillus* using Hagem's Agar as the most suitable nutrient medium. More recently similar to our studies Santiago-Martinez et al. (2003) evaluated seven culture media for the *Laccaria bicolor* growth and recorded that out of seven culture media tried Malt Agar Extract showed highest growth rate and colony diameter. Garcia-Rodriguez et al. (2006) recorded higher average growth in diameter of the three strains of *Pisolithus tinctorius* in Potato Dextrose Agar Medium and Melin Norkran's Modified Medium than in the Malt Extract Medium.

Effect of Temperature

To record the effect of temperature on growth of mycelium, the fungus was inoculated on the basal solid medium i.e. Potato Dextrose Agar 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 the mean colony diameter of mycelium at 20°C (8.90±0.14 cm) was maximum and followed by 25°C (7.48±0.17 cm). The minimum mycelial growth was observed at 10°C (3.44±0.24 cm) whereas growth ceased completely at 5°C and 40°C. It can be concluded from the results that 20°C was the optimum temperature for growing *A. ceciliae* in culture for further studies.

In the similar studies Hacskaylo et al. (1965) observed 24°C as the optimum temperature for the mycelial growth of *Amanita rubescens* and *Suillus luteus*. Optimal temperature for the mycelial growth lies between 18°C and 27°C for majority of mycorrhizal fungi (Harley, 1959). The growth was ceased above 35°C and below 5°C in case of many mycorrhizal fungi (Hacskaylo et al., 1965). Hung and Chien (1978) conducted physiological studies on two ectomycorrhizal fungi (i.e. *Pisolithus tinctorius* and *Suillus bovines*) and revealed that the optimum temperatures for maximum mycelial growth were 28°C and 20°C respectively. Peng and Chien (1988) observed 30°C, 20°C and 25°C as optimum temperatures for the growth of *Boletus* sp., *B. qriseus* and *Suillus grevillei* respectively.

Effect of Hydrogen Ion Concentration (pH)

To record the effect of pH on linear growth of mycelium, the pH of the basal solid medium (Potato Dextrose Agar) 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 20°C in different incubators.

It is evident from the Table-3 and Fig. 1B that the maximum mycelial growth was supported at pH 5.0 (9.06±0.10 cm) followed by pH 5.5 (8.30±0.26 cm). The minimum growth was recorded in pH 8.5 (3.66±0.14 cm). Thus pH 5.0 was recorded as best for mycelial growth of *A. ceciliae*, hence subsequent experiment to see the effect of light and darkness on the mycelial growth of this mushroom was carried out at this pH only.

These results are consistent with early observations made by Modess (1941) who determined the pH requirements of a large number of ectomycorrhizal Hymenomycetes. Optima varied between 3.5 and 5.9 with little growth of many species below 2.5 or above 6.5. Most of the ECM fungi are acidophilic (Mikola, 1973; Slankis, 1974). Peng and Chien (1988) reported that optimum pH for mycelial growth of *P. tinctorius* and *Suillus bovines* was 6.6 and 5.5 respectively. Sanchez et al. (2001) observed that the strains of some edible ectomycorrhizal fungi *Lactarius deliciosus*, *Suillus granulatus* and *Suillus luteus*, from regions of the Mediterranean, increased their biomass when pH of solid media increased

from 4.5 to 5.5. Moreover, other ectomycorrhizal fungus, such as *Tricholoma focale*, diminished its biomass with similar increments in pH.

Effect of Light and Darkness

To record the effect of light and darkness on the growth of *A. ceciliae* mycelium, petriplates containing basal solid medium (Potato Dextrose Agar) adjusted at pH 5.0 were inoculated and incubated at 20°C in light and darkness.

It is clear from the results that the growth of mycelium was better in dark (9.10±0.22 cm) than in light (8.32±0.19 cm) Table-4 and Fig. 1B.

These results are in agreement with the results of Hung and Chien (1978) who also reported the inhibition in the growth of *Pisolithus tinctorius* and *Suillus bovines* under light conditions. Light has inhibitory effect on the vegetative growth of most fungi (Cochrane, 1958). Raman and Thiagarajan (1988) also reported inhibitory effect of light on the growth of *Laccaria laccata* and *Amanita muscaria*.

Table 1: Effect of different solid media on mycelial growth of *Amanita ceciliae*. 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)	8.80 ± 0.16^{a}
2.	Hagem's Agar (HM)	8.60 ± 0.23^{a}
3.	Modified Melin Norkran's Medium (MMN)	8.26 ± 0.24^{b}
4.	Yeastal Potato Dextrose Agar (YPDA)	7.86 ± 0.22^{b}
5.	Malt Yeast Agar Extract (MYAE)	7.60 ± 0.14^{c}
6.	Pridham Yeast Malt Dextrose Medium (PYMD)	7.50 ± 0.24^{c}
7.	Wheat Grain Extract (WGE)	7.12 ± 0.23^{d}
8.	Martin's Medium (MM)	6.90 ± 0.14^{d}
9.	Maize Grain Extract (MGE)	5.04 ± 0.19^{e}
10.	Dimmick Agar Extract (DAE)	1.20 ± 0.14^{f}

Table 2: Effect of different temperature on mycelial growth of *Amanita ceciliae*. 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^{f}
2.	10	3.44 ± 0.24^{e}
3.	15	4.50 ± 0.14^{c}
4.	20	8.90 ± 0.14^{a}
5.	25	7.48 ± 0.17^{b}
6.	30	7.24 ± 0.27^{b}
7.	35	4.78 ± 0.17^{c}
8.	40	0.00 ± 0.00^{f}

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

Sr. No.	pH	Colony Diameter (cm) (Mean ± S.D.)
1.	4.0	5.22 ± 0.19^{i}
2.	4.5	$7.32\pm0.27^{c,d}$
3.	5.0	9.06 ± 0.10^{a}
4.	5.5	8.30 ± 0.26^{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^{g}
10.	8.5	3.66 ± 0.14^{h}

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

Sr. No.	Treatments	Colony Diameter (cm) (Mean ± S.D.)
1.	Light	8.32 ± 0.19^{b}
2.	Dark	9.10 ± 0.22^{a}

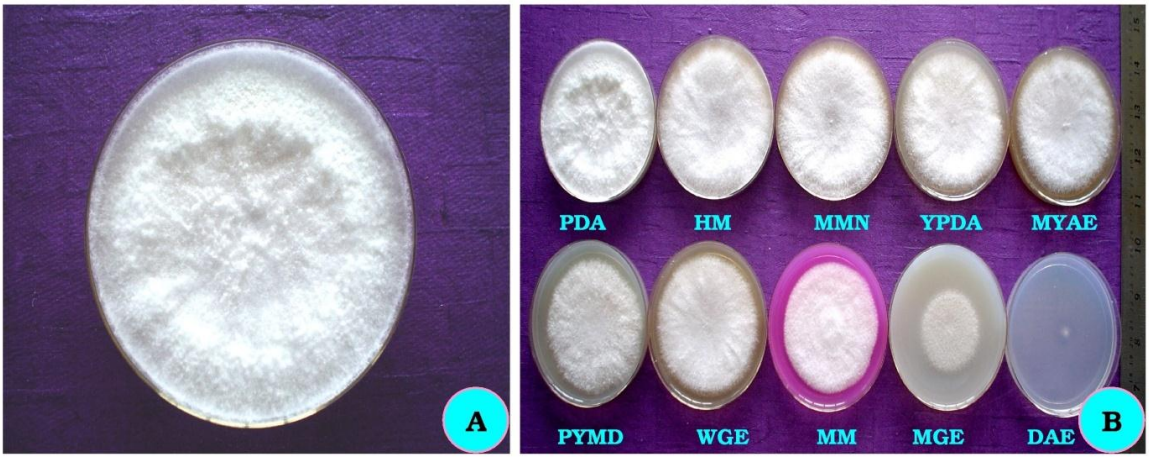

Fig. 1: (A) Petri plate containing pure culture of *Amanita ceciliae* (B) Petri plates showing cultural characteristics of *A. ceciliae* on different solid media

CONCLUSIONS

Ten solid media were tested for the mycelial growth of *Amanita ceciliae*, out of ten solid media tested, *A. ceciliae* showed maximum mycelial growth on Potato Dextrose Agar Medium. The data on the effect of different temperatures on the mycelial growth of this fungus clearly indicate that maximum growth was recorded at 20°C. To determine the optimum pH, the mycelium was grown in best suited solid media at optimum temperature and at different pH levels. 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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