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In vitro* Isolation and Influence of Nutritional Conditions on the Mycelial Growth of the Entomopathogenic and Medicinal Fungus *Cordyceps militaris

Amit Kumar Sehgal and Anand Sagar

Department of Biosciences, Himachal Pradesh University, Summer Hill, Shimla-171005 India

Abstract: *Cordyceps militaris* is an entomopathogenic and medicinal fungus. Therefore *in vitro* isolation and influence of nutritional conditions on the mycelial growth of this fungus was carried out in this study. A small bit of tissue from stipe and stroma was used as starting material for *in vitro* isolation. The present study revealed that this fungus showed optimum growth in Yeastal Potato Dextrose Agar Medium and Glucose Asparagine Solution. The maximum mycelial growth was observed at 25°C and pH 7.5 and 5.5 in solid and liquid medium, respectively. Among 12 carbon and 9 nitrogen sources tested, the best growth was supported by Sucrose and Ammonium phosphate, respectively. All the four mineral sources supported good growth of the fungus, however 2 ppm calcium chloride and manganese sulphate, 1 ppm ferrous sulphate and 5 ppm ammonium molybdate proved superior as compared to higher or lower concentrations of the same mineral source tried. Among the 4 growth regulators, Gibberellic acid supported the maximum mycelial growth at 100 ppm concentration.

Key words: *Cordyceps militaris*, *in vitro*, nutritional conditions, entomopathogenic, medicinal fungus

INTRODUCTION

The genus *Cordyceps*, a known group of entomopathogenic fungi, forms fruiting bodies in their insect hosts and 750 species in the genus have been recognized (Sung, 1996). *Cordyceps militaris* is parasitic on larvae and pupae of Lepidoptera. If the Ascospores alight on the integument of susceptible larvae and pupae, germ tubes may penetrate, possibly aided by their ability to hydrolyse chitin (Huber; 1958, McEwen, 1963). After infection, cylindrical hyphal bodies appear in the haemocoel of the pupa. The hyphal bodies increase by budding and the buds are distributed within the insect's body. After death, mycelial growth follows and the body of the insect becomes transformed into a sclerotium, from which the perithecial stromata later develop (Webster, 1980).

Several *Cordyceps* species being regarded to have antitumour, antiaging, antidiabetic, immunomodulating, hypoglycemic, aphrodisiac and antimalarial activities (Chen *et al.*, 1997; Isaka and Tanticharoen, 2001; Kiho *et al.*, 1996; Kim *et al.*, 2001; Kittakoop *et al.*, 1999; Kuo *et al.*, 1996; Mizuno, 1998; Zhao *et al.*, 2002; Zhu *et al.*, 1998). The cost of 1 kg of wild collected *Cordyceps* in the market varies from 30,000 to 60,000 Nepali Rupees in Nepal and about Rs 1 Lac in India. The high prize of this fungus is because of its medicinal

benefits and difficulty associated with its harvesting due to its small size (Sharma, 2004).

Medicinal properties, high prize and difficulty associated with its harvesting due to small size of this fungus stress the need for its artificial cultivation and steps in this direction have been taken by *in vitro* isolation and to study influence of nutritional conditions on the mycelial growth of this fungus.

MATERIALS AND METHODS

Specimens collection and *in vitro* isolation: The material used in the present studies was *Cordyceps militaris*, a fascinating Ascomycetous fungus. Fruiting bodies of the fungus have been collected from Glen forests of District Shimla of Himachal Pradesh, India (Fig. 1a-c). Fruiting bodies have been preserved dry as well as wet (Ainsworth, 1971) and the specimens have been deposited in the Herbarium, Department of Biosciences, Himachal Pradesh University Shimla, India.

The cultures of *C. militaris* were raised from the stipe and stroma portion of healthy, sun-dried and fresh specimens. The specimens were first washed with distilled water and then the tissues from the stipe and stroma 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 sec

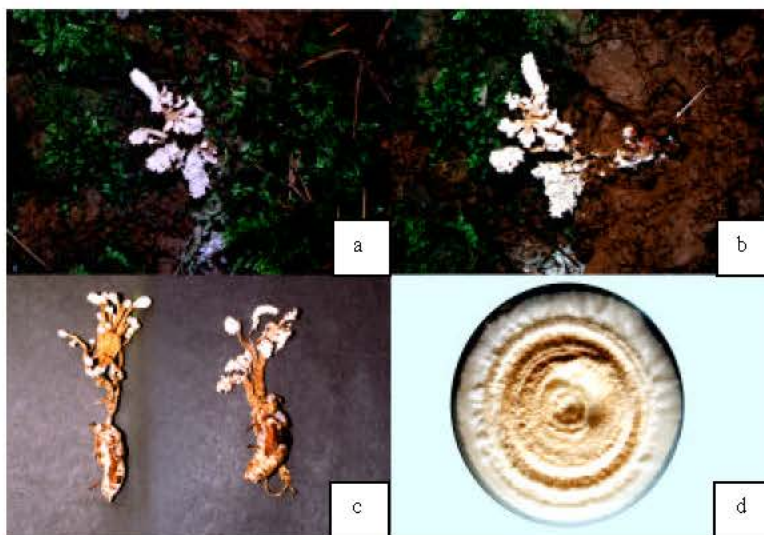


Fig. 1: (a) Fruiting bodies of *Cordyceps militaris* in its natural habitat. (b) Association of *C. militaris* with the host insect buried in soil. (c) Fruiting bodies of *C. militaris* emerging out from the head of the insect host. (d) Petri plate containing pure culture of *C. militaris* in Yeastal Potato Dextrose Agar Medium

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 the petri plates containing Potato Dextrose Agar (PDA) 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. Twelve solid and 5 liquid media have been tried during the present studies. All the media were prepared following (Tuite, 1969).

Inoculum preparation: Mycelial discs of 5 mm diameter were taken out with a presterilized borer under aseptic conditions, to be used as inoculum in solid media. In liquid media the inoculum preparation was different. Mycelial disc of 5 mm was transferred to 250 mL flask containing 50 mL of liquid medium and incubated at 25°C for 8-10 days. After 10 days, there appeared a ball of mycelium, which was homogenised in medium by a sterilized rod. The 5 mL of this homogenised mycelium was added to each of different liquid media as an inoculum used for further studies.

Recording of vegetative growth in solid and liquid media: Vegetative growth of mycelium in the solid media was measured by taking the diameter of colony in two directions at right angles. In liquid media, the mycelial mats were filtered through Whatman No. 1 filter paper

discs of 7.5 cm diameter. The filter papers were previously oven dried at 80°C until constant weight and weighed after keeping in moisture free desiccator. After filtration, the mycelium along with filter paper was again oven dried as described above for 12 h and finally weighed to give dry weight of the mycelium. Three replicates of each medium were used and average values were taken for comparison of growth in different media. The medium with best mycelial 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 in solid and liquid media, inoculated petri plates and flask 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 and liquid medium.

Effect of hydrogen ion concentration (pH): To record the effect of different pH on the growth of this fungus, the best solid medium 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, 9.0, 9.5, 10.0. The best liquid medium 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 and N/10 HCl. The pH was checked with the help of digital type Phillips pH meter. The inoculated petri plates and flasks were incubated for 10 days at optimum temperature and after that the growth was measured.

Effect of light and darkness: Best selected solid and liquid medium with optimum pH was inoculated and was given light and dark treatment at optimum temperature. Growth was observed after 10 days of incubation.

Effect of different carbon and nitrogen sources: In order to find the best carbon source for the mycelial growth of the fungus, the amount of carbon present in best liquid medium was substituted with twelve carbon sources on the basis of their molecular formulae so as to provide equal amount of carbon, whereas, starch was added at the rate of 5 g L⁻¹ of medium. Similarly the nitrogen present in best liquid medium was also replaced with nine nitrogen sources.

Effect of different mineral sources: Best liquid medium was used for studying the response of various mineral sources on the growth of the fungus. The mineral sources used were calcium chloride, ferrous sulphate, manganese sulphate and ammonium molybdate. Each mineral source was added in amount of 1, 2 and 5 mg L⁻¹ of medium.

Effect of different growth regulators: Effect of various growth regulators Gibberelic acid, NAA (Naphthalene acetic acid), Kinetin and IBA (Indole butyric acid) all at 20,50,100 mg L⁻¹ concentration were also evaluated for mycelial growth of fungus in best liquid medium.

Statistical analysis of the data: Data was statistically analysed for Standard Deviation (SD) and Critical Difference (CD). Critical Difference was calculated by Analysis of Variance (ANOVA) one-way classification model.

RESULTS

Mycelial characteristics: Mycelium was initially creamish white, becoming densely matted. As soon as the colony matures the colour of mycelia changes from creamish white to light brown. At approximately 14 days of incubation, the mycelium begins to form small nodules at the centre on the surface of the medium, appearing light brown while peripheral mycelia remain creamish white. As the medium is completely consumed, the mycelia become increasingly mud-like (Fig. 1d). When the mycelial growth was observed at periodic intervals, maximum growth of this fungus was attained at 10 days of incubation. After that there is no significant increase in mycelial growth. Keeping this information in mind in subsequent experiments, the final data related to mycelial growth was recorded only after 10 days of incubation. The cultures were deposited in the Herbarium,

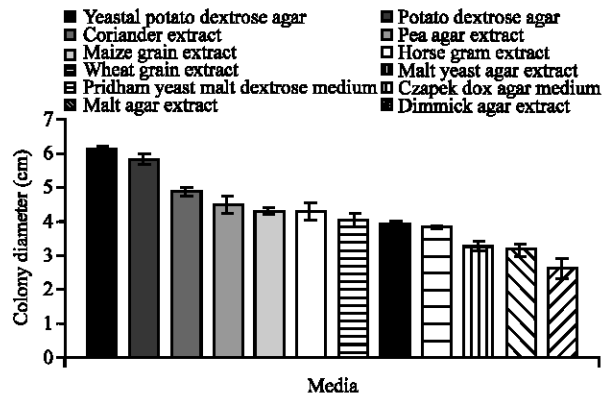


Fig. 2: Effect of different solid media on the growth of *C. militaris*

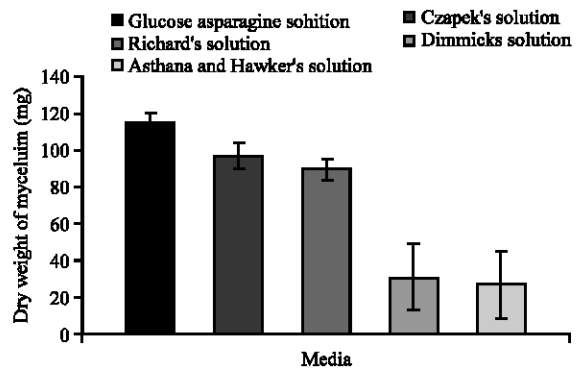


Fig. 3: Effect of different liquid media on the growth of *C. militaris*

Department of Biosciences, Himachal Pradesh University, Shimla India.

Growth of mycelium on different solid and liquid media:

It is evident from (Fig. 2) that out of twelve solid media tested for mycelial growth, the maximum colony diameter (6.12 cm) was recorded on Yeastal Potato Dextrose Agar Medium followed by Potato Dextrose Agar Medium (5.83 cm). The minimum colony diameter (2.63 cm) was observed in Dimmick Agar Extract. Since the best growth of the fungus was recorded on Yeastal Potato Dextrose Agar Medium, it has been used as basal solid medium in subsequent studies and for the maintenance of cultures.

Out of five liquid media used to record the mycelial growth of the fungus. It is clear from the (Fig. 3) that the maximum dry weight (115.23 mg) of mycelium was recorded in glucose asparagine solution followed by that in Czapek's solution (96.78 mg). Asthana and Hawker's solution showed the least growth of mycelium (27 mg). Since Glucose Asparagine Solution showed the best growth of mycelium, hence it was used as the basal liquid medium for the subsequent studies.

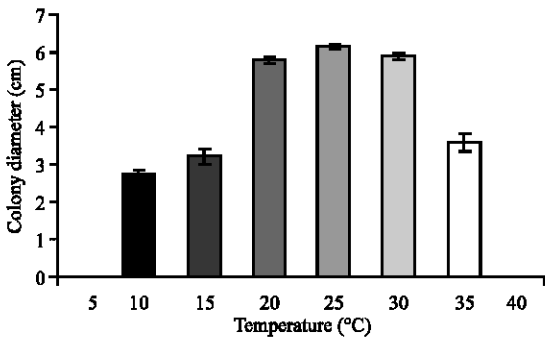


Fig. 4: Effect of different temperature on the growth of *C. militaris* in basal solid medium

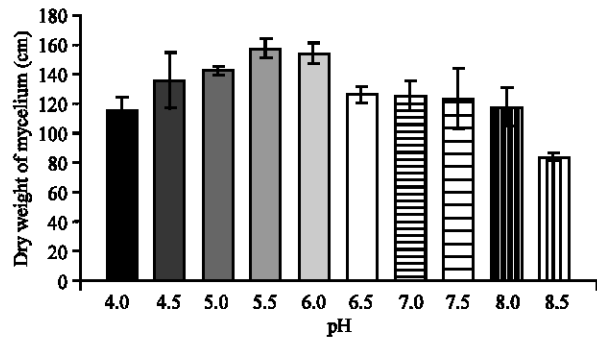


Fig. 7: Effect of different pH on the growth of *C. militaris* in basal liquid medium

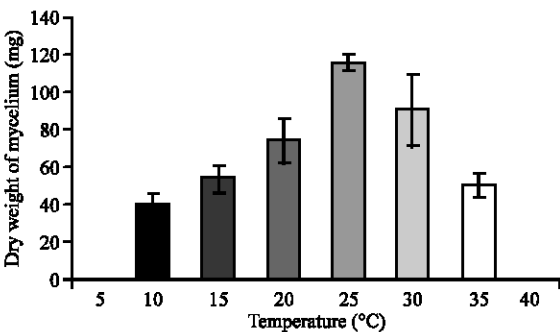


Fig. 5: Effect of different temperature on the growth of *C. militaris* in basal liquid medium

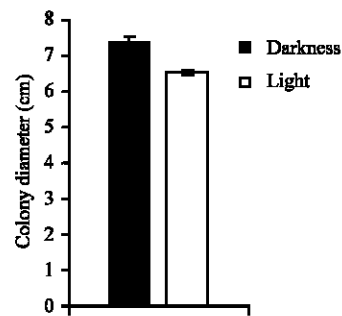


Fig. 8: Effect of light and darkness on the growth of *C. militaris* in basal solid medium

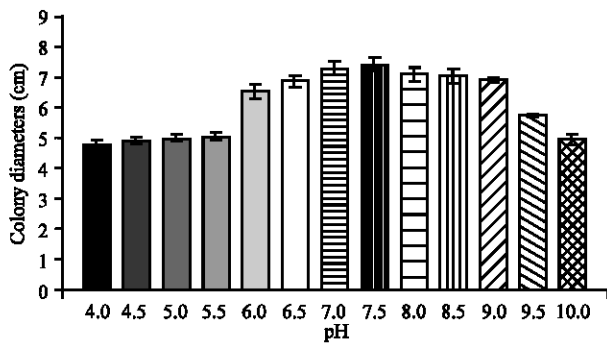


Fig. 6: Effect of different pH on the growth of *C. militaris* in basal solid medium

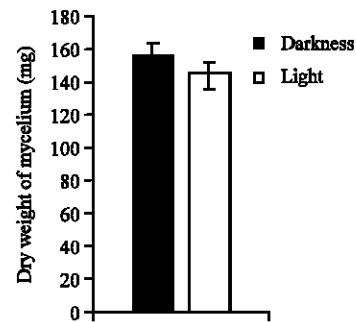


Fig. 9: Effect of light and darkness on the growth of *C. militaris* in basal liquid medium

Effect of temperature: It can be seen from (Fig. 4 and 5) that the maximum colony diameter (6.15 cm) and maximum dry weight (115.23 mg) of mycelium was recorded at 25°C in basal solid and liquid medium respectively. The growth ceases completely at 5 and 40°C in both solid and liquid medium. Since the optimum temperature is 25°C, hence the subsequent studies were conducted at this temperature only.

Effect of hydrogen-ion concentration (pH): It is clear from (Fig. 6) that maximum growth of the mycelium (7.41 cm)

was achieved at pH 7.5 followed by 7.0 (7.26 cm), 8.0 (7.08 cm) and 8.5 (7.03 cm) in descending order. Since, slightly alkaline pH of 7.5 was recorded to be optimum for the best growth of mycelium in basal solid medium.

It is evident from (Fig. 7) that the maximum dry weight (157.32 mg) of mycelium was recorded at pH 5.5 followed by pH 6.0 (154.15 mg) and pH 5.0 (142.85 mg) in descending order. Hence 5.5 is the optimum pH for the growth of *C. militaris* in basal liquid medium.

Effect of light and darkness: Basal solid and liquid medium were incubated at optimum temperature and

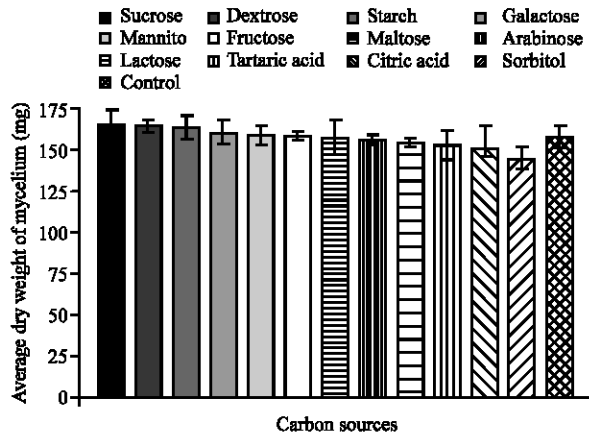


Fig. 10: Effect of various carbon sources on the growth of *C. militaris*

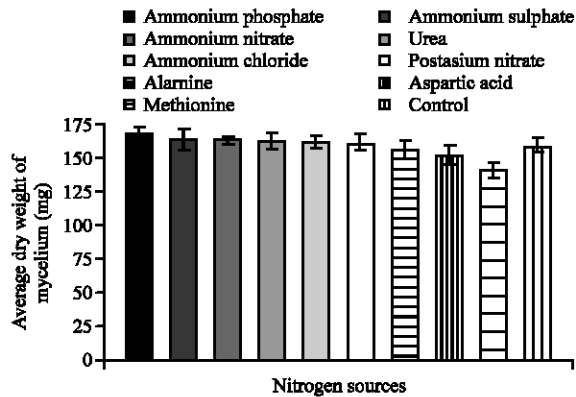


Fig. 11: Effect of various nitrogen sources on the growth of *C. militaris*

optimum pH in light and darkness, growth was observed after 10 days of incubation. It is clear from (Fig. 8 and 9) that, in both basal solid and liquid medium the better mycelial growth was observed in darkness in comparison to light.

Effect of different carbon and nitrogen sources: Out of twelve carbon sources tested for mycelial growth of *C. militaris*. Maximum growth was observed in sucrose (165.02 mg) followed by dextrose (164.35 mg) whereas minimum growth was observed in sorbitol (145.02 mg) (Fig. 10). It is also clear from Fig. 11 that out of 9 nitrogen sources evaluated for maximum mycelial growth of fungus, ammonium phosphate (167.53 mg) was found to be best nitrogen sources followed by ammonium sulphate (163.22 mg). In methionine least growth (140.02 mg) was observed.

Effect of different mineral sources: It is evident from the data presented in Fig. 12 that mineral sources tested

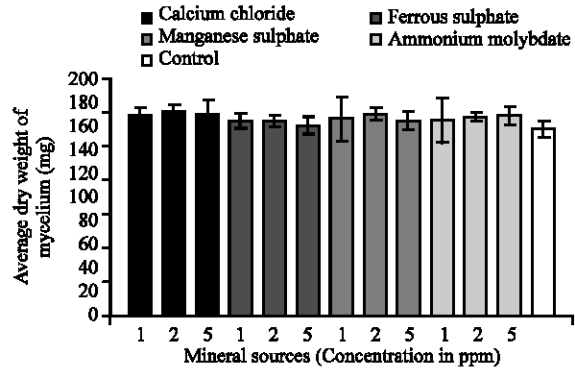


Fig. 12: Effect of different mineral sources on the growth of *C. militaris*

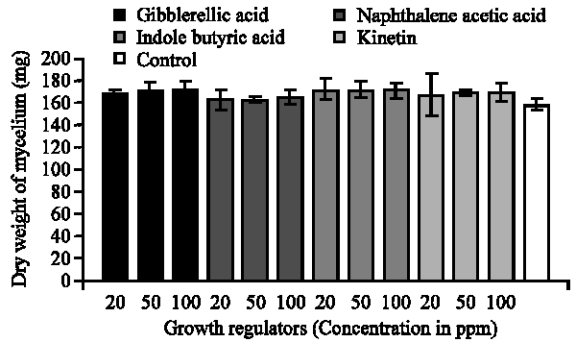


Fig. 13: Effect of different growth regulators on the growth of *C. militaris*

supported more mycelial growth of *C. militaris* as compared to control. It was also revealed that 2 ppm concentration of calcium chloride and manganese sulphate was optimum requirement of the fungus, whereas it was 1 and 5 ppm for ferrous sulphate and ammonium molybdate respectively. An increase or decrease in the concentration beyond the optimum one resulted in decreased growth.

Effect of different growth regulators: Out of four growth regulators tested at three concentrations. All the concentrations of different growth regulators supported more growth of the fungus as compared to control. However gibberellic acid at 100 ppm concentration supported maximum mycelial growth than the lower concentration tested (Fig. 13).

DISCUSSION

In the present study *in vitro* isolation of *Cordyceps militaris* was done on Potato Dextrose Agar Medium initially. Similarly *C. militaris*, *C. sinensis* and *C. unilateralis* were grown on PDA successfully (Kim

and Yun, 2005; Kim *et al.*, 2003; Wongsu *et al.*, 2005). Out of 12 solid and 5 liquid media tried for evaluation of maximum mycelial growth. Yeastal potato dextrose agar medium and glucose asparagine solution were found to be best solid and liquid medium, respectively. The exact reason for the maximum growth of mycelium in yeastal potato dextrose agar medium and glucose asparagine solution is not known, but it appears that the reason for such growth is probably the presence of Yeast and Asparagine, which as nitrogen source can enhance the growth of mycelium. Similarly maximum mycelial growth of *C. militaris* and *C. sinensis* was recorded in media containing nitrogen sources in comparison with media containing carbon sources (Kim *et al.*, 2003; Kim and Yun, 2005). The maximum growth of *C. militaris* was recorded at 25°C. According to Kim *et al.* (2003) and Xu and Yun (2003) the most of the entomopathogenic fungi have similar temperature optima, ranging from 20-25°C. The maximum mycelial growth of *C. militaris* was observed at pH 7.5 and 5.5 in solid and liquid medium respectively. Sung *et al.* (1995) also observed that *Cordyceps nutans* and *Phytocordyceps ninchukiospora* grow best on pH 6.0 and *Paecilomyces tenuipes* on pH 7.0 and *Cordyceps scarabaeicola* on pH 9.0. The optimum pH for mycelial growth of entomopathogenic fungi is presumably dependent on the strain even though most of the fungi grow well at pH 5.0-pH 6.0 in their submerged cultures (Bae *et al.*, 2000; Kim *et al.*, 2002, 2003; Park *et al.*, 2001). Mycelium was found to grow better under the condition of darkness in comparison to light.

The results of present study also revealed that sucrose and ammonium phosphate were the best carbon and nitrogen sources, respectively. Kim and Yun (2005) also reported the Sucrose and corn steep powder as carbon and nitrogen sources respectively for maximum mycelial growth of *C. militaris* and *C. sinensis*. Bae *et al.* (2000) observed a high level of mycelial growth of *P. japonica* in media containing maltose. Out of four mineral sources tested it was also revealed that 2 ppm concentration of calcium chloride and manganese sulphate was optimum requirement of the fungus, whereas it was 1 and 5 ppm for Ferrous sulphate and Ammonium molybdate respectively. Kim and Yun (2005) also reported CaCl₂ as best mineral source for maximum mycelia growth of *C. militaris* and *C. sinensis*. Jonathan and Fasidi (2001) observed a high level of mycelial growth by *Psathyrella atroumbonata* in media containing calcium and magnesium. Chardonnet *et al.* (1999) found that external Ca²⁺ can play an indirect role in fungal growth by altering internal Ca²⁺, which controls the cytoplasmic Ca²⁺ gradient and the activity of fungal enzymes involved in cell wall expansion. *Flammulina velutipes* showed best

mycelial growth at 1 ppm FeSO₄, 2 ppm ZnSO₄ and MnSO₄ and 10 ppm ammonium molybdate (Sharma *et al.*, 2005). Among the four growth regulators Gibberellic acid supported maximum mycelial growth at 100 ppm concentration. Similarly, Sharma *et al.* (2005) also observed the maximum mycelial growth of *Flammulina velutipes*.

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