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In vitro propagation technique for large scale propagation of *Cordyceps militaris*- a medicinal mushroom

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Cordyceps militaris is an insect pathogenic fungus with medicinal value. It belongs to ascomycetes group. *Cordyceps militaris*propagation involves various steps of propagation viz. pure culture, master culture, spawn liquid culture and spawn solid culture. It takes approximately 14 weeks to for complete growth of stroma *in vitro*. The fruiting body was inoculated on Potato Dextrose Agar (PDA) medium for 15 days for mycelial growth (pure culture). Pure cultures were multiplied on PDA for 15 days in dark and 2 days in light (master culture). The fungal cultures were cultured on liquid medium with potato starch and glucose as a main component for 5 to 7 days. The fungal cultures were cultured in a brown rice-based medium at 20°C for 15 days in dark for stromatal induction, and for 55-60 days at 12 hours light for fruiting body formation.

Key words: Ascomycetes, *Cordyceps militaris*, spawn culture

INTRODUCTION

An arthropod pathogenic mushroom includes over 500 species distributed globally (Zheng *et. al.* 2011). Pharmacological active components of *Cordyceps militaris*are Cordycepin, Cordycepic acids,macrolides andpolysaccharides (Xiao and Zhong, 2007). Cordycepin possesses therapeutic potential in immunomodulation, induction of apoptosis, combat hyperlipidemia and useful in cancer therapy (Wen *et. al.* 2014; 2017). Medicinal value of Cordyceps is due to Cordyanhidrides, Cordypyridones, cycloaspeptide, epicoccin, gliocladicillin, trichocladinol, trichothecanes and spirotenuipesines. (Sharma 2004, Isaka*et al.* 2005).

According to globe news wire (2019) report The Global *Cordyceps sinensis* and *militaris* extract market, by formulation (tablet/capsule, liquid, powder), by application (medicine, Dietary Supplements, Food Additives), and by Strain Type (*Cordyceps sinensis* and *Cordyceps militaris*), is estimated to be valued at US$ 473.4 Mn in 2018 and is expected to exhibit a Compound Annual Growth Rate (CAGR) of 10.4%, over the forecast period (2018-2026), as highlighted in a new report published by Coherent Market Insights.The growing demand for herbal medicine in primary healthcare is projected to contribute to the development of the market for *Cordyceps sinensis* and *C. militaris*. The demand for herbal cordyceps extract increased substantially in 2016 and 2017, due to its use in nutritional supplements for respiratory function, sports, strength, sexual health and many other supplementation. The active principles of *C. militaris* are beneficial to act as pro-sexual, anti-inflammatory, anti-oxidant/anti-aging, anti-tumour/ anti-cancer/anti-leukemic, anti-proliferative, anti-metastatic, immunomodulatory, anti-microbial, anti-bacterial, anti-viral, anti-fungal, anti-protozoal, insecticidal, larvicidal, anti-fibrotic, steroidogenic, hypoglacaemic, hypolipidaemic, anti-angiogenetic, anti-diabetic, anti-HIV, anti-malarial, anti-fatigue, neuroprotective, liver-protective, reno-protective as well as pneumo-protective, let alone their other synergistic activities, which let it be marketable in the western countries as over-the-counter medicine (Das *et al.* 2010).

The rising prevalence of chronic diseases including diabetes, cardiovascular disease, respiratory and

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cancer diseases is expected to drive the adoption of cordyceps sinensis extracts preventive healthcare products, which is further anticipated to grow the growth of the cordyceps sinensis extract market in the upcoming years.

Amongst all the species, *C. militaris* is considered as the oldest source of some useful chemical constituents. Besides their popular applications for tonic medicine by the all stairs of the community, the constituents of *C. militaris* are now used extensively in modern systems of medicine. The current survey records the mysterious potentials of *C. militaris* are boosting up the present herbal treatments, as well as gearing up the green pharmacy revolution, in order to create a friendly environment with reasonable safety. (Das *et al.* 2010)

Due to its high market demand, need of the mass multiplication of *C. militaris* has increased. The price of *Cordyceps militaris* has continued to increase over the past few years because of

the growing worldwide demand and resource limitations. Artificial cultivation of the fruiting bodies to substitute natural *C. militaris* is urgently needed for the effective protection of a valuable bioresource and environment and for commercial trade.Present study aims to set the technique of in vitro propagation of whole fruiting body of *C. militaris*.

MATERIALS AND METHODS

Stroma of the *Cordyceps militaris* are used as an Explant. Stroma of the *Cordyceps militaris* were collected from natural habitat from Himalayan region India.

Pretreatment

It is pretreated with 0.1 % of $HgCl_2$ and 3 % of Bavistin for 10 minutes subsequently. Each treatment is followed by the 3- 4 washes of double distilled water.

Pure Culture

Very small piece (2 –3 mmsized) stroma are used as an explant. It is inoculated onPotato Dextrose agar medium at 5.5 pH in Petri plate. Petriplates are sealed with parafilm tape. Cultures are incubated under dark for 13 to 15 days at 20 to 25ºC. It results in the formation of white mycelium mat of *Cordyceps militaris* (Fig.1 a & b). It is followed by light shock for 3 to 4 days. Light stimulates the pigmentation in the pure cultures (Fig. 1 c).

Master Culture

Pigmented pure cultures were inoculated onPotato Dextrose agar medium in Petri plates to multiply the mycelial cultures. The similar process is followed for the growth of the master culture as like the pure cultures.

Liquid Spawn/seed Culture

Liquid culture is prepared in distilled water with potato starch and glucose asmain components. Other components of liquid cultures are peptone, Yeast extract, KH_2PO_4, K_2HPO_4, and $MgSO_4$ (Lin *et al.* 2018). The initial pH was adjusted to 5.5-5.7 using 1 N HCl or 1 N NaOH. The medium (300 ml) was added to a 500 ml Erlenmeyer flask and autoclaved at 121ºC for 30 min. It is allowed to cool for 5 to 7 hours in air laminar flow hood under UV light. The medium was inoculated with fungal patches of master cultures incubated on a rotary shaker at 150 rpm and 18ºC for 7 days. Liquid cultures were become mature to grow on substrate culture when the ball like orange mycelium is developed in conical flask.

Substrate Culture

Main component of the substate culture is brown rice. 35 g rice and 85 ml liquid medium (22 g/L glucose, 11 g/L peptone, 0.1 g/L $MgSO_4.7H_2O$, 1.5 g/L K_2HPO_4, with 1000 ml D.W.) in 700 ml cylindrical glass bottle and autoclaved for 25 min at 121ºC. The pH of the medium is adjusted 6 (Lee *et al.* 2019). Medium was cooled to room temperature and inoculated with 5 ml seed culture, incubated at 20 ºC for 12 to 17 days was given dark treatment for promoting vegetative growth. Primordial fruiting body formation began after 12-15d after incubation with relative humidity 90-95%. After 15 days cultures are transferred in 12 hours light period at 18 ºC. After one week of light exposure fruiting bodies of *Cordyceps* starts to develop. Light not exceed 12 h per day (Wen *et al.* 2014). The cultures with well-developed fruiting bodies within 50-60 d after inoculation.

RESULTS AND DISCUSSION

The present study focuses on the *in vitro* culture technique of *Cordyceps militaris*. There are four

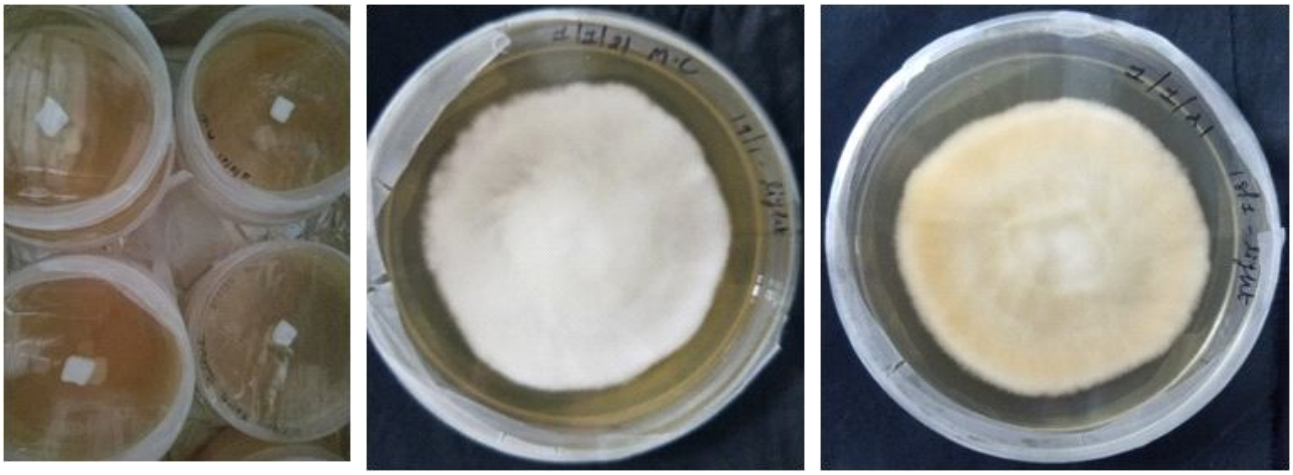

Fig 1.: a.Development of mycelium on PDA medium, b. Development of mycelial mat, c. Pigmentation of mycelium after light shock

Fig. 2: a. Spawn culture after five days of incubation on rotary shaker, b. Formation of blastospores in liquid spawn

Fig. 3: a. Induction of stroma after 15 days dark period, b. Yellow pigmentation after two days light treatment, c. Orange pigmentation after 4 days of light treatment

main phases of *in vitro* cultivation of *Cordyceps militaris* for large scale propagation viz. Pure culture, Master culture, Spawn or seed culture and Substrate culture. In all the different phases temperature, pH and incubation time was found to have a direct effect on cordycepin production. The best possible combination of temperature, pH and incubation time was found to be 25°C, 5.5 and 21 days, respectively, for maximum cordycepin production (Adnan *et al.* 2017). In this study brown rice was basal substrate for the fruiting body, as it increases the cordycepin, adenosine level and dry weight of *C. militaris* cultures as observed by Wen *et al.* (2017). Glucose was added as mail carbon

source and peptone as a nitrogen source. When the mycelial cultures are multiplied in liquid culture it develops bar shaped blastopores. It is inoculated on rice culture under dark it develops into the white mycelial growth of the *Cordyceps militaris*. It results into the pigmented mycelium, when it is subjected to light shock. Raethong (2020) has also optimized conditions for fast growth and cordycepin production from *C. militaris*.

All fungi are reported to grow better *in vitro*, if cultures are provided with mineral salts. Among all mineral salts K_2HPO_4, KH_2PO_4 and $MgSO_4$yielded good fruiting body yield. Similar results were obtained by Chiang *et al*. (2014).

The other factors responsible for the better growth of the *C. militaris* is pH of the medium. During all the stages of *in vitro* cultivation 5.5 to 6.0 pH was maintained. Similar effect of pH were noticed by Shih *et al.* (2007) and Wen *et al* (2011). Brown rice was used as a grain source in this study. Brown rice was found to be the most favourable to the mycelium and fruiting body growth (Dang *et. al.* 2018).

CONCLUSION

In the present work, nutritional requirements for the in vitro fruiting body cultivation of *C. militaris* were studied. We had got the best fruiting body growth in 5.5 pH and 20 C temperature. Other components for the liquid spawn culture and substrate cultures are peptone, Yeast extract, KH_2PO_4, K_2HPO_4, and $MgSO_4$.

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