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EFFECT OF VARIOUS SOLID SUBSTRATE COMPOSITIONS ON PRODUCTION EFFICIENCY OF *CORDYCEPS MILITARIS* UNDER A CONTROLLED ENVIRONMENT SYSTEM

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ABSTRACT
Commercial cultivation of *Cordyceps militaris* has shown a tremendous increase in the last few years due to its biochemical constituents and pharmacological properties. Its large scale cultivation is conducted under controlled environment system to fulfill the demand for scientific investigations and product development for pharma industry. For laboratory scale cultivation brown rice is commonly used as a basal solid substrate. In the present study six solid substrate variables were used to study their production potential for *Cordyceps militaris*. The substrate compositions were 10% and 20% each of bajara, soya bean and insect cuticle along with 90% and 80% brown rice respectively were used as six replicates. Liquid culture inoculated glass jars of 500 ml each, which were placed in the culture room for 55 days and after the maturation of fruiting bodies production parameters were recorded.

Highest average number, length, wet and dry weights of the fruiting bodies were recorded in variable II (80% brown rice and 20% Bajara grains), its total production was 59.93% higher than control and significantly higher than other variables. Variable IV (20% soya bean and 80% brown rice), showed lowest growth and production. Variable V & VI (insect cuticle and brown rice) showed lower production as compare to control. Some more locally available solid substrate compositions may be tried to find out the most suitable economically viable composition for commercial production.

Keywords: *Cordyceps militaris, Solid Substrates*

INTRODUCTION
Medicinal mushrooms have been known from hundreds of years for their bio-metabolites (Tuli *et al*., 2014a), the Fungi of the genus *Cordyceps* are among the most important "traditional medicines" since they contain bioactive compounds of high pharmacological value. Artificial cultivation of *Cordyceps sinensis* is difficult (Guo and Yang, 1999; Jiang and Yao, 2003), however some attempts have been made to cultivate this fungus artificially on the using host insect or artificial media with limited success (Cao *et al*., 2015; Tao *et al*., 2016). It was reported by several workers that most of the active constituent of *Cordyceps militaris* were similar to those of *Cordyceps sinensis* like cordycepin, polysaccharides, ergosterol, and mannitol. (Das *et al*., 2010; Patel and Ingalhalli, 2013; Yu *et al*., 2006 and Zhou *et al*., 2009). The ethnopharmacological importance of *Cordyceps militaris* has been widely analyzed by many researchers like Olatunji *et al*., 2018; Chiu *et al*., 2016; Cui *et al*., 2015; Das *et al*., 2010; Reis *et al*., 2013 and Chen *et al*., 2017, since this fungus is considered to be a valuable source of

metabolites that act directly on various human metabolic pathways, for example, the alcoholic extract of *Cordyceps militaris* possess antioxidant, antibacterial, antifungal, and anti-proliferative properties in different human tumor cell lines (Reis *et al*., 2013), while cordycepin, the major bioactive compound of *Cordyceps militaris*, presented potent anti-inflammatory, anticancer, anti-metastatic, and immune-modulator activities (Chen *et al*., 2017; Lee *et al*., 2020; Nakamura *et al*., 2015; Yoon *et al*., 2018). *Cordyceps militaris* can be cultivated artificially and its natural compounds are similar to cultured ones (Xiaolu and Yue, 1999).

Cordyceps militaris is the most studied member of the genus popularly referred to as "Bei Chong Cao" in China. It ranks second (after *Cordyceps sinensis*) among the most commercialized *Cordyceps* species in East Asia, where it is used as folk tonic medicine. *Cordyceps militaris* is sexually heterothallic but its single mating-type can also fruit without mating and meiosis to produce sexual perithecia (Lu *et al*., 2016). *Cordyceps militaris* has been commercially produced using solid-state fermentation (SSF) and submerged fermentation for cordycepin production (Das *et al*., 2010; Lin *et al*., 2017; Lee *et al*., 2016; Cui, 2015). Several studies for in vitro stroma production of *Cordyceps militaris* had been conducted (Kobayasi, 1941; Basith and Madelin, 1968) similarly, different insect larvae and pupae have been used for study of the infection process and stromata formation of *Cordyceps militaris* (Harada *et al*., 1995; Chen and Ichida, 2002; Sato and Shimazu, 2002; Hong *et al*., 2010). Its mycelia and ascomata can be generated ex-situ with or without the addition of alive or dead insect tissues (Kontogiannatos *et al*., 2021). The mass production of *Cordyceps militaris* has long been successful by inoculation of fungal propagates on artificial rice media (Lin *et al*., 2006). However, commercial exploitation of *Cordyceps militaris* is still in need of improvement in solid substrate composition related to fruiting body production and content of bioactive compounds.

The aim of this study was to investigate the effect of various solid substrates combinations on the production of *Cordyceps militaris* in a controlled environment system to find out a cheaper substrate composition for its commercial production.

MATERIALS AND METHODS

The pure culture of *Cordyceps militaris* was procured from Directorate of Mushroom Research, Solan, Himachal Pradesh and following steps were used for its propagation

Step I: Culture Plates/Slants Preparation

Culture from master culture Plate/Slant was collected to inoculate it in Petri dishes on PDA Medium to produce working cell culture then agar plates incubated at 21 ± 1°C temp for 17-21 days in dark and after it transfer in light followed by keeping in the fridge at 4 °C.

Step II: Liquid Culture Medium Preparation

(A) Preparation of Liquid Culture Medium

The liquid culture medium consisted of Potato starch 1 Litter, Dextrose 30 g L^{-1}, Peptone 10 g L^{-1}, Yeast extract 5 g $L^{-1}$and $MgSO_4$ 0.5 g L^{-1}, pH 6.0. Mix all material in Flask and Five hundred milliliter of liquid add into each 1 Lit. Flask and Autoclave at 121°C & 15 PSI for 50 min. after autoclaving, place the conical flask in the laminar air flow chamber to cool it down.

(B) Liquid Culture Preparation

Small pieces of pure culture (around 1cm x 1cm) from PDA Plate/Slant containing pre-cultured *Cordyceps militaris* were sub-cultured into Liquid culture medium and grow for 5-7 days at 22 °C temp with shaking at 121 rpm.

Step III: Substrate Preparation

(A) Preparation of Substrate

The liquid culture medium consisted of Brown Rice 20 g and Liquid Medium 40 ml in each jar.

(B) Liquid Medium for Substrate

The liquid medium consisted of Peptone 10 g L^{-1}, Yeast extract 5 g L^{-1}, Dextrose 20 g L^{-1}, $MgSO_4$ 0.5 g L^{-1}, KH_2PO_4 2 g, Tri Ammonium Citrate 1 g L^{-1}, Vitamin B1 0.05 g L^{-1} and Vitamin B12 0.01 g L^{-1} pH 6.0. Mix all material in 1 litter Distilled Water and 40 ml of liquid add into each jar, containing brown rice then Autoclave all jars at 121°C and 15 PSI For 50 min., after autoclaving, place the Culture Jars in the laminar air flow chamber to cool it down for 8 h.

(C) Inoculation

Take the Culture from liquid culture containing pre-cultured *Cordyceps militaris* were inoculated in all the variable substrate, then cultured Jars were incubated in culture room at 22 °C for 12 days in the dark, followed by transfer to light for 43 days. The primordia of the fruiting bodies began to form 12 to 15 days after lowering the incubation temperature at night with culture temperature maintained 22 °C during the day hours and relative humidity was maintained at 80-85%. Light cycles were controlled using timers and the CO_2 level was maintained by circulating sufficient fresh filtered air into the incubation room. The culture bottles were placed in 20 °C and 65% relative humidity. After the maturation of fruiting bodies production parameters were measured (Number of fruiting bodies, Maximum length, Wet and Dry Weight of fruiting bodies per jar).

Substrate variables and their combinations for the study:

Control: Brown rice (*Oryza sativa*) 20 gm and Liquid Medium 40 ml.
Variable I: Brown rice 18 gm, Bajra (*Pennisetum glaucum*) 2 gm and Liquid 40 ml.
Variable II: Brown rice 16 gm, Bajra 4 gm and Liquid 40 ml.
Variable III: Brown rice 18 gm, Soya bean (*Glycine max*) 2 gm and Liquid 40 ml.
Variable IV: Brown rice 16 gm, Soya bean 4 gm and Liquid 40 ml.
Variable V: Brown rice 18 gm, insect (*Philosamia risini*) cuticle 2 gm and Liquid 40 ml.
Variable VI: Brown rice 16 gm, insect cuticle 4 gm and Liquid 40 ml.

For each Variable 50 jars of 500 ml were used and the experiment was repeated thrice. The production results are the average of these observations.

RESULTS AND DISSCUSION

The results of the study are summarized in table 1 where the solid substrate brown rice was treated as control for fruiting body production and six variables, Bajra grain 10% and 20% with 90% and 80% of brown rice (Variable I & II) Soya bean 10% and 20% with 90% and 80% of brown rice (Variable III & IV) and Insect Cuticle 10% and 20% with brown rice 90% and 80% (Variable V & VI) were selected to study their effect on the production of fruiting bodies. After the maturation of fruiting bodies in all jars, their average number, length, wet and dry weight, were compared with the control.

The brown rice is usually considered as a better substrate for higher fruiting body and cordycepin production (814.2 mg/g) as compared to wheat (638.8 mg/g) and oat substrate (565.2 mg/g) as reported by Mohd Adnam (2017).

During the present study average number of fruiting bodies in brown rice substrate remained 22.7 per jar as compared to 27.1 and 30.1 in Variable I & II, 32.4 and 30.1 in Variable III & IV, and 18.2 and 13.4 in Variable V & VI respectively. Average length of fruiting bodies per jar was

Figure 1: Photograph showing the Substrate jars of Control Group with mature fruiting bodies (average length 7.03 cm and average number 22.7) after 55 days of inoculation

Figure 2: Photograph showing the Substrate jars of Variable II (Brown Rice 80% or Bajra 20%) with mature fruiting bodies (average length 8.53 cm and average number 30.1) after 55 days of inoculation

Figure 3: Photograph showing the Substrate jars of Variable IV (Brown Rice 80% or Soybean 20%) with mature fruiting bodies (average length 4.7 cm and average number (30.1) after 55 days of inoculation

Table 1: Showing Results Production of Fruiting body of *Cordyceps militaris* in different solid substrate Variables.

Grains	Variable	Average No. of Fruiting Bodies per Jar	Average Length of Fruiting Bodies (cm)	Average Wet Weight of Fruiting Bodies per Jar (gm)	Average Dry Weight of Fruiting Bodies per jar (gm)	Percentage Increase Or Decrease
Brown Rice	**Control**	22.7	7.03	16.9	2.77	
Brown Rice + Bajra	**Variable I** Brown Rice (90%) + Bajra (10%)	27.1	8.01	21.2	3.61	+30.32
	Variable II Brown Rice (80%) +Bajra (20%)	30.1	8.53	26.1	4.43	+59.93
Brown Rice + Soybean	**Variable III** Brown Rice (90%) + Soybean (10%)	32.4	5.22	16.1	2.70	-2.5
	Variable IV Brown Rice (80%) + Soybean (20%)	30.1	4.7	14.5	1.80	-35.0
Brown Rice + Soybean	**Variable V** Brown Rice (90%) + Insect cuticle (10%)	18.2	5.94	18.16	2.70	-2.5
	Variable VI Brown Rice (80%) + Insect cuticle (20%)	13.4	5.70	15.23	2.55	-8.0

highest in Variable II, about 21% higher than Control. In Variable IV it was about 32% less than the Control

The maximum wet weight of the fruiting bodies was significantly higher (54.4%) in Variable II as compared to control while it was lowest 14.5 g in Variable IV which was 14% lower than control. The average dry weight in Variable II was 59.9% higher than control. It was also significantly higher than other variables. The lowest dry weight was recorded in Variable IV. In Variable V & VI insect cuticle was used with Brown rice but the total production was lower than the control.

Pathania and Sagar (2014) have successfully grown *Cordyceps militaris* by using Maize grains, wheat grains and sorghum as solid substrates, but no clear methodology for commercial production was provided of this fungus.
Adnan *et al.,* (2017) reported brown rice as the best Basal substrate for cordeycipin production. Gregori (2014) reported the production of cordeycipin over spent between grains using different strain of *Cordyceps militaris* in the range 100-800 mg/g. depending upon the concentration of solid substrate, however this method was not found cost effective so low-cost grains like bajra and soya bean may be used for commercial production of fruiting bodies of *Cordyceps militaris*. Some more solid substrates may be tried to find out cost effective substrate compositions to increase fruiting bodies and Cordycepin production.

CONCLUSION

Cordyceps militaris has gained importance as a functional food because of its bioactive macromolecules and its medicinal significance. The results obtained in this study could have significant impact on industrial scale production, using combination of cost effective grains, out of six Variables. Variable II showed highest production of fruiting bodies under controlled conditions. The lowest production was recorded in Variable IV which was 35% lower than the control. Further research would be for the precise determination of physicochemical compounds responsible for higher production of Cordycepin and Adenosine Contents. Some more cost effective Solid Substrates and optimized fermentation methods may also be explored to improve production and their impact on the active ingredients.

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REFERENCES

Adnan M, Ashraf SA, Khan S, Alshammari E and Awadelkareem AM (2017). Effect of pH, temperature and incubation time on cordycepin production from *Cordyceps militaris* using solid-state fermentation on various substrates. *CyTA -Journal of Food* **15**(4) 617-621
Basith M and Madelin MF (1968). Studies on the production of perithecial stromata by *Cordyceps militaris* in artificial culture. *Canadian Journal of Botany* **46** 473-80.
Cao L, Ye Y and Han R (2015). Fruiting body production of the medicinal Chinese caterpillar mushroom, *Ophiocordyceps sinensis* (Ascomycetes), in artificial medium. *International Journal of Medicinal Mushrooms* **17** 1107–1112.
Chen A, Wang Y, Shao Y and Huang B (2017). A Novel Technique for Rejuvenation of Degenerated Caterpillar Medicinal Mushroom, *Cordyceps militaris* (Ascomycetes), a Valued Traditional Chinese Medicine. *International Journal of Medicinal Mushrooms* **19** 87–91.
Chen R and Ichida M (2002). Infection of the silkworm, *Bombyx mori*, with *Cordyceps militaris*. *Journal of Insect Biotechnology and Sericology* **71** 1-3.
Chiu CP, Liu SC, Tang CH, Chan Y, El-Shazly M, Lee CL, Du YC, Wu TY, Chang FR and Wu YC (2016). Anti-Inflammatory Cerebrosides from Cultivated *Cordyceps militaris*. *Journal of Agricultural and Food Chemistry* **64** 1540–1548.
Cui JD (2015). Biotechnological Production and Applications of *Cordyceps militaris*, a Valued Traditional Chinese Medicine. *Critical Reviews Biotechnology* **35** 475–484.

Das SK, Masuda M, Sakurai A and Sakakibara M (2010). Medicinal Uses of the Mushroom *Cordyceps militaris*: Current State and Prospects. *Fitoterapia* **81**(8) 961–968.
Gregori A (2014). Cordycepin production from *Cordyceps militaris* cultivation on spent brewery grains. *Acta Biologica Slovenica* **57**(2) 45-52.
Guo HP and Yang ZM (1999). Progress in research of pharmacological of *Cordyceps sinensis*. *Traditional Herbal Drugs* **30** 231-233.
Harada Y, Akiyama N, Yamamoto K and Shirota Y (1995). Production of *Cordyceps militaris* fruit body on artificially inoculated pupae of *Mamestra brassicae* in the laboratory. *Transactions of the Mycological Society of Japan* **36**(2) 67-72.
Hong IP, Kang PD, Kim KY, Nam SH, Lee MY, Choi YS, Kim NS, Kim HK, Lee KG and Humber RA (2010). Fruit body formation on silkworm by *Cordyceps militaris*. *Mycobiology* **38** 128-32.
Jiang Y and Yao YJ (2003). Anamorphic fungi related to *Cordyceps sinensis*. *Mycosystema* **22** 161-176.
Kobayasi Y (1941). The genus *Cordyceps* and its allies. *Science Reports of the Tokyo Bunrika Daigaku Section B* **5** 53-260.
Kontogiannatos D, Koutrotsios G, Xekalaki S and Zervakis GI (2021). Biomass and Cordycepin Production by the Medicinal Mushroom *Cordyceps militaris*—A Review of Various Aspects and Recent Trends towards the Exploitation of a Valuable Fungus. *Journal of Fungi* **7**(11) 986.
Lee CT, Huang KS, Shaw JF, Chen JR, Kuo WS, Shen G, Grumezescu AM, Holban AM, Wang YT, Wang JS, Hsiang YP, Lin YM, Hsu HH and Yang CH (2020). Trends in the Immunomodulatory Effects of *Cordyceps militaris*: Total Extracts, Polysaccharides and Cordycepin. *Frontiers in Pharmacology* **11** 1824.
Lee J, Cho K, Shin SG, Bae H, Koo T, Han G and Hwang S (2016). Nutrient Recovery of Starch Processing Waste to *Cordyceps militaris*: Solid State Cultivation and Submerged Liquid Cultivation. *Applied Biochemistry and Biotechnology* **180** 274–288.
Lin Q, Long L, Wu L, Zhang F, Wu S, Zhang W and Sun X (2016). Evaluation of Different Agricultural Wastes for the Production of Fruiting Bodies and Bioactive Compounds by Medicinal Mushroom *Cordyceps militaris*. *Journal of the Science of Food and Agriculture* **97** 3476–3480.
Lin QY, Zhong YJ, Li TH, Shen YH and Song B (2006). Recent research advances in *Cordyceps* biology. *Acta Edulis Fungi* **13**(2) 97-102.
Lu Y, Xia Y, Luo F, Dong C and Wang C (2016). Functional convergence and divergence ofmating-type genes fulfilling in *Cordyceps militaris*. *Fungal Genetics and Biology* **88** 35–43.
Nakamura K, Shinozuka K and Yoshikawa N (2015). Anticancer and Anti metastatic effects of Cordycepin, an Active Component of *Cordyceps sinensis*. *Journal of Pharmacological Sciences* **127** 53–56.
Olatunji OJ, Tang J, Tola A, Auberon F, Oluwaniyi O and Ouyang Z (2018). The Genus *Cordyceps*: An Extensive Review of Its Traditional Uses, Phytochemistry and Pharmacology. *Fitoterapia* **129** 293–316.
Patel KJ and Ingalhalli RS (2013). *Cordyceps militaris* (L.: Fr.) Link An important medicinal mushroom. *Journal of Pharmacognocy and Phytochemistry* **2** 315-319.
Pathania P and Sagar A (2014). Studies on the biology of *Cordyceps militaris*: A medical mushroom from North West Himalaya. *Kavaka* **43** 35-40.

Reis FS, Barros L, Calhelha RC, Ciric A, Van Griensven LJ, Sokovic M and Ferreira IC (2013). The Metabolic Extract of *Cordyceps militaris* (L.) Link Fruiting Body Shows Antioxidant, Antibacterial, Antifungal and Antihuman Tumor Cell Lines Properties. *Food and Chemical Toxicology* **62** 91–98.

Sato H and Shimazu M (2002). Stromata production for *Crodyceps militaris* (Clavicipitales: Clavicipitaceae) by injection of hyphal bodies to alternative host insects. *Applied Entomology and Zoology* **37** 85-92.

Tao Z, Cao L, Zhang Y, Ye Y, and Han R. (2016). Laboratory rearing of Thitarodes armoricanus and Thitarodes jianchuanensis (Lepidoptera: Hepialidae), hosts of the Chinese medicinal fungus *Ophiocordyceps sinensis* (Hypocreales: Ophiocordycipitaceae). *Journal of Economic Entomology* **109**(1) 176–181.

Tuli HS, Sharma AK and Sandhu SS (2014a). Optimization of fermentation conditions for cordycepin production using *Cordyceps militaris* 3936. *Journal of Biological and Chemical Sciences* **1** 35–47.

Xiaolu J and Yue S (1999). The determination of active components in various *Cordyceps militaris* strains. *Acta Edulia Fungi* **6**(1) 49-52.

Yoon SY, Park SJ and Park YJ (2018). The Anticancer Properties of Cordycepin and Their Underlying Mechanisms. *International Journal of Molecular Science* **19** 3027.

Yu HM, Wang BS, Huang SC and Duh PD (2006). Comparison of preventive effects between cultured *Cordyceps militaris* and natural *Cordyceps sinensis* against oxidative damage. *Journal of Agricultural and Food Chemistry* **54**(8) 3132-3138.

Zhou X, Gong Z, Su Y, Lin J and Tang K (2009). Cordycepin fungi: natural products, pharmacological functions and developmental products. *Journal of Pharmacy and Pharmacology* **61**(3) 279-291.