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Morphological, Physiological and Molecular studies on wildly collected *Cordyceps militaris* from North West Himalayas, India

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Abstract
Cordyceps militaris (L.) Link is an entomopathogenic fungus, which enjoyed an extensive praise for its medicinal functions. In this study, taxonomic details, isolation of pure culture, influence of different physiological requirements on the mycelia growth of this fungus its chemical components and molecular characterization has been carried out. A small bit of tissue from stipe and stroma was used as material for *in vitro* isolation. The present investigation revealed that this fungus showed optimum growth in YPDA (yeastal potato dextrose agar) and GAS (glucose asparagine solution). The maximum mycelial growth was observed at 25 ºC and pH 7.5 and 5.5 in solid and liquid media respectively. Among various carbon, nitrogen, mineral and vitamin sources tested, sucrose, beef extract, zinc chloride and folic acid produced the maximum mycelial yield respectively. The fungus was found rich in various chemical components like vitamins, proteins trace elements, cordycepin and cordycepic acid. Molecular characterization of fungus resulted in amplified product of 540 basepairs, which is submitted in NCBI database having submission ID KJ004029. *C. militaris* has been successfully cultivated under lab scale cultivation trials under standardized nutritional and climatic conditions.

Keywords: *Cordyceps militaris*, medicinal mushroom, entomopathogenic fungus, physiological requirement, molecular characterization

1. Introduction
Medicinal fungi have long been an important part of human civilization, and species in the genus *Cordyceps* are especially valued [1]. *Cordyceps*, a macrofungus that is parasitic on insects, is used as a source for functional food development and new drug discovery [2]. It belongs to phylum Ascomycota, class Ascomycetes, order Hypocreales and family Clavicipitaceae [3]. While not actually a mushroom in the taxonomic sense, it has been regarded as, a medicinal mushroom throughout history. The name comes from the Latin words: *cord* and *ceps*, meaning “club” and “head”, respectively. The Latin conjugation describes the appearance of the club fungus, *Cordyceps sinensis*, whose stroma or fruitbody extends from the mummified carcasses of insect larvae, usually caterpillar larva of the Himalayan bat moth, *Hepialis armoricanus. Cordyceps* mushroom has a long history as medicinal fungus. It has been regarded as a cornerstone of traditional Chinese medicine for centuries; that apparently have a number of far reaching medicinal effects [4, 5]. *Cordyceps* mushrooms have been used to treat conditions including respiration and pulmonary diseases, renal, liver, cardiovascular diseases, hyposexuality and hyperlipidemia. It is also used in the treatment of immune disorders, and as an adjunct to modern cancer therapies [3, 6].

2. Materials and Methods
2.1. Survey, collection, taxonomic studies and isolation of pure culture
Fruiting bodies of *C. militaris* (L.) Link have been collected from Glen forest and Tara Devi (Distt. Shimla, H.P.) and its adjoining areas during the months of June to September. Fruiting bodies have been preserved dry as well as wet [7] and the specimens have been deposited in the Herbarium of department of Biosciences, Himachal Pradesh University, Shimla.

2.2. Macroscopic studies
Various characters, which help in the identification of specimens e.g. shape, size and colour of the stipe and stroma, association with insect larvae and pupae were recorded by examining the specimens with naked eye. The specimens were identified by following Lincoff [8].

2.3. Microscopic studies
For microscopic studies both dried as well as wet preserved specimens were used. The dried parts of specimen were kept for few minutes in 95% ethyl alcohol (to expel out the air) and then in water. The anatomical details of the specimen was worked out by cutting free hand sections of the material. Microscopic details of the specimen was worked out in laboratory with the help of research microscope. This included the study of mycelium and spores. For clarity the sections were stained with 1% cotton blue and lactophenol. The sections were observed under the microscope. Photomicrographs of slides of mycelium and spores were taken and measurements were recorded with micrometer.

2.4. SEM Studies
Surface of mycelium and spores were imaged with the help of Scanning Electron Microscope (SEM). For scanning electron microscopy, samples were mounted on carbon tape and were placed on the stub, then placed in Environmental Scanning Electron Microscope Mode (ESEM MODE) under vacuum and desired pressure, the images of the samples were obtained on screen.

2.5. Isolation of pure culture of *Cordyceps militaris*
The cultures 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 tissue from the stipe and stroma portion were cut with the help of a sterilized blade. The bits of tissue (2-3 mm) were taken up with a sterilized forceps and dipped in 0.1% mercuric chloride solution for 5-10 seconds. Now the tissue was placed on filter paper to remove the excess moisture. The small bits of *Cordyceps* tissues were then transferred aseptically into the petriplates containing potato-dextrose agar (PDA) medium with the help of a sterilized forceps. These were then incubated at 25 ^{0}C for at least 8-10 days and observed regularly for appearance of culture. The actively growing mycelial colonies were sub cultured to obtain pure cultures.

2.6. Physiological Studies
Twelve solid and five liquid media have been tried during the present studies. All media were prepared following Tuite [9].

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

2.7. 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 filter paper No.1 and weighed to give dry weight of the mycelium. The medium with best mycelial growth was used for further studies.

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

2.9. 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 also 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 NaOH and HCl. The pH was checked with the help of digital type Philips pH meter. The inoculated petri plates and flasks were incubated for 10 days at optimum temperature and after that the growth was measured.

2.10. 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.

2.11. Effect of different Carbon, nitrogen, minerals and vitamin sources
To find out the best carbon, nitrogen, mineral and vitamin sources for the growth of fungus, the best liquid basal medium was substituted by different nutritional sources. Observations on dry mycelia weight were recorded after 10 days of incubation.

2.12. Determination of different Chemical Components
The various nutritive and chemical components like proteins, vitamins, trace elements, cordycepin, cordycepic acid, polysaccharides and superoxide dismutase were determined following standard techniques and methods [10].

2.13. Molecular Characterization
For DNA extraction, mycelia cultures were raised in liquid potato broth. The DNA was extracted using HiPura TM Fungal DNA Purification Kit. Amplification of genomic DNA was performed by PCR. Amplification of 5.8s rRNA gene for assessing ITS length variation was done using universal primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCCCGCTTATTGATATTGC) [11].

2.14. Artificial Cultivation Trials
Mass inoculum was prepared on wheat grains [12]. Lab scale cultivation trials were performed on grains and potato broth medium supplemented with different nutrients and subjected to different conditions (Dark, Light and in BOD) in laboratory.

3. Results

3.1. Macroscopic characters

The fruiting body of *C. militaris*, was creamish white in colour. Fruiting body shows association with insect larvae and pupae. The associated insect's body becomes mummified by the growth of the mycelium. The mycelium of the fungus forms fruiting bodies, which interestingly, always emerge from the head of the larva. The size of fruiting body varied from 4.2 to 7.8cm (Plate 1, a-b).

3.2. Microscopic characters

Conidiophore: Sub cylindrical, 210.2-212.4 x 45.3-46.3 µm
Phialide : Flask Shaped, 223.3-226.1 x 48.7-49.8 µm
Conidia : Barrel Shaped, 217.2-223.2 x 47.6-49.9 µm
Hyphae : Thin walled, branched, 14.8-16.1 µm broad (Plate1, c-d)

3.3. SEM Studies

The mycelia and spores of *C. militaris* were observed under Scanning Electron Microscope (SEM) at different magnification at pressure 2.9e-1 Torr. The diameter of hyphae generally ranged from 3.18 µm to 2.49 µm. Spore surface of fungus was smooth and velvety, its size ranged from 5.89 µm x 3.82 µm to 5.82 µm x to 3.20 µm (Plate 1, e-f).

3.4. Mycelial characteristics

Mycelial growth of *C. militaris* was longitudinally radial, aerial initially, creamish white, becoming densely matted and wooly in texture. As soon as the colony matures the colour of mycelium changed from creamish white to light brown. As the medium was completely consumed, the mycelia became increasingly mud-like and granular in texture. At approximately 16 days of growth or a bit later, the mycelium of *C. militaris* began to form small nodules (perhaps sclerotia) at the centre on the surface of the medium, appearing light brown while peripheral mycelia remained creamish white (Plate-2, a-b).

3.5. Physiological Studies

Among the twelve solid and five liquid media tried, Yeastal Potato Dextrose Agar and Glucose asparagine solution were found to be the best solid and liquid medium respectively (Plate-2, b-c and Fig.1-2). The best mycelial growth of *C. militaris* was observed at 25 ºC both in solid and liquid media (Fig. 3-4). The best mycelial growth of *C. militaris* was observed at pH 7.5 and 5.5 in solid and liquid medium respectively (Fig. 5-6). With regard to the effect of light and darkness on solid medium, the mycelium was found to give better growth under darkness in comparison with light (Fig.7). Among five carbon, six nitrogen, six mineral and six vitamin sources tested, sucrose, beef extract, zinc chloride and folic acid produced the maximum mycelial yield, respectively (Fig.8-11).

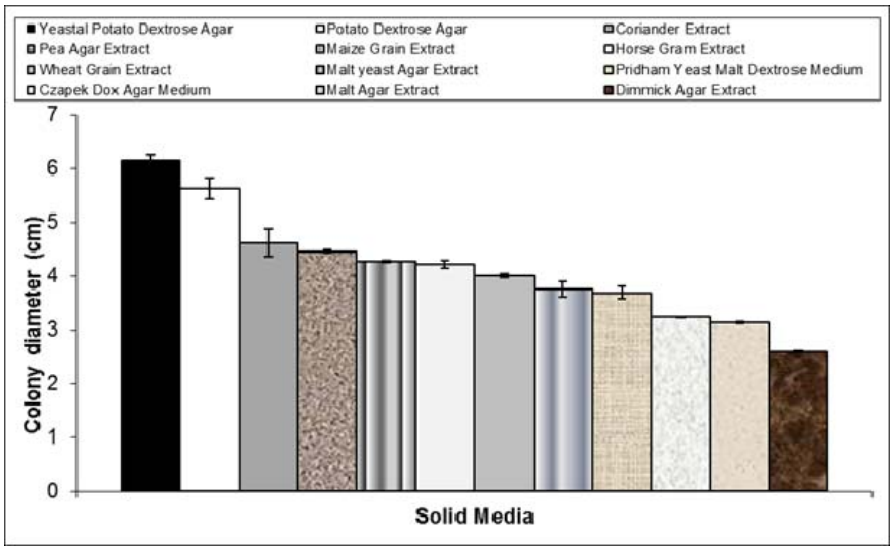

Fig 1: Effect of different solid media on the growth of *C. militaris*

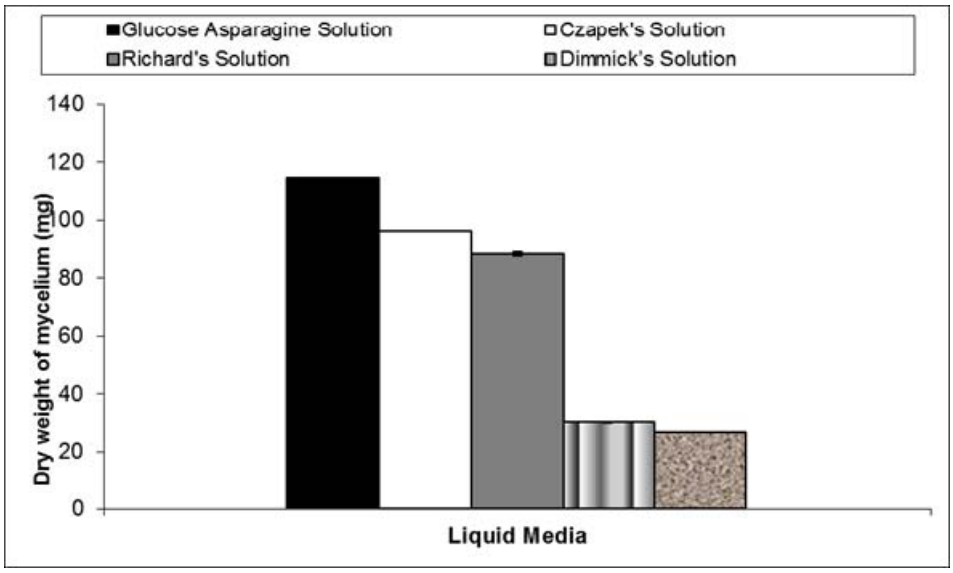

Fig 2: Effect of different liquid media on the growth of *C. militaris*

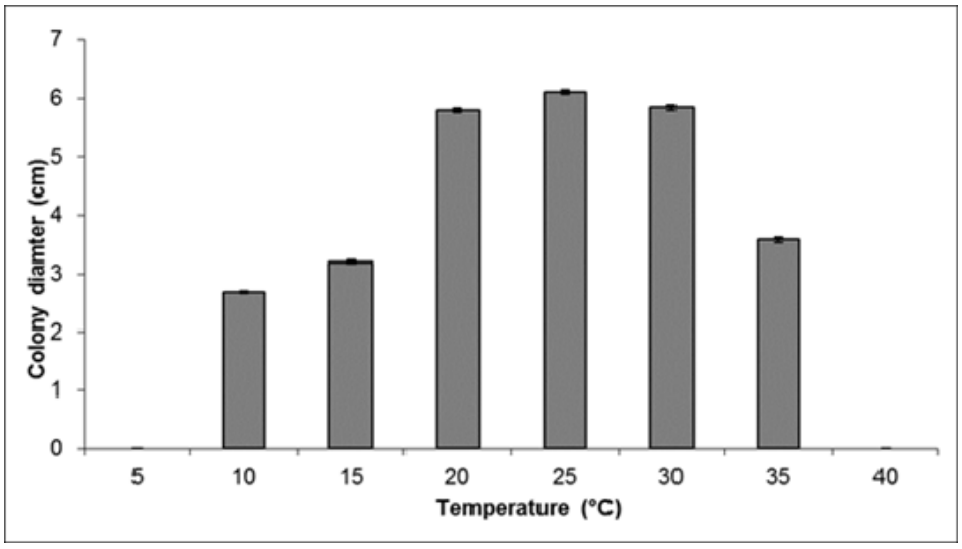

Fig 3: Effect of temperature on the growth of *C. militaris* in basal solid medium

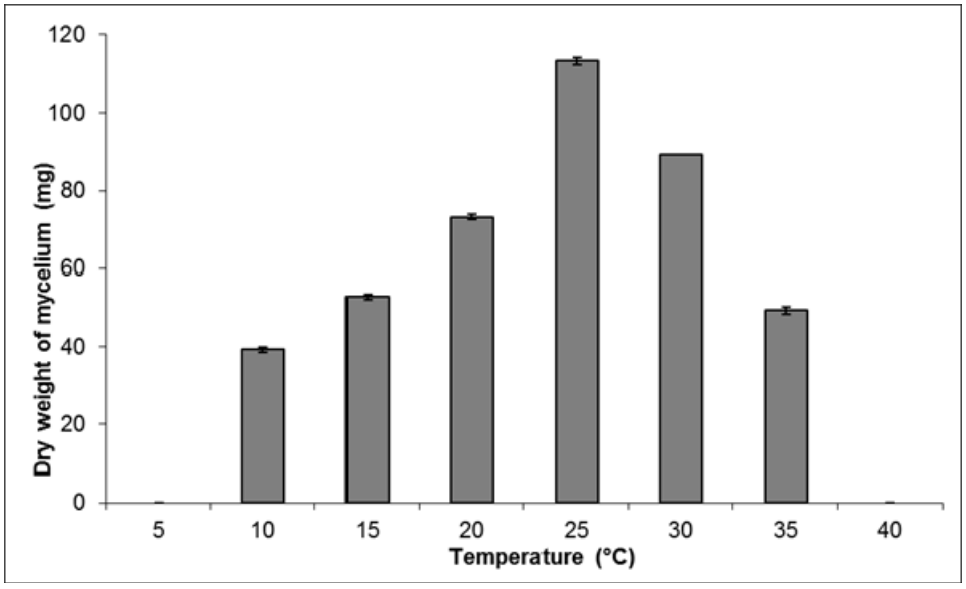

Fig 4: Effect of temperature on the growth of *C. militaris* in basal liquid medium

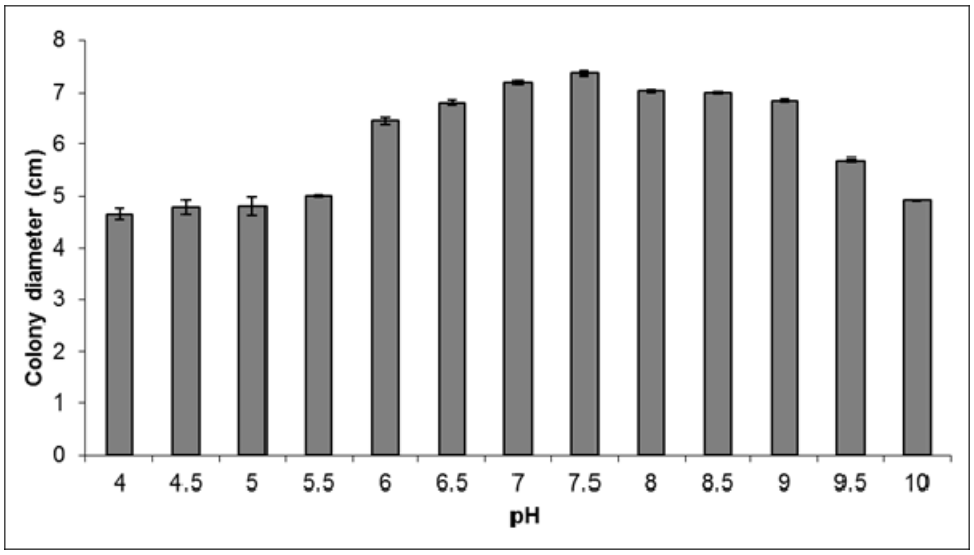

Fig 5: Effect of different pH levels on the growth of *C. militaris* in basal solid medium

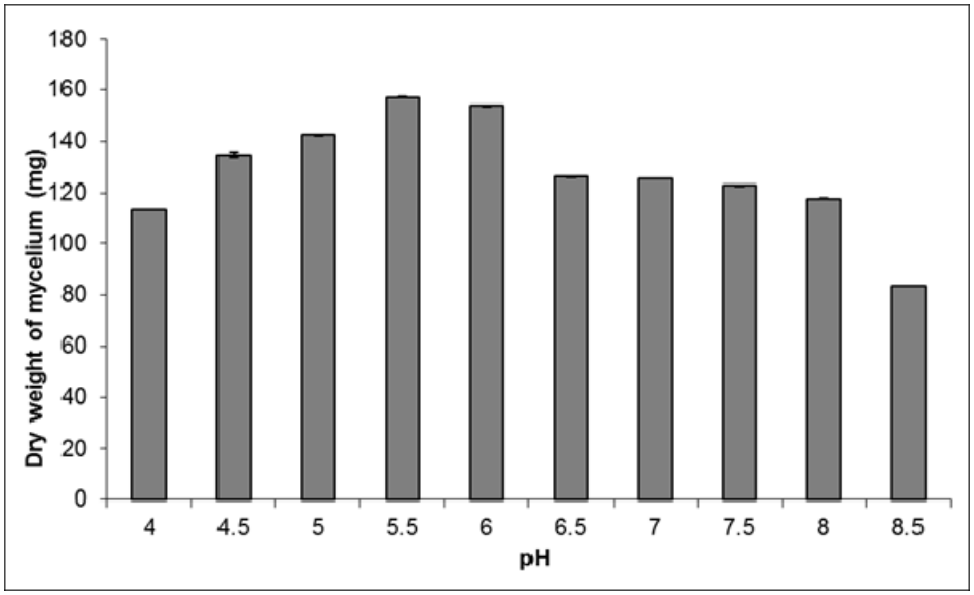

Fig 6: Effect of different pH levels on the growth of *C. militaris* in basal liquid medium

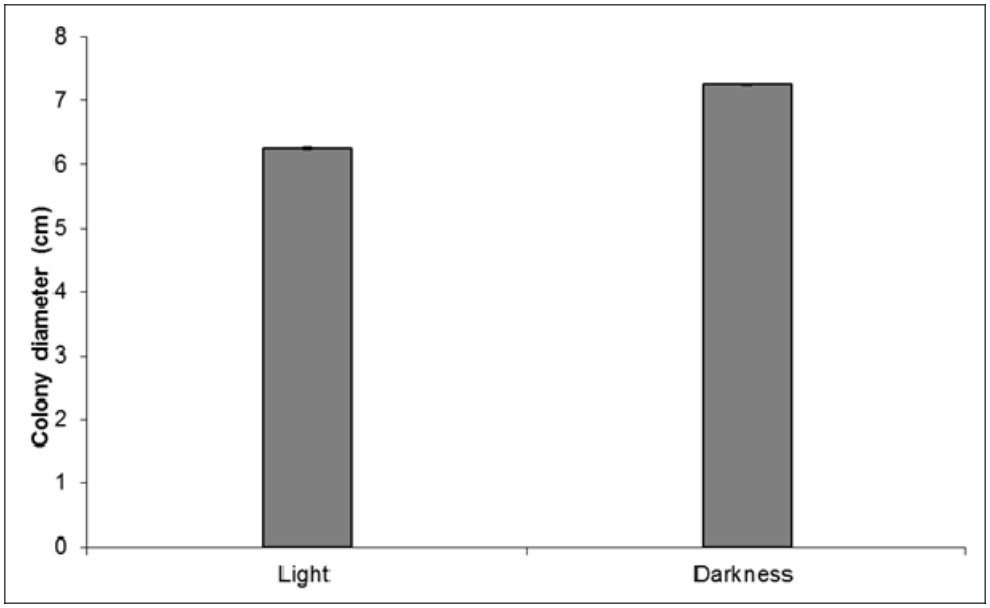

Fig 7: Effect of light and darkness on the growth of *C. militaris* in basal solid medium

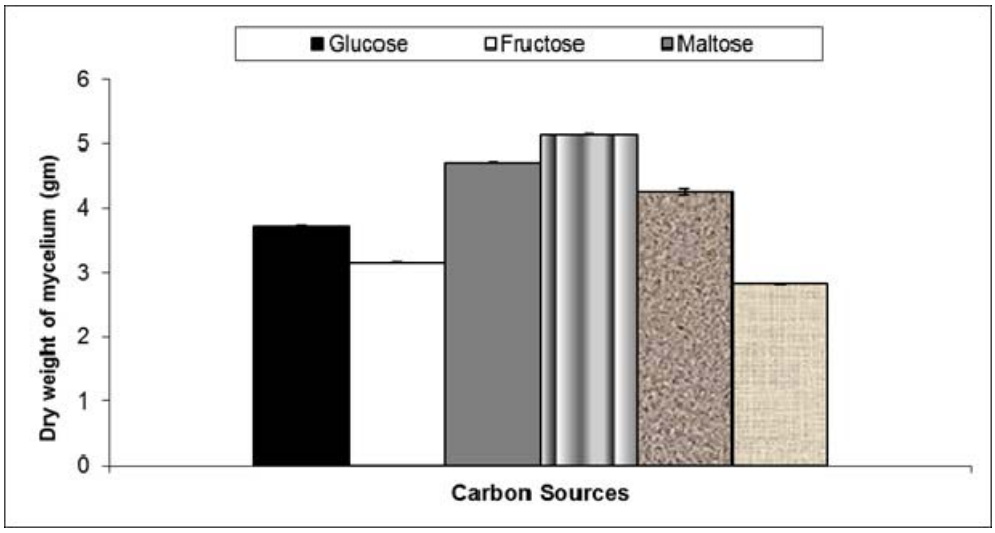

Fig 8: Effect of different carbon sources on the growth of *C. militaris* in basal liquid medium

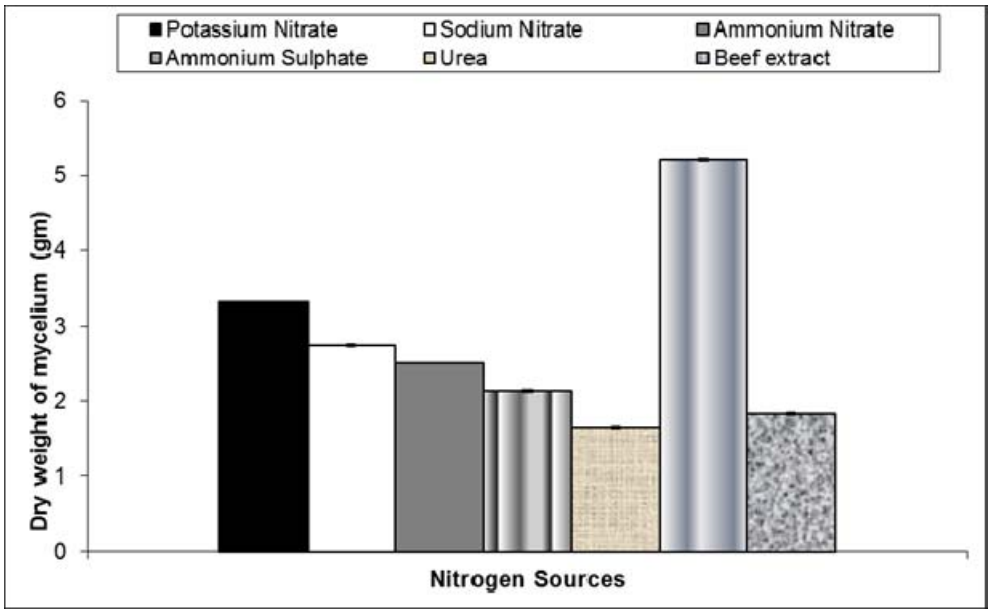

Fig 9: Effect of different nitrogen sources on the growth of *C. militaris* in basal liquid medium

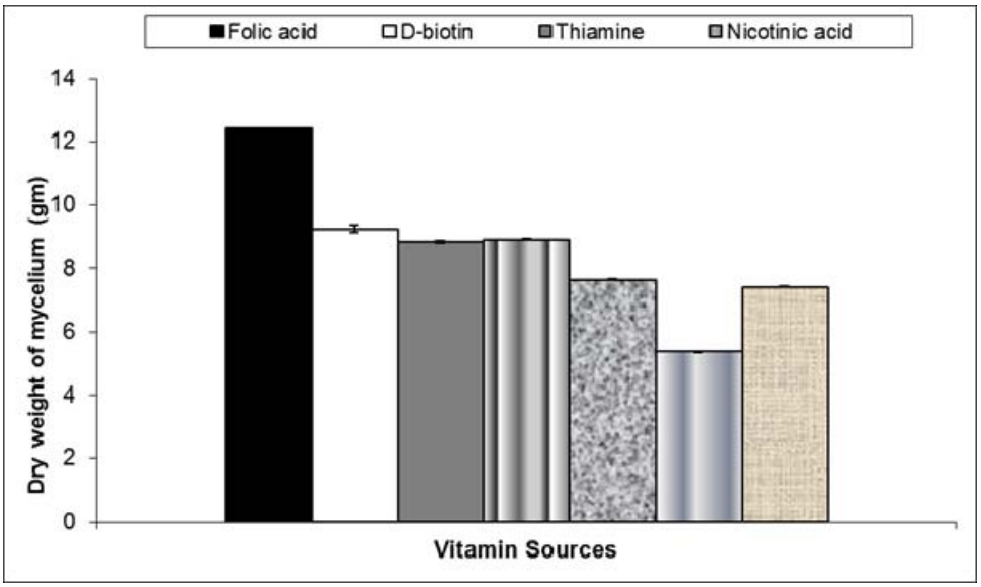

Fig 10: Effect of different vitamin sources on the growth of *C. militaris* in basal liquid medium

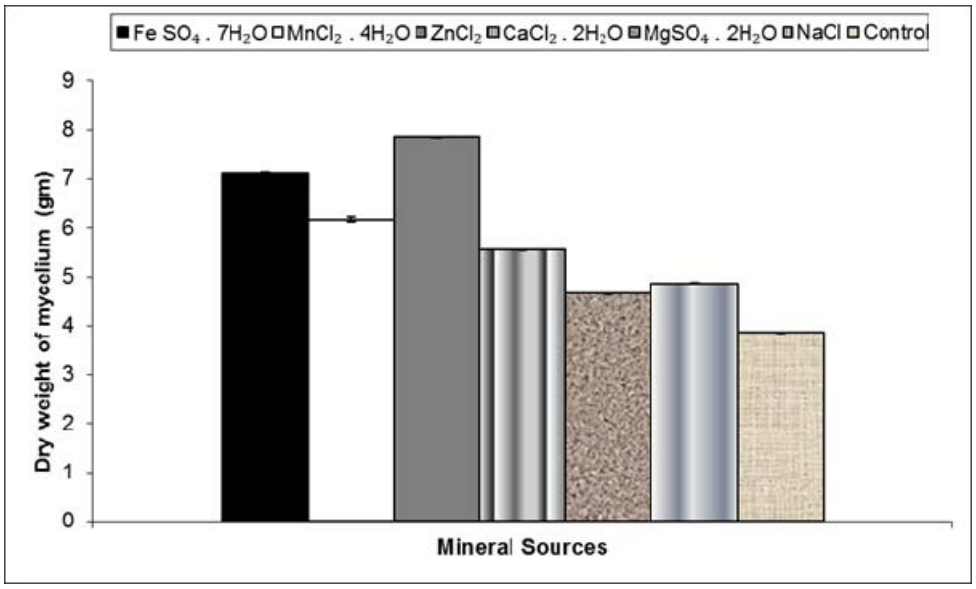

Fig 11: Effect of different mineral sources on the growth of *C. militaris* in basal liquid medium

3.6. Chemical components of *C. militaris*

Fruiting bodies of *C. militaris* have been found to be rich in the following biochemical components:

Protein (%)	Vitamins (mg/g)					Trace elements (ppm)			Cord-ycepin (%)	Cordyc-epic acid (%)	Polysacc-harides (%)	SOD (Units/mg protein)
	A	B_1	B_6	B_{12}	B_3	Se	Zn	Cu				
39.15	32.7	12.4	58.7	69.3	40.6	0.35	127.1	27.7	1.24	10.79	28	51

3.7. Molecular Characterization: Using genomic DNA isolated from the mycelial culture, an approximately 500 basepair fragments of the rDNA-ITS region was amplified using ITS1 and ITS4 primers and subjected to nucleotide sequencing. The amplified product was found to have 540 basepairs and total base count as 118 a, 187 c, 143 g and 92 t. In NCBI BLAST submitted sequence found 100% similarity with *Isaria tenuipes* (anamorphic stage). The aligned sequence was deposited in GeneBank named as *Isaria tenuipes* ASPPHP1 having submission ID KJ004029. Here AS stands for Anand Sagar, PP for Pooja Pathania and HP1 for Himachal Pradesh 1 sequence only. Based on the above results, it is ascertained that this wild mushroom a member of family Clavicipataceae in the Hypocreales (Ascomycota).

BASE COUNT 118 a 187 c 143 g 92 t
ORIGIN
1 tcccaccctt ctgtgaccta cccatagttg cttcggcgga cccgccccag cgtccggacg

61 gcccagcgcc ggcccgcgac ctggacccag gcggccgccg gggaccacgc aaccctgtat

121 ctgtcagcct ctctgaatcc gccgcaaggc aacacaaacg aatcaaaact ttcaacaacg

181 gatctcttgg ttctggcatc gatgaagaac gcagcgaaat gcgatacgta atgtgaattg

241 cagaattccg tgaatcatcg aatctttgaa cgcacattgc gcccgccagc attctggcgg

301 gcatgcctgt tcgagcgtca tttcaaccct cgacgtcccc cgggacgtcg gccttgggga

361 ccggcagcac cccgccggcc ctgaaatgga gtggcggccc gtccgcggcg acctctgcgc

421 agtacaagca ctcgcaccgg gaacccgacg cggcccgccg tgaaaccccc aacctctgaa

481 cgttgacctc ggatcaggta ggactacccg ctgaacttaa gcatatcaat aagcggagga

3.8. Mass inoculum and lab scale cultivation trials

Mass inoculums was prepared on different substrates (wheat grains, maize grains and sorghum) following standard method as described in methodology for lab scale cultivation trials of *Cordyceps militaris* on different substrates (Plate 2, d-f). Polypropylene bags containing sterilized wheat grains and sorghum were inoculated with pure culture of *C. militaris.* These bags were incubated at 25°C for 15 days in a dark room. Entire grain surface was colonized by the fine thread like mycelium of fungus. These polypropylene bags were then opened and subjected to different treatments like moisture, humidity, temperature and light variations. It was noticed that bags subjected to moderate day light (2-4 hours), optimum temperature (25 ^{0}C), 50%-70% atmospheric humidity started showing vertical stromata which resulted in the formation of complete fruiting bodies in 50-60 days (Plate 2, g-i).

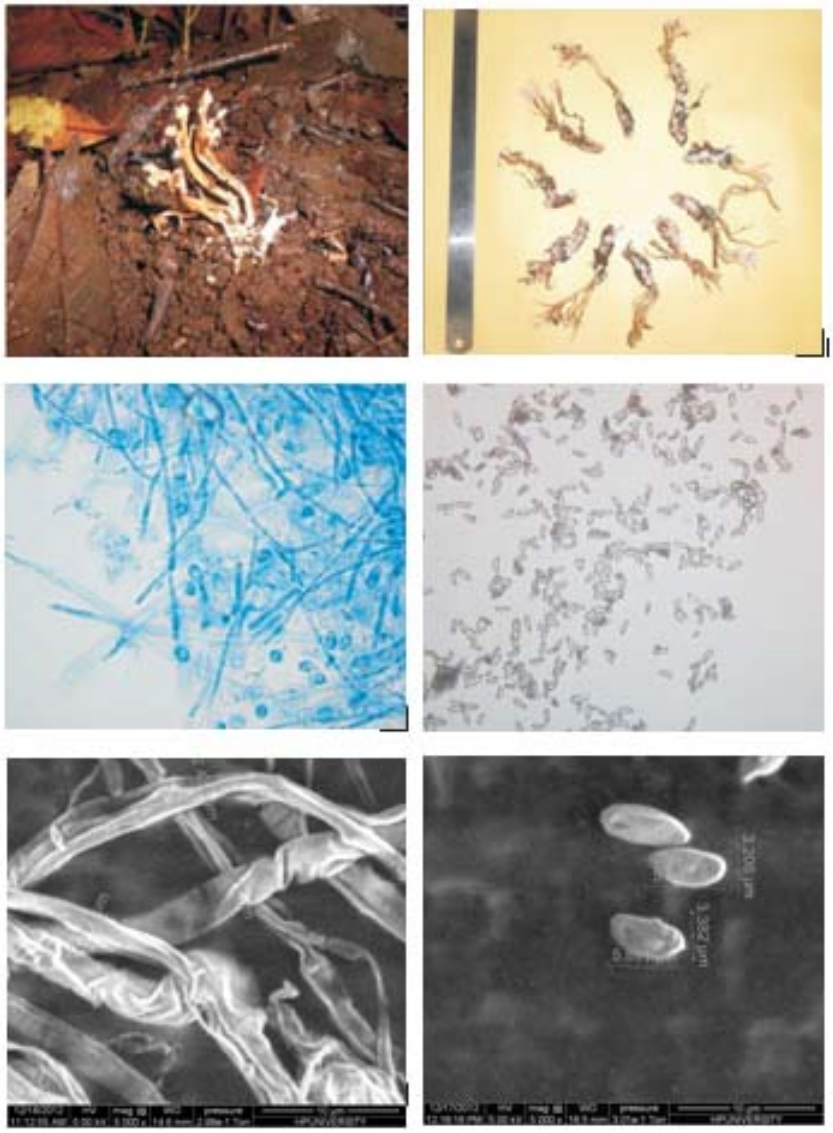

Plate 1:
a) *Cordyceps militaris* in its natural habitat.
b) Fruiting bodies of
C. militaris showing association with its insect host.
c) Microphtograph of mycelium of *Cordyceps militaris.*
d) Microphtograph of spores of *Cordyceps militaris.*
e) SEM images of hyphae of *C. militaris.*
f) SEM images of spore of *C. militaris.*

Plate 2:
a) Pure culture of *Cordyceps militaris* .
b) Growth of *Cordyceps militaris* on Yeastal Potato Dextrose Agar (Best Solid Medium).
c) Growth of *Cordyceps militaris* on Glucose Asparagine Solution (Best Liquid Media).
d) Mass inoculum on Wheat grains.
e) Mass inoculum on Sorghum grains.
f) Mass inoculum on Maize grains.
g) Artificially cultivated fruiting bodies of *C. militaris* on Wheat grains.
h) Artificially cultivated fruiting bodies of *C. militaris* on Maize grains.
i) Artificially cultivated fruiting bodies of *C. militaris* on Broth medium.

4. Discussion

During present study specimens of *C. militaris* have been collected from different sites of Shimla district of Himachal Pradesh. The macroscopic and microscopic details have been worked out. Sehgal and Sagar [13] have also conducted study on morphology and anatomy of *C. militaris* and our results pertaining to measurement are in agreement with them with slight variations. SEM study on *C. militaris* has been conducted for the first time in India.

Among the twelve solid and five liquid media tried, yeastal potato dextrose agar and glucose asparagine solution were found to be best solid and liquid media at 25 ºC, respectively. The best mycelial growth of *C. militaris* was observed at pH 7.5 and 5.5 in solid and liquid media, respectively. With regard to light and darkness, the mycelium was found to show better growth under darkness. In case of different carbon, nitrogen, minerals and vitamins tried, sucrose, beef extract, zinc chloride and folic acid produced the maximum mycelial yield respectively. All results are in agreement with earlier reports with minor variations which can be attributed to strain specific behavior of the fungus.

Present investigations primarily confirmed the presence of carbohydrates, proteins, fats and different minerals in the fruiting bodies of *C. militaris* collected from Himachal Pradesh. There lies a scope for extending such study to the analysis of individual types of Carbohydrates, amino acides, minerals and vitamins.

Molecular characterisation studies on pure culture of *Cordyceps militaris* have identified this fungus as *Isaria tenuipes* (having 100% similarity). Madelin [14] have also reported that condial state of *C. militaris* (telomorphic stage) produced in pure culture resembles *Paecilomyces* which is now known as *Isaria tenuipes* (anamorphic stage). Molecular techniques have been successfully used for identifying fungi. However, molecular analysis alone has limitations. The use of ITS sequences also has limitations because the non-coding ITS sequence is fast evolving with many variable characters, it is usually difficult to achieve a perfect sequence alignment at high taxonomic levels. Moreover it has been shown that 20-30% of sequence downloaded from genebank for comparative analysis may

not be accurate in the identification [15, 16]. But as per the matching of descriptions of morphological, macro and microscopic characters of fruting body, conidia and ascospores with the details given by Lincoff [8] the specimen is *Cordyceps militaris.* This is the first report from Himachal Pradesh on molecular characterization of *Cordyceps militaris.*

C. militaris has been artificially grown successfully under lab scale cultivation trials on wheat and maize grains substrates providing it suitable standardized climatic conditions. There are few reports [17, 18] in literature which claim artificial cultivation of *C. militaris* using different substrates including insect dead bodies. But none of such claim provides a clear and long term methodology for commercial cultivation of this mushroom.

In the Indian mushroom scenario, this is the first report of successful artificial cultivation of this costliest (Rs 1 lakh/Kg) and highly medicinal mushroom. Nearly 80–85% of all medicinal mushroom products are extracted from their fruiting bodies while only 15% are derived from mycelium culture [19]. As all physical and chemical requirements for the growth of *C. militaris* have been standardized in the present investigation, there is a scope to extend this work towards commercial cultivation of *C. militaris* using agro-wastes which will reduce pollution and pressure on the yield from natural forests.

5. Conflict of Interest: The authors declare that there are no conflicts of interest.

6. Acknowledgement: Thanks are due to the University Grants Commission, New Delhi for providing financial assistance (F.No. 37-391/2010(SR)) and Chairman, Department of Biosciences, Himachal Pradesh University, Shimla for providing laboratory facilities.

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