CHARACTERIZATION OF MEDICINAL MUSHROOM, *Cordyceps* sp. FROM KASARGOD DISTRICT

By

LAYA P. K.
(2016-11-024)

THESIS

Submitted in partial fulfillment of the requirement for the degree of

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DEPARTMENT OF PLANT PATHOLOGY
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DECLARATION

I, hereby declare that the thesis entitled **'Characterization of Medicinal Mushroom, *Cordyceps* sp. from Kasargod District'** is a bonafide record of research work done by me during the course of research and that this thesis has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara,
Date: 29/08/2018

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CERTIFICATE

Certified that this thesis entitled **"Characterization of Medicinal Mushroom, *Cordyceps* sp. from Kasargod District"** is a bonafide record of research work done independently by **Ms. Laya P. K.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

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Introduction

1. INTRODUCTION

Medicinal mushrooms existed on earth for millions of years and have been utilized by humans along with the evolution of ancient folk medicine. The research on medicinal mushroom is at primitive stage in India, especially in Kerala, which offers a great hope and also holds a promise for the control of fatal diseases. The genus of fungi *Cordyceps* includes more than 500 unique species of parasitic types (Stensrud *et al.* 2007; Sung *et al.*, 2007), which have been traditionally used in herbal medicine for centuries. These "club head" medicinal mushrooms, not real mushrooms in real taxonomic sense, have a wide range of chemical components that mimic current pharmacological substances in a natural way. Recently, the price of *Cordyceps* is equal to gold, which reached 13-15 lakh rupees per kg in international markets. *Cordyceps* has been used to treat conditions including respiration and pulmonary diseases, renal, liver, and cardiovascular diseases, hyposexuality, and hyperlipidemia (Arora, 2015). *Cordyceps* has proven to be a nontoxic fungus which is also used in the treatment of immune disorders and as an adjunct to modern cancer therapies *viz.*, chemotherapy, radiation treatment and surgery. The distribution of members of the genus *Cordyceps* is cosmopolitan in nature, but mostly confined to, China, Japan, Korea, Thailand and Asia. In Asia, *Cordyceps* sp. is endemic to the Tibetan Plateau including the adjoining high altitude areas of the Central and East Himalayas that includes Nepal, Bhutan and India's Uttaranchal, Sikkim, Himachal Pradesh and Arunachal Pradesh (Panicker, 2017).

Reports of *Cordyceps* sp. from Kerala are very few and from the surveys conducted in coastal sandy areas of Kasargod district, it was observed that *Cordyceps* sp. attacks the coconut root grubs (*Leucopholis coneophora* Burm.) (Kumar and Aparna, 2014). In India an organized approach for isolation, identification, characterization and artificial culturing of this particular species of *Cordyceps* under *in-vitro* conditions and its further exploitation for medicinal benefits was not carried out. Most species of *Cordyceps* have a restricted host range and rigid

host specificity. Due to scarcity in availability and threats by environmental factors, the resource of *Cordyceps* sp. in nature faces an ecological disaster. The artificially cultivated source of *Cordyceps* sp. will be the sustainable alternative to overcome this crisis. *Cordyceps* sp. is only found in cohabitation with a specific host, and it is this unique growth parameter that has made it challenging to produce in artificial cultivation. Based on molecular phylogenetic study by Sung *et al.* in 2007 the genera *Cordyceps*, which attack Coleopteran group of insects, were transferred to the genus *Ophiocordyceps* and therefore in the present study the genus name of the target fungus is renamed as *Ophiocordyceps.* The present study of 'Characterization of medicinal mushroom, *Cordyceps* sp. from Kasargod district' deals with the characterization and identification of *Cordyceps* sp. growing on coconut root grub from Kasargod district of Kerala, based on morphological, physiological and phylogenetic studies. This is the primary endeavor in India, where experiments on artificial culturing and assessment of nutritional status of this fungus was also done.

The study comprises of the following objectives,

- Survey for the collection of *Cordyceps* sp.
- Isolation of *Cordyceps* sp.
- Cultural, morphological and physiological characterization of *Cordyceps* sp.
- Artificial culturing of *Cordyceps* sp.
- Proximate analysis of *Cordyceps* sp.

Review of literature

2. REVIEW OF LITERATURE

Cordyceps are entomopathogenic fungi on arthropods which on germination, kills and mummifies their larva, and then grows from the body of the host. Due to its low availability in nature and increase in demand, it needs systematic study in the identification and characterization of this mysterious fungus. Natural incidence of *Cordyceps* has been found on coconut root grub (*Leucopholis coneophora* Burm.), which is a serious polyphagous pest in sandy loam tracts of Northern Kerala. Therefore this work of 'Characterization of medicinal mushroom, *Cordyceps* sp. from Kasargod district' is a first attempt to document the morphological, cultural, physiological and nutritional characteristics of this fungus.

2.1. HISTORY AND SIGNIFICANCE

Cordyceps sinensis attracted the attention of the general public and the health profession in 1993, when a group of Chinese runners broke nine world records in the world outdoor track and field championships in Germany. The team was first accused of using performance-enhancing drugs but the coach attributed success to the use of a *C. sinensis* based Traditional Chinese Medicinal (TCM) tonic (Steinkraus and Whitfield, 1994). Herdsmen of Tibet region noticed that their livestock became active and energetic after eating a certain mushroom present in the high altitude graze lands, which was later identified as *Cordyceps* sp. (Sharma, 2004). Holliday and Cleaver (2004) reported that the first mention of *Cordyceps* was from the Tang Dynasty (AD 618-AD 907) as a creature which transformed into a plant in the summer then back to an animal in the winter. Early records of *Cordyceps* as medicine are as old as the Qing Dynasty in China and recorded in *Ben-Cao-Cong-Xin* (New Compilation of *Materia Medica*) around 1757. *C. sinensis* is the most important member of the genus *Cordyceps*. In 1843, Berkely, the British mycologist first described the fungus as *Sphaeria sinensis* Berk. Later in 1878, Saccado renamed *S. sinensis* as *Cordyceps sinensis* and later modified as *C. sinensis* (Berk.) Sacc.

Berkeley described *S. sinensis* in Latin as "fusca, stipite cylindraceo deorsum subincrassato; capitulo cylindrico cum stipite confluente apiculato; apiculo sterili" which means "it is dark, the stem is cylindrical, somewhat thicker downwards, the head is cylindrical and pointed, confluent with the stem and the tip being sterile" (Devkota, 2006). Recently *Cordyceps* has also become a fashionable luxury product, often given as a gift, and a culinary status symbol in China, especially during the Chinese New year. However, most of the *C. sinensis* consumed in the Western markets are pills made from the ground mycelium, artificially grown on grains with a fraction of the price of natural product (Winkler, 2008). About 3000 years ago, the fungus was mentioned as 'Bhu-Sanjivani' in Astreya Samhita and Charak Samhita. For the last 2000 years, this fungus is being used as a medicine in China and research on this product was started around the middle of the nineteenth century (Shrestha *et al.*, 2010).

2.2. GEOGRAPHIC DISTRIBUTION

The distribution of *Cordyceps* sp. is cosmopolitan, but mostly confined to high Himalayan mountains in China, Tibet, Nepal and India, at an altitude of 3000 to 5000 meters (Sharma, 2004) or in Asian high altitude grass land ecosystems (Stensrud *et al.*, 2007). According to Sung and Spatafora (2004) *C. cardinalis* was first reported from Japan and Eastern United States. *Cordyceps* represents more than 500 species of endoparasites on insects and other arthropods in varied climatic conditions prevailing in six continents (Stensrud *et al.*, 2007; Sung *et al.*, 2007). Li *et al.* (2011) stated that in China the natural *C. sinensis* fruiting body-caterpillar complexes are mostly distributed on the high plateaus of 3500-5000 m above sea level in Tibet, Qinghai, Sichuan, and Yunnan Provinces. Cock and Allard. (2013) reported the occurrence of *Ophiocordyceps barnesii* infecting third instar larvae of sugarcane root grub *Brachylepis werneri* in Southwestern Somalia. Sangdee and Sangdee (2013) isolated *Ophiocordyceps longissima* from cicada larva

collected from Maha Sarakham Province in Northeastern Thailand. Srivilai *et al.* (2013) also reported two new species of *Cordyceps* from Northeastern Thailand. Arora *et al.* in 2013 collected *Cordyceps sinensis* from higher altitudes of Pithoragarh district of Uttaraghand. Chen *et al.* (2013) reported a new species, *Ophiocordyceps lanpingensis* from Lanping country of Yunnan Province in Southwestern China. From the recent surveys conducted in coastal sandy areas of Kasargod district, it was observed that *Cordyceps* sp. is attacking the coconut root grubs (*Leucopholis coneophora* Burm.). This was reported by Kumar and Aparna in 2014 and found that *Cordyceps* sp. is capable of suppressing tumour cells and inhibiting the proliferation of lung cancer cells. Pathania *et al.* (2015) reported the presence of *Cordyceps militaris* from Northwest Himalayan regions of India. According to Shrestha *et al.* (2016) 27 species of *Cordyceps* including *Ophiocordyceps neovolkiana* were recorded, attacking the family Scarabidae of order Coleoptera. Sridhar and Karun (2017) surveyed forest locations in different altitudinal ranges of Western Ghats and reported the occurrence of *Ophiocordyceps nutans* in association with the stink bug *Halyomorpha halys* infecting the bark of one of the tree species, *Cassine glauca* at high altitude forests ranging from 845-935 m above MSL.

2.3. MEDICINAL PROPERTIES

Cordycepin is an important bioactive compound first extracted from *Cordyceps militaris* (Cunningham *et al.*, 1950), and was identified as 3′-deoxyadenosine (Kaczka *et al.*, 1964). Bok *et al.* (1999) isolated antitumor sterols like 5a, 8a-epidioxy-24(R)-methylcholesta-6,22-dien-3b-D-glucopyranoside and 5,6-epoxy-24(R)-methylcholesta-7,22-dien-3b-ol from the methanol extract of mycelium of *Cordyceps sinensis*. They also isolated two previously known compounds, ergosteryl-3-O-b-D-glucopyranoside and 22-dihydroergosteryl-3-O-b-D-glucopyranoside. Zhou *et al.* (1998) reviewed many

studies and reported that the main activities of *C. sinensis* were oxygen-free radical scavenging, anti-senescence, anti-atherosclerotic actions and sexual function-restoration. Koh *et al.* (2003) found out that hot water fraction of mycelia of *C. sinensis* has anti-stress and anti-fatigue effects on rats and mice. Kuo *et al.* (2005) mentioned that many fungi belonging to the genera *Cordyceps* have been used as food and herbal medicines in Asia. According to Koc *et al.*, 1996; Ahn *et al.*, 2000 and Nakamura *et al.*, 2006 bioactivities of this fungus include: anti-viral, anti-fungal, anti-bacterial, anti-leukemic activities, and anti-metastatic action on some cancer cell lines. Later cordycepin was found to be present in *Cordyceps sinensis* and *Cordyceps kyushuensis* (Mao *et al.*, 2005; Masuda *et al.*, 2006 and Das *et al.*, 2010). Nam *et al.* (2006) reported that *Cordyceps* sp. is used as roborant, sedatives, and supplementary therapy for jaundice, tuberculosis and cancer. They also noted that in China, some *Cordyceps* sp. has successfully been used in immunity modulation, fatigue resistance, longevity elongation. It is well known as cardiotonic, aphrodisiac and expectorant (Baral and Kurmi, 2006). In Nepal, powdered *O. sinensis* is taken along with honey, milk or water as a traditional drink. Alcoholic drink is also known to be prepared with dried *O. sinensis* (Devkota, 2006).

C. sinensis has been widely used as a general tonic for protecting and improving lung and kidney functions (Liang *et al.*, 2009). *C. sinensis* is commonly used for the treatment of fatigue, night sweating, hyposexualities, hyper glycemia, hyper lipidemia, respiratory disease, renal disfunction and renal failure, arrhythmias, cardiovascular diseases and liver disease (Kumar and Spandana, 2013). According to Singh *et al.* (2014) many bioactive compounds present in *Cordyceps* like polysaccharides, cordycepin, adenosine, cordymin, ergosterol etc. imparts anti-bacterial, anti-diabetes, anti-inflammatory, anti-fatigue, anti-oxidant, anti -tumour, anti-aging and immune modulatory properties. It is also well known as "Panacea of all Illness" due to its high efficacy and potency. Arora (2015) reported that *Cordyceps sinensis* (Berk.) Sacc. has several therapeutic and pharmacological

actions and also used to cure respiratory and immune disorders, renal, liver and cardiovascular diseases, hyposexuality and hyper lipidema, due to the presence of bioactive ingredients like cordycepin, adenosine, ergosterol etc.

2.4. NOMENCLATURE

The name *Cordyceps* comes from the two Latin words *viz.*, *cord* and *ceps*, meaning "club" and "head" respectively (Holliday and Cleaver, 2004). In the Himalayan regions of India, this is popularly known as 'Keera jhar' (insect herb). The Chinese name for the fungus is 'Dong Chong Xia Cao' meaning winter worm, summer plant' and the Japanese name is 'Tochukaso' (Sharma, 2004). Yartsa gumba (yartsa means winter and gumba means summer) is the Tibetan name for *Ophiocordyceps sinensis* given by Nyamnyi Dorje (1439-1475) and during 15th centaury the local name became yarsagumba in Sikkim also. This is also the most common name in Nepal, Bhutan and India. Other common Nepali names are Jivan buti, Saram buti, Kira chhyau, Kira jhar, Jingani, etc. (Shrestha *et al.* 2010). Barseghyan *et al.* (2011) reported that based on macro and micro morphological characteristics, the anamorph of two strains *O. sinensis* were identified as *Hirsutella sinensis* and *Tolypocladium sinensis*. Chioza and Ogha (2014) reviewed much literature and concluded that *Hirsutella sinensis* is currently receiving general acceptance as a true anamorph of *Ophiocoryceps sinensis*.

2.5. ISOLATION OF *Cordyceps* sp.

Qinqin *et al.* (2009) isolated *Cordyceps ophioglossoides* from parasitized *Elaphomyces* sp. collected from Yunnan province of Southern China, on solid potato sucrose agar medium. Sung *et al.* (2010) isolated *Cordyceps* by discharging ascospores from fresh specimens over 2% water agar plates, and then the agar blocks containing ascospores were cut and inoculated on Sabouraud's dextrose agar plus yeast extract medium. *Ophiocordyceps longissima* isolate Cod-MK1 was isolated by

Sangdee and Sangdee in 2013 on potato dextrose agar (PDA) medium. Pathania *et al.* (2015) reported that the isolation of *Cordyceps militaris* can be done from stipe and stroma portion of both fresh and sun dried fruiting bodies. Isolation of *Ophiocordyceps sinensis* was done by Ko *et al.* (2017) by placing small pieces of sterilized fruiting body in a bottle containing 5 ml of sterile water. They homogenized the solution with a blender prior to dilution in sterile water and 100 µl of the diluted solution was inoculated onto potato dextrose agar medium and cultured the fungus successfully by incubating at various temperatures for several days.

2.6. CULTURAL CHARACTERS

The cultural characters of *Cordyceps* were studied by many workers. Holliday and Cleaver in 2004 observed that the mycelium of *C. sinensis* was longitudinally radial and non-aerial and the colour was initially white and later on, densely matted and appeared as orange-brown to tan in colour. Sehgal and Sagar (2006) found that mycelium of *C. sinensis* was initially creamish white, becoming densely matted. He also noted that when the colony matured the colour of mycelia changes from creamish white to light brown. Small nodules of light brown colour were formed at the centre, on the surface of the medium, while peripheral mycelia remain creamish white. As the medium was completely consumed, the mycelia become increasingly mud-like. According to Sung *et al.* (2011) the cultures of *Cordyceps* sp. were usually established from ascospores, and the germination rates and growth rates differ depending on the species. Arora *et al.* (2013) reported that colony of *C. sinensis* on Sabourad's dextrose agar yeast extract medium was initially cream with lined depressions, later turning to dark orange and the reverse side of the culture showed dark tan colour.

2.7. SELECTION OF MEDIA

Several scientists tried culturing of the fungus, *C. sinensis* on different artificial media such as potato dextrose agar, beef extract dextrose agar, casein hydrolysate dextrose agar, soyabean extract dextrose agar, rice extract dextrose agar and finger millet medium (Das *et al.*, 2005; Harsahay *et al.*, 2010). Study done by Sehgal *et al.* (2006) revealed that among the different solid media tested yeastal potato dextrose agar medium was found to be the optimum medium for the growth of *Cordyceps militaris* and among the five liquid media tested *i.e.*, glucose aspargine solution, richard's solution, asthana and hawlker's solution, czapek's solution and dimmiccks solution, glucose asparagine solution was found to be the best for the growth of *Cordyceps militaris*. Nam *et al.* (2006) examined *Cordyceps sphecocephala* for its telemorphic characteristics and found that its colony diameter was 32 mm on potato dextrose agar for 30 days at a temperature of 24 ± 1°C. Amin *et al.* (2008) reported that among the different media tested *i.e.,* potato dextrose agar, malt extract agar, potato dextrose yeast agar and yeast extract agar *C. sinensis* completed its full growth in potato dextrose agar medium within 47 days, which was significantly lower than that of other media. Bharseghyan *et al.* (2011) examined different strains of *O. sinensis* and reported that most of the strains preferred sabouraud's dextrose agar and some of the strains preferred potato dextrose agar as the medium for optimal development. The least favorable nutrient for all strains was czapek solution agar. Arora *et al.* (2013) inoculated *C. sinensis* in five different media *viz.*, potato dextrose agar, sabouraud's dextrose agar, malt extract agar, czapek dox agar and oat meal agar and among these media, maximum mycelial growth was found in sabouraud's dextrose agar medium. Pathania *et al.* (2015) reported that among the 12 solid and five liquid media tried for the growth of *Cordyceps militaris*, yeastal potato dextrose agar and glucose asparagine solution were found to be the best solid and liquid medium respectively. Adnan *et al.* (2017) reported that among the different solid substrates tested for

cordycepin production in *Cordyceps militaris*, rice medium was found to be the best substrate producing maximum cordycepin (814.60 mg/g) followed by oat and wheat medium producing 638.85 and 565.20 mg/g cordycepin respectively.

2.8. MORPHOLOGICAL CHARACTERS

The stroma of fruiting body of *Ophiocordyceps* were in various shapes like circle, club, cotton swab stick, coral reef and noodles (Hye-Young, 1999). The fruiting bodies were dark brown to black and the larval body parasitized by the mushroom's mycelium, appears yellowish to brown in color (Holliday and Cleaver, 2004). Shin *et al.* (2004) reported that *Cordyceps pruinosa* produces 3 to 4 stromata having a distinct club shaped head and long stalk with numerous semi immersed perithecia at the apex. According to Sung *et al.* (2007) the genus *Ophiocordyceps* is characterized by stromata or subiculum darkly pigmented or rarely brightly coloured, tough, fibrous, and pliant to wiry, rarely fleshy, often with aperithecial apices or lateral pads. *C. cardinalis* is characterized by gregarious, one to 26 stromata per host, orange reddish to reddish, coloured fruiting bodies growing on lepidopteran larva (Sung *et al.*, 2010). Winkler in 2010 noted a special behavior that *Cordyceps sinensis* directs the infected ghost moth larva of the genus *Thitarodes* to crawl into a position ideal for fungal fruiting body emergence and spore dispersal. This phenomenon was also reported in *Ophiocordyceps unilateralis* which attacks carpenter ants (*Camponotus* sp.), and directs them to climb up on vegetation and clamp down onto a leaf or twig before death for optimum fruiting body emergence and spore dispersal (Evans *et al.*, 2011; Shang *et al.*, 2015). Shrestha *et al.* (2010) mentioned the macro morphological characters of *O. sinensis*, and he described that the basal part is the caterpillar containing fungal endosclerotium and the upper fungal part is the stroma, the fertile region which is slightly swollen, sublanceolate or fusiform and the surface of the head is granular due to projecting openings or ostioles of perithecia. Pathania *et al.* (2013) stated that the fruiting body of *C. militaris*, was

creamish white in colour and which always emerge from the head of the mummified larva. Arora *et al.* (2013) studied about *C. sinensis* and reported that ascocarp of the fungus is on an average four to seven cm long emerging out of the mummified caterpillar. The ascocarp was usually erect, stalked, somewhat swollen at tip, emerge mostly single, double or rarely triple. Stalks were similar to grass straw, slightly broader at the base and tapered towards the end. Kumar and Aparna (2014) found that 80% of the grubs collected from 1 m^2 area of different locations of Kasargod district of Kerala were infected with *Cordyceps* fungus and the fruiting bodies have an average length of seven cm. They also mentioned that it produces stroma, the colour of which may vary from orangish brown to black.

As reported by Yan (2014) *Cordyceps (Ophiocordyceps) sinensis* (Berk.) Sacc. infects the ghost moth larvae in the late summer or early autumn and gradually consume the internal tissues and make the host into ''stiff worms'' completely filled with fungal mycelia in winter. In spring and early summer of the subsequent year, fruiting body emerges out from the larval head like a grass (Zhou *et al.*, 1998; Li *et al.*, 2011; Holliday and Cleaver, 2008; Winkler, 2010; Zhang *et al.*, 2017; Chen *et al.*, 2013; Lo *et al.*, 2013;). Chen *et al.* (2013) described a new species of *Ophiocordyceps i.e.*, *Ophiocordyceps lanpingensis*, which was having thinner stroma, smaller perithecium, and thinner ascospores. The phylogenetic analysis showed that *O. lanpingensis* had the closest evolutionary relationship with *O. sinensis* and *O. robertsii*.

2.9. MICROSCOPIC CHARACTERS

Detailed studies were conducted by researchers on the microscopic characters of the fruiting body of *Cordyceps.* Alexopolous *et al.* (1996) reported that *Cordyceps* was having ovoid to cylindrical unitunicate asci. Gwangpo (2000) studied the presence of perithecium of *C. sinensis* as either oval-shaped or egg-shaped, consisting of numerous numbers of thin, long ascus and ascospores. Shin *et al.* (2004) observed

that the apex of the stroma contains numerous perithecia with several ascospores inside the asci. The ascospores were characterized by a thread like structure in the middle with non disarticulating part spores attached on both sides. They also observed that the imperfect forms produces spherical or spindle shaped conidia on phialides. Shreshta *et al.* (2005) reported that under submerged condition in sterile water, the majority of ascospores of *Cordyceps militaris* (Part spores) germinated unidirectionally and conidia produced directly from the tips of germinating hyphae of part-spores within 36 h after ascospore discharge, exhibiting microcyclic conidiation. The first formed conidia were cylindrical or clavate followed by globose and ellipsoidal ones. They also noted that conidiogenous cells were more slender than vegetative hyphae, having attenuated tips. The characteristic features of asexual stage of *C. militaris* mentioned by them were microcyclic conidiation, undifferentiated conidiogenous hyphae (Phialides), polymorphic conidia and solitary, opposite or whorled type of phialidic arrangement. Nam *et al.* (2006) studied the morphology of perithecia, asci and ascospores of *Cordyceps sphecocephala.* Chen *et al.* (2006) observed that the hyphae of *C. sinensis* were ramose, septate and hyaline and conidiophores were erect, hyaline, glabrate, having one or two ramification. The phialide is simplicial or acrogenous in nature. According to Sung *et al.* (2007), perithecia of *Ophiocordyceps* were superficial to completely immersed, ordinal or oblique in arrangement. Asci were hyaline, cylindrical, usually with thickened ascus tip, rarely fusoid to ellipsoid. Ascospores were usually cylindrical, multiseptate, disarticulating into part-spores or non-disarticulating. According to Xie *et al.* (2010) and Zhong *et al.* (2010) the hyphae of *C. sinensis* were white and stout and ascospores were present in ascus. Microscopic observations on *Cordyceps cardinalis* by Sung *et al.* (2010) revealed that perithecia were ovoid and semi-immersed, asci have a specific cap and the ascospores were unevenly septate but do not disarticulate into part-spores. Arora *et al.* (2013) reported that perithecia of *C. sinensis* were found to be oval-shaped or egg shaped filled with numerous

elongated, unitunicate, capitate, cylindrical and hyaline ascus and the hypha was aerial, cottony white to creamish or yellowish, septate, branched and fast growing. According to the study by Pathania *et al.* (2015) *Cordyceps militaris* has barrel shaped smooth to velvety conidia on sub cylindrical conidiophores with flask shaped phialides and the hyphae is thin walled, branched around 14.8 - 16.1 µm broad.

2.10. TAXONOMIC HISTORY AND MOLECULAR IDENTIFICATION

With increased demand and less availability of *Cordyceps* sp. in world markets, fermented products of its anamorph have been used as substitutes. This had raised questionable quality control challenges and as a result the need for molecular methods to identify *Cordyceps sinensis* became a necessity. Chen *et al.* (2001) used Random Amplified Polymorphic DNA (RAPD) markers to investigate genetic variation and evolutionary relationships of 29 samples of *C. sinensis* collected from different geographical populations on the Qinghai-Tibet plateau. They concluded that out of 137 RAPD bands a correlation can be derived between geographical distance and genetic distance and the molecular phylogenetic tree also suggests that the 29 samples were divided into three notable clusters, corresponding to the geographical populations, *i.e.*, the North Population (NP), Middle Population (MP) and South Population (SP). Tamura *et al.* (2004) studied the prospects for inferring very large phylogenies of *Cordyceps* by using the neighbor-joining method. Kuo *et al.* (2005) followed the neighbor-joining method using the ITS1, 5.8S rRNA, and ITS2 regions to construct a phylogenetic tree of *Cordyceps sinensis* with 17 *Cordyceps* isolates. Molecular systematics on *Cordyceps sinensis* and its related taxa were done by Wei *et al.* in 2006.

Based on molecular phylogenetic study, Sung *et al.* (2007) separated the mega genus *Cordyceps* into four genera, *viz. Cordyceps*, *Ophiocordyceps*, *Metacordyceps* and *Elaphocordyceps* as a result; many members which were early placed in the genera *Cordyceps* were transferred to the genus *Ophiocordyceps.*

Chen *et al.* (2009) reported that 18 Snr DNA and NS5/NS6 region sequences were almost identical and have potential for molecular characterization and diversity analysis of the isolates of *Cordyceps* sp. They also used *Cfo* I and *Rsa* I restriction determined signature sequences to characterize 12 fermented products of *Cordyceps* sp. Peng *et al.* (2013) established a protocol for DNA extraction, purification, and quantification of *O. sinensis* from Tibet, with quantitative real-time PCR targeting the Internal Transcribed Spacer (ITS) region. The method was assessed using 34 soil samples from Tibet. A real-time quantitative PCR (q PCR) system, including a pair of species - specific ITS primers and its related program, was developed for *O. sinensis* assay with high reliability and efficiency by Li *et al.* in 2000. Ko *et al.* in 2017 conducted molecular identification of anamorph of *Cordyceps sinensis*, *ie.*, *Hirsuitella sinensis* by multi-locus sequence typing of several fungal genes (ITS, nrSSU, nrLSU, RPB1, RPB2, MCM7, β-tubulin, TEF-1α, and ATP).

2.11. PHYSIOLOGICAL CHARACTERS

Effect of major physiological characters *viz.*, temperature, pH, light, darkness, carbon, nitrogen, vitamins and mineral sources on growth of *Cordyceps* sp. was studied by various researchers.

2.11.1. Effect of Temperature and pH

Sasaki *et al.* (2005) observed the mycelial growth of three strains of *Cordyceps nutans* Pat. at different temperatures and pH, and found that mycelial growth was greater at 20°C and 25°C, but at 5°C, 30°C, and 35 °C, growth was absent in all the three strains. Strains grew relatively well in neutral to weak alkaline pH levels (pH 7.0 - 9.0) compared with acidic pH levels. Amin *et al.* (2008) observed that mycelial growth of *Cordyceps sinensis* was best in potato dextrose agar under alkaline condition with a pH level 9 at 20-25°C. Hung *et al.* in 2009 evaluated

fifteen *Cordyceps militaris* strains for the production of cordycepin (3'- deoxyadenosine) by static culture in Erlenmeyer flasks and observed the effect of temperature on mycelial growth and found that optimum temperature for mycelial growth and cordycepin production was 15 - 20° C and 25° C, respectively. According to Sung *et al.* (2010) a temperature of 25°C and a pH of 7 resulted in the highest mycelial growth of *Cordyceps cardinalis.* Mei *et al.* (2013) reported that for the growth of *Ophiocordyceps sinensis* the ideal temperature range is 4 - 25^{0}C. According to Arora *et al.* (2013) mycelial growth of *Cordyceps sinensis* inoculated on Sabourad's dextrose agar for temperature study was maximum (44.87mm) at 15°C and the minimum (34.27mm) at 25°C under an optimum pH level 6. Study conducted by Sehgal *et al.* (2006) and Pathania *et al.* (2015) revealed that the best mycelial growth of *C.militaris* was observed at 25°C both in solid media (yeastal potato dextrose agar) and liquid media (glucose asparagine solution) and the optimum level of pH was found to be 7.5 and 5.5 in solid and liquid medium respectively.

Adnan *et al.* (2017) reported that 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 in *Cordyceps militaris*. Ko *et al.* (2017) reported that optimum temperature for growth of the anamorph of *Cordyceps sinensis*, *i.e.*, *Hirsuitella sinensis* was 16^{0}C.

2.11.2. Effect of light and darkness

According to Sehgal *et al.* (2006) and Pathania *et al.* (2015) the mycelial growth of *C. militaris* in both solid medium (Yeastal potato dextrose agar) and liquid medium (Glucose aspargine solution) was highest under darkness in comparison to light.

2.11.3. Effect of sources of carbon, nitrogen, minerals and vitamins

Shin *et al.* (2004) observed that *Cordyceps pruinosa* showed maximum mycelial growth in the medium where dextrin or sucrose or saccharose was used as

carbon source, and peptone or yeast extract or tryptone as nitrogen source at a C/N ratio of 1:1 with KH_2PO_4 as mineral source.

Nutritional requirements for the mycelial growth of *Cordyceps sinensis* in submerged culture was studied by Dong and Yao in 2005 and reported that among 17 carbohydrates, 16 nitrogen sources, nine vitamins, four macro-elements, four trace elements and eight ratios of carbon to nitrogen tested, sucrose, peptone, folic acid, calcium, zinc and a carbon to nitrogen ratio of 12:1 were found to be the optimum requirements for mycelial growth of *Cordyceps sinensis*. They also optimized the concentrations of sucrose, peptone and yeast extract and the effects of medium composition on the growth of mycelium were found to be in the order sucrose > yeast extract > peptone. The optimal concentration for mycelial growth was determined as 50 g l^{-1} sucrose, 10 g l^{-1} peptone and 3 g l^{-1} yeast extract. Sehgal *et al.* (2006) stated that among the different carbon, nitrogen and mineral sources tested, sucrose (165.02mg), ammonium phosphate (167.53mg), calcium chloride (170mg) and manganese sulphate (165mg) was optimum requirement of the *C. militaris* to yield maximum dry weight. Sung *et al.* (2010) observed that among carbon sources, cereals, and nitrogen sources tested, the growth of *Cordyceps cardinalis* was best in maltose, oatmeal, and peptone. Mei *et al.* (2013) observed better conidial germination on solid medium containing 2.5 per cent malt extract, one per cent soluble starch and 0.9 per cent agar at pH 6.0 at a temperature of 14°C. However more nitrogen and iron sources inhibited yields of the conidia. Singh *et al.* (2014) worked on the optimization of nutritional necessities for *in vitro* culture of *Ophiocordyceps sinensis*. This study revealed that among the carbon sources tested, mycelial yield was significantly higher on sucrose and among the nitrogen sources beef extract and yeast extract showed significantly higher mycelial yield. They also found out that the organic nitrogen sources were significantly more productive than the inorganic nitrogen sources. Among the vitamins tested, yield obtained with folic acid was significantly higher than the other vitamins. Among all micro nutrients and

macro nutrients tested, significantly higher mycelial yield was obtained from calcium chloride and zinc chloride respectively. According to Pathania *et al.* (2015) among five carbon, six nitrogen, six mineral and six vitamin sources tested, sucrose, beef extract, zinc chloride and folic acid respectively produced the maximum mycelial yield of *Cordyceps militaris*. He *et al.* (2016) observed that the yield of *Ophiocordyceps sinensis* was maximum in medium containing glucose (40 g/L), yeast powder (65 g/L), KH_2PO_4(1.5 g/L), $MgSO_4$ (3 g/L) incubated at 17°C for12 days.

2.12. ARTIFICIAL CULTIVATION.

Artificial cultivation of *Cordyceps* sp. is the most difficult task as reported by many scientists. Several factors should be taken into consideration for development of fruiting bodies. The former Sichuan traditional Chinese medicine institute (Currently the Chongqing Academy of Chinese Materia Medica) achieved the cultivation of *O. sinensis*. Due to the high cost and low stability, commercial production is laborious. Padiyath (2002) observed that the best substrate for artificial culturing of *Hirsutella thompsonii* was wheat grains, which is a member of the genus *Hirsutella* where the anamorph of *Cordyceps* belongs to.

According to Liang *et al.* (2009), *O. sinensis* has to be successfully cultivated by considering the factors like heredity of strains, suitable culture temperature, right amount of light, and culture medium with plenty of biologicaly active substances and organic nitrogen sources. Kim *et al.* (2010) observed that 50 - 60 g of brown rice and 10-20 g of silk worm pupa mixed with 50-60 ml of water with liquid inoculums in 1000 ml polypropylene (PP) bottle incubated at 25°C temperature under continuous light was found to be optimum conditions for fruiting body production of *Cordyceps cardinalis*. Great progress on the cultivation of *O. sinensis* was reported by Xie (2010) at the Tibetan Plateau Characteristic Resource Science Work station of Sun Yat-Sen University. Kaszak (2014) also reported the industrial production of

Ophiocordyceps sinensis for its medicinal and pharmaceutical properties in different artificial media. Zhou *et al.* (2009) reported two different patterns of current artificial cultivation of *O. sinensis* which were complete artificial cultivation and semi-natural cultivation. In complete artificial cultivation, reared larvae were inoculated with cultured strains thereafter the infected larvae were fed indoors. After 1 – 2 years, *O. sinensis* can be harvested. With regard to semi-natural cultivation, the infected larvae were released to natural habitats, allowing them to grow freely. After 3 – 5 years, *O. sinensis* can be harvested in the releasing areas.

2.13. PROXIMATE ANALYSIS

Proximate analysis of fruiting bodies and mycelia will give a picture of nutritional values of *Cordyceps*. Chan *et al.* (2015) conducted the proximate analysis of fruiting bodies and cultured mycelia of caterpillar medicinal fungus, *Cordyceps militaris* and reported that protein content was 59.8 per cent and 39.5 per cent in the fruiting body and mycelium respectively and the mycelium was distinguished by its carbohydrate content (39.6 %), which was higher than that of the fruiting body (29.1%). They also studied the vitamin composition and the most abundant vitamins were found to be vitamin A, vitamin B_3, and vitamin E. The ash content of fruiting body was 5.1 ± 0.06 per cent and that of mycelium was 5.7 ± 0.22 per cent. The moisture content was higher in mycelium which was 13.1 ± 1.1 per cent than in fruiting body which was 5.7 ± 0.77 per cent. Rakhee *et al.* (2016) evaluated chemical composition of *C. sinensis* and they reported that the carbohydrate content was 55.68 per cent, the crude fiber content was found to be 6.40 per cent, the fat content was 1.80 per cent, moisture content was 7.18 per cent, protein content was 21.46 per cent and total ash content was 7.48 per cent.

Materials & methods

3. MATERIALS AND METHODS

The present study on 'Characterization of medicinal mushroom, *Cordyceps* sp. from Kasargod district' was carried out in the Department of Plant pathology at College of Agriculture, Padannakkad and College of Horticulture, Vellanikkara during the period of 2016-18. The detailed account of materials used and methods followed during the course of experiment are given below.

3.1. SURVEY FOR THE COLLECTION OF *Cordyceps* sp.

Purposive sampling surveys were conducted in three locations of coastal sandy tracts of Kasargod district during June to September months of 2017 and 2018 and the fruiting bodies of *Cordyceps* sp. were collected. The three locations selected were Instructional Farm, College of Agriculture, Padannakkad, Valiyaparamba area of Nileshwar and Regional Agricultural Research Station, Pilicode. Observations on the fruiting bodies *viz.,* average population, and depth of occurrence were taken by collecting the sporocarps from these three locations. Soil analysis was also done for these three locations to find out the variation in population of this fungus. The fruiting bodies were collected in brown paper covers and brought to the laboratory for further observations. These fruiting bodies were preserved fresh as well as in dry forms under refrigerator for future studies. For proper interpretation of results, soil data analysis of samples collected from the three locations was done.

3.2. ISOLATION OF *Cordyceps* sp.

Isolation of fungus was done from different parts of the fungal structure *viz.*, sclerotia, stipe and stroma of both fresh and dry specimens by following standard tissue culture technique. The samples were washed under running tap water and from all the three parts *viz.*, sclerotia, stipe and stroma inner and outer portions of tissues were taken for isolation. These tissues were cut into small pieces, and were disinfected with sodium hypochlorite solution (1%) for one minute. After three

washings using sterilized distilled water, the samples were placed on solidified potato dextrose agar (PDA) medium aseptically in sterile Petri dishes under a laminar air flow chamber. All the dishes were incubated at room temperature (28 ± 2°C) and observed from next day onwards. The fungal growth obtained was subsequently sub cultured to solidified PDA in sterile Petri dishes. The isolate was named as CD1. Periodic sub culturing and maintenance of the isolate was also done in PDA slants under refrigerated condition at 4°C for further studies.

3.3. CHARACTERIZATION OF *Cordyceps* sp.

3.3.1. Cultural characterization

The cultural characters like colour, shape, texture and the growth rate of the mycelium were studied in different media. Five different media *viz.,* potato dextrose agar (PDA), yeast potato dextrose agar (YPDA), malt extract agar (MEA), oatmeal agar (OMA) and czapek dox agar (CDA) were selected to find out the optimum medium for the growth of fungus. Composition of different media is given in the Appendix -1.

The different media were prepared and 150 ml each of the media were transferred to 250 ml conical flask and plugged tightly with cotton and sterilized in an autoclave under 15 psi pressure and 121°C for 30 minutes. After completion of sterilization media were melted in a microwave oven, cooled and poured aseptically in sterile Petri dishes under laminar air flow chamber. After solidification of media the plates were inoculated with 5 mm mycelial disc of one week old culture of the fungus. The Petri dishes were sealed, properly labeled and incubated under room temperature (28 ± 2°C). Four replications were maintained for each treatment in completely randomized design. Mycelial growth (cm) was measured. Observations were taken until the fungal culture completed full growth in each media.

3.3.2. Morphological characterization

Various morphological characters like shape, size and colour of sclerotia, stipe and stroma and branching pattern of the stroma associated with the fruiting bodies were studied.

Microscopic slides were prepared and mounted using lactophenol cotton blue stain (Composition of stain was given in the appendix 2) and observed under compound microscope at 5x, 10x, 40x and 100x magnifications to study the anamorphic stage (Asexual stage) including hyphae, synnemata, conidiophores, conidia and teleomorphic stage (Sexual stage) including perithecia, asci, ascospores of the fungus. Measurements and photomicrographs were taken using Zeiss Axiolab image analyzer.

3.3.3 Molecular characterization

Molecular characterization of the isolate was done to identify the isolate at species level by DNA barcoding using universal primers of ITS sequences. For this, isolate was sent to Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum.

3.3.3.1. DNA isolation using NucleoSpin® Plant II Kit (Macherey-Nagel)

About 100 mg of the tissue/mycelium was homogenized using liquid nitrogen and the powdered tissue is transferred to a microcentrifuge tube. Four hundred microlitres of buffer PL1 is added and vortexed for 1 minute. Ten microlitres of RNase A solution was added and inverted to mix. The homogenate was incubated at 65°C for 10 minutes. The lysate was transferred to a Nucleospin filter and centrifuged at 11000 x g for 2 minutes. The flow through liquid was collected and the filter was discarded. Four hundred and fifty microlitres of buffer PC was added and mixed well. The solution was transferred to a Nucleospin Plant II column, centrifuged for 1 minute and the flow through liquid was discarded. Four hundred

microlitre buffer PW1 was added to the column, centrifuged at 11000 x g for 1 minute and flow though liquid was discarded. Then 700 µl PW2 was added centrifuged at 11000 x g and flow through liquid was discarded. Finally 200 µl of PW2 was added and centrifuged at 11000 x g for 2 minutes to dry the silica membrane. The column was transferred to a new 1.7 ml tube and 50 µl of buffer PE was added and incubated at 65°C for 5 minutes. The column is then centrifuged at 11000 x g for 1 minute to elute the DNA. The eluted DNA was stored at 4°C.

3.3.3.2. Agarose Gel Electrophoresis for DNA Quality check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad)

3.3.3.3. PCR Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X Phire PCR buffer (contains 1.5 mM $MgCl_2$), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers.

Primers used

Target	Primer Name	Direction	Sequence (5' → 3')
ITS	ITS-1F	Forward	TCCGTAGGTGAACCTGCGG
	ITS-4R	Reverse	TCCTCCGCTTATTGATATGC

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

PCR amplification profile

ITS & LSU

98 °C - 30 sec

98 °C - 5 sec
60 °C - 10 sec } 40 cycles

72 °C - 15 sec

72 °C - 60 sec

4 °C - ∞

3.3.3.4. Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the

bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.3.3.5. ExoSAP-IT Treatment

ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product was mixed with 2 µl of ExoSAP-IT and incubated at 37°C for 15 minutes followed by enzyme inactivation at 80°C for 15 minutes.

3.3.3.6. Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufactures protocol.

The PCR mix consisted of the following components:

PCR Product (ExoSAP treated)	- 10-20 ng
Primer	- 3.2 pM (either Forward or Reverse)
Sequencing Mix	- 0.28 µl
5x Reaction buffer	- 1.86 µl
Sterile distilled water	- make up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96°C for 2 minutes followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 minutes for all the primers.

3.3.3.7. Post Sequencing PCR Clean up

1. Make master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction
2. Add 12µl of master mix I to each reaction containing 10µl of reaction contents and are properly mixed.
3. Make master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol per reaction.
4. Add 52 µl of master mix II to each reaction.
5. Contents are mixed by inverting.
6. Incubate at room temperature for 30 minutes
7. Spin at 14,000 rpm for 30 minutes
8. Decant the supernatant and add 100 µl of 70% ethanol
9. Spin at 14,000 rpm for 20 minutes.
10. Decant the supernatant and repeat 70% ethanol wash
11. Decant the supernatant and air dry the pellet.

The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

3.3.3.8. Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al.,* 2010).

3.3.4. Physiological characterization of *Cordyceps* sp.

3.3.4.1. Effect of temperature on the mycelial growth of Cordyceps sp.

To find out the optimum temperature for the mycelial growth of *Cordyceps* sp., seven different temperatures were selected viz., 5°C, 10°C, 15°C, 20°C, 25°C, room temperature (26 ± 2°C), 30°C and 35°C. 20 ml of sterilized medium which was selected as the best medium in the experiment 3.3.1 for the growth of *Cordyceps* sp. was poured into sterile Petri dishes under laminar air flow chamber. After solidification of medium mycelial discs of 5mm diameter were inoculated at the centre of each Petri dishes, sealed and labeled. Four replications were maintained for each treatment in completely randomized design. The plates were incubated at selected temperatures. The growth rate of mycelia was measured at 10 days intervals and observations were taken.

3.3.4.2. Effect of pH on the mycelial growth of Cordyceps sp.

To find out optimum pH for growth of the fungus, best medium selected in the experiment 3.3.1 was prepared and the pH was adjusted to 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10 by adding either 0.1N hydrochloric acid (HCl) or 0.1N sodium hydroxide (NaOH) and sterilized. The media were inoculated with five mm mycelial discs of one week old culture. Four replications were maintained for each treatment in completely randomized design. The plates were incubated at the temperature which was selected as the optimum temperature for the growth of

Cordyceps sp. in the experiment 3.3.4.1. The growth rate of mycelia was measured at 10 days intervals and colony diameter was recorded.

3.3.4.3. Effect of light and darkness on the mycelial growth of Cordyceps sp.

To find out the optimum condition of light and darkness for the growth of the fungus, four condition of light and darkness were selected *viz.*, 24 h of light under room temperature (27 ± 2 °C), 24 h of light under incubator (30°C), 24 h of darkness under room temperature (27 ± 2 °C) and 24 h darkness under incubator (30°C). Best medium selected in the experiment 3.3.1 with optimum pH selected under experiment 3.3.4.2 was sterilized and inoculated with 5 mm mycelial discs of one week old culture. Four replications were maintained for each treatment in completely randomized design. The plates were incubated under the selected conditions of light and darkness. The growth rate of mycelia was observed at 10 days intervals and colony diameter was measured.

3.3.4.4. Effect of carbon sources on the mycelial growth of Cordyceps sp.

To study the effect of different carbon sources on the mycelial growth of *Ophiocordyceps* sp. four sources of carbon were selected *viz.,* dextrose, sucrose, fructose and maltose. Each sources of carbon was substituted to the best medium selected in the experiment 3.3.1 at the rate of 20g/L. The prepared media with optimum pH selected under experiment 3.3.4.2 were sterilized. Each medium was inoculated with 5 mm mycelial discs of one week old culture. Four replications were maintained for each treatment in completely randomized design. The plates were incubated at optimum conditions selected under experiment 3.3.4.3. The growth rate of mycelia was observed at 10 days intervals and colony diameter was measured.

3.3.4.5. Effect of nitrogen sources on the mycelial growth of Cordyceps sp.

To study the effect of different nitrogen sources on the mycelial growth of *Cordyceps* sp. four sources of nitrogen were used *viz.*, yeast extract, beef extract, potassium nitrate and ammonium nitrate. Best medium selected in the experiment 3.3.1 substituted with best carbon source selected in the experiment 3.3.4.4 was substituted with each sources of nitrogen at the rate of 5g/L. The prepared media with optimum pH selected under experiment 3.3.4.2 were sterilized and inoculated with 5 mm mycelial discs of one week old culture and the plates were incubated at optimum conditions selected under experiment 3.3.4.3. Four replications were maintained for each treatment in completely randomized design. The growth rate of mycelia was observed at 10 days intervals and colony diameter was measured.

3.3.4.6. Effect of macro mineral sources on the mycelial growth of Cordyceps sp.

To study the effect of different macro minerals on the mycelial growth of *Cordyceps* sp. four sources of macro minerals were selected *viz.,* $CaCl_2$, KH_2PO_4, K_2HPO_4 and $NaCl_2$. Best medium with the optimum sources of carbon and nitrogen selected in the previous experiments was used as basal medium and which was prepared by adding one g/L each of the macro minerals in the basal medium. The media were prepared; pH was adjusted to optimum and sterilized. The media were inoculated with 5 mm mycelial discs of one week old culture. Four replications were maintained for each treatment in completely randomized design. The plates were incubated at optimum conditions selected under experiment 3.3.4.3. The growth rate of mycelia was measured at 10 days intervals and colony diameter was recorded.

3.3.4.7. Effect of micro minerals on the mycelial growth of Cordyceps sp.

To study the effect of different micro minerals on the mycelial growth of *Cordyceps* sp. four sources of micro minerals were used *viz,* $ZnCl_2$, $MnCl_2$, $CuSO_4$ and $FeSO_4$. Best medium selected in the experiment 3.3.4.6 was selcetd as basal

medium. Media were prepared by adding 500 mg/L each of the micro minerals in the basal medium. The prepared media with optimum pH selected under experiment 3.3.4. were sterilized and inoculated with 5 mm mycelia discs of one week old culture. Four replications were maintained for each treatment in completely randomized design. The plates were incubated at optimum conditions selected under experiment 3.3.4.3. The growth rate of mycelia was measured at 10 days intervals and colony diameter was recorded.

3.3.4.8. Effect of vitamin on the mycelial growth of Cordyceps sp.

To study the effect of different sources of vitamins on the mycelial growth of *Cordyceps* sp. six sources of the vitamins were used *viz*, folic acid, nicotinic acid, riboflavin, thiamine, biotin and pyridoxine. Best medium with the optimum sources of carbon and nitrogen, micromieral, and macro mineral was selcetd as basal medium. Media were prepared by adding 10 mg/L each of the vitamin in the basal medium. The prepared media with optimum pH was sterilized in autoclaved under 15 psi pressure and 121°C for 30 minutes (folic acid and thiamine was sterilized using 0.22µm aperture filter). Then the media were inoculated with 5 mm mycelial discs of one week old culture. Four replications were maintained for each treatment in completely randomized design. The plates were incubated at optimum conditions selected under experiment 3.3.4.3. The growth rate of mycelia was observed at 10 days intervals and colony diameter was measured.

From the observations of the above experiments, one optimum media for the growth of *Cordyceps* sp. was formulated and the medium was named as yeast extract potato fructose agar (YEPFA).

3.3.5. Comparison of YEPFA with conventional media.

To evaluate the performance of the optimum medium formulated for the growth of *Cordyceps* sp. (YEPFA) a comparative study was done with the basic

medium PDA and YPDA which was selected as the best medium in the experiment 3.3.1., both in solid and liquid media.

3.3.5.1. Growth rate of Cordyceps sp.

YEPFA, YPDA and PDA were prepared, and pH was adjusted to optimum level and sterilized. The media were inoculated with 5 mm mycelial discs of one week old culture. Seven replications were maintained for each treatment in completely randomized design. The plates were incubated at optimum conditions. The radial growth of fungal colony was measured at 10 days intervals until the fungal culture completed full growth in each media.

3.3.5.2. Mycelial weight of Cordyceps sp.

Mycelial weight of *Cordyceps* sp. in broth of the three media *viz.,* YEPFB, YPDB and PDB was also observed. For this 100 ml broth of each medium was prepared and pH was adjusted to optimum level and sterilized and poured in 250 ml conical flasks and sterilized. Five mm culture disc was inoculated in the broth of each medium and the flasks were incubated at optimum conditions. Observations were taken at 10 days intervals. After completion of growth, mycelial mat from each broth was taken and dried at 60^0C for 24 h in a hot air oven and filtered on Whatman No.1 filter paper and the weight was recorded.

3.4. ARTIFICIAL CULTURING OF *Cordyceps* sp.

Different cereal grains *viz.*, rice, wheat and sorghum were tried as the substrates for artificial culturing of *Cordyceps* sp. under *in vitro* conditions. 50g of each cereal mixed with 2.5 g of $CaCO_3$ was filled in 100 ml conical flask and sterilized under 15 psi pressure at 121°C for 95 minutes in an autoclave. The flasks were inoculated with 10 mm discs of actively growing one week old culture of *Cordyceps* sp. and incubated at 30°C. Days to complete the mycelial growth

throughout the substrate were noted to find out the best substrate for artificial culturing of *Cordyceps* sp.

3.5. PROXIMATE ANALYSIS OF *Cordyceps* sp.

Analysis of proximate constituents *viz.*, carbohydrate, protein, fibre content, total minerals, moisture, ash content and vitamin C was conducted. The sporocarps collected from the field and mycelial growth obtained on YEPFB under *in vitro* conditions were used for this study. The samples were dried and powdered for the proximate analysis. The moisture content was estimated on wet weight basis whereas the rest of the parameters were analysed on dry weight basis following standard methods of analysis (Sadashivam and Manikkam, 2008)

3.5.1. Determination of carbohydrate

Carbohydrate was determined by anthrone method (Hedge and Hofreiter, 1962), in which 100 mg of the powdered sample was weighed and transferred to a boiling tube. Hydrolyzation of the sample was done with 5ml 2.5N hydrochloric acid by keeping in boiling water bath for 3 hrs and then cooled to room temperature. Then neutralization was done with solid sodium carbonate until the effervescence ceases. The volume was made up to 100ml and centrifugation was done. The supernatant was collected and 1 ml aliquot was taken for analysis. The standards were prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard. Zero served as the blank. The volume was made up to 1ml in all tubes including the sample tubes by adding distilled water. Then 4ml of the anthrone reagent was added to all the tubes and heated for 8 min in a boiling water bath. Samples were cooled rapidly and read the green to dark green colour at 630 nm using spectrophotometer. A standard graph was drawn by plotting concentration on x axis and absorbance on y axis. From the graph the amount of carbohydrate was calculated in the sample tube using the following formula

$$\text{Amount of carbohydrate/100mg of sample} = \frac{\text{mg of glucose}}{\text{Volume of test sample}} \times 100$$

3.5.2. Determination of protein

Protein estimation was performed by Lowry's method (Lowry *et al.*, 1951).

3.5.2.1. Extraction of protein

500mg of the sample was weighed and ground well with a pestle and mortar in 5-10 ml of buffer. This was centrifuged and supernatant was used for the for protein estimation.

3.5.2.2. Estimation of protein

Into a series of test tubes, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standards were pipetted out and then 0.1ml and 0.2 ml of the sample extracts were also pipetted out in two other test tubes. The volume was made upto 1 ml in all the test tubes. A tube with 1 ml water served as the blank. 5 ml of alkaline Cu solution were added to each tube, mixed well and allowed to stand for 10 min. Then 0.5ml of Folin – ciocalteaure agent was added, mixed well and incubated at room temperature in dark for 30 min, for the development of blue coloration. The readings were taken at 660nm using a spectrophotometer. A standard graph was drawn, and the amount of protein was calculated and expressed as mg/g of the sample.

3.5.3. Determination of crude fibre content

Estimation of crude fibre was done by following methods described by Maynard (1970) and Misra *et al.* (1975). Two gram of ground material was extracted with ether or petroleum ether to remove the fat. After extraction, 2g of dried the sample was boiled with 200ml of sulphuric acid for 30 min with bumping chips.

Filtration was done through muslin and washed with boiling water until washings were no longer acidic. Then boiling was done with 200ml of sodium hydroxide solution for 30 min. Again filtration was done through muslin cloth and washed with 25ml of boiling 1.25 per cent sulphuric acid, three 50ml portion of water and 25ml alcohol. The residue was transferred to preweighed ashing dish (W1) and then dried for 2 hrs at 13 ± 2^0C. The dish was cooled in a desiccator and weighed (W2). Then ignition was done for 30min at 600 ± 15^0C, cooled in a desiccator and reweighed (W3).The per cent of crude fibre in ground sample was calculated by using the formula,

$$\text{Per cent crude fibre} = \frac{\text{Loss in weight on ignition } [(W2-W1) - (W3-W1)]}{\text{Weight of sample}} \times 100$$

3.5.4. Determination of moisture

Five grams of the sample (W1) was taken in a preweighed crucible and dried in hot air oven until a constant weight was obtained (W2). The difference between the initial and final weight gives the moisture content, which is then converted and expressed in percentage.

$$\text{Per cent moisture content} = \frac{W1 - W2}{W1} \times 100$$

3.5.5. Determination of total ash

One gram of the sample was transferred to a weighed silica crucible (W1). It was heated on a Bunsen burner at low flame and when the substrate charred the crucible was transferred to a muffle furnace. It was heated at 500 to 550°C for about 2 hours till a white coloured ash was obtained. It was then cooled in a desiccator and

weighed (W2). The difference between the weights gives the ash content, which is then converted and expressed in percentage.

$$\text{Per cent ash} = \frac{\text{W2- W1}}{\text{Weight of the sample}} \times 100$$

3.5.6. Determination of Ascorbic acid

Volumetric method was followed for Ascorbic acid estimation (Harris and Ray, 1935). Initially 5 ml of working standard solution was pipette out into a 100 ml conical flask. 10 ml of 4 per cent oxalic acid was added and titrated against the 2, 6-dichlorophenol indophenol dye (V1 ml). End point was determined by the appearance of pink colour, which persists for a few minutes. The amount of dye consumed was calculated as the amount of ascorbic acid. Then the sample (0.5-5 g (W), depending on the sample) was extracted in 4 per cent oxalic acid and made up to 100ml and centrifugation was done. 5 ml of the supernatant was pipette out and10 ml of 4 per cent oxalic acid was added to it. Then titration was done against 2, 6- dichlorophenol indophenoldye (V2ml). The amount of ascorbic acid was calculated and expressed in percentage.

$$\text{Ascorbic acid content} = \frac{0.5\text{mg}}{\text{V1ml}} \times \frac{\text{V2ml}}{5\text{ ml}} \times \frac{100\text{ml}}{\text{W}} \times 100$$

3.6. STATISTICAL ANALYSIS

Data were subjected to analysis of variance (ANOVA) using the statistical package WASP. 2 developed by ICAR GOA. Levels of significance, means, coefficient of variation and critical difference were obtained for various data sets.

Results

4. RESULTS

The present study on 'Characterization of medicinal mushroom, *Cordyceps* sp. from Kasargod district' was carried out to identify and characterize the fungus parasitizing the coconut white root grub *Leucopholis coneophora* (Burm.) by studying the morphological, cultural, physiological and phylogenetic characters, its artificial culturing and to analyze the proximate composition of the fungus. Based on molecular phylogenetic study the genera *Cordyceps* which attack Coleopteran group of insects were transferred to the genus *Ophiocordyceps* and therefore in the present study the genus name of the target fungus is renamed as *Ophiocordyceps*. The results of the investigation carried out during 2016-18 are presented below.

4.1. SURVEY FOR THE COLLECTION OF *Cordyceps* sp.

4.1.1. Collection of *Cordyceps* sp.

Purposive sampling surveys were conducted in three locations of coastal sandy tracts of Kasargod district during June to September months of 2017 and 2018. The fruiting bodies of *Cordyceps* sp. emerged from the parasitized grub were collected from three locations *viz.*, Instructional farm of College of Agriculture, Padannakkad, Valiyaparamba area of Nileshwar and Regional Agricultural Research Station, Pilicode (Plate 1), where the coconut root grub is an endemic polyphagous pest. The fruiting bodies of the fungus were found in the soil under cashew trees in Instructional farm of College of Agriculture, Padannakkad, under mango trees in Valiyaparamba and under coconut palms in Pilicode.

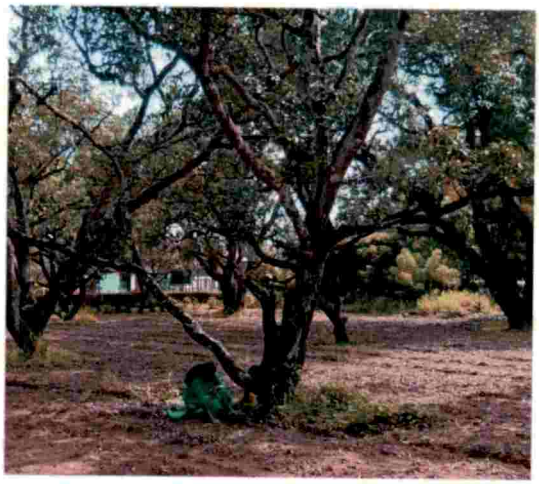
The fruiting bodies were found to be emerged from the soil after summer showers usually during 2nd week of May and after the onset of monsoon showers during 1st week of June. The population of fruiting bodies in one square meter area of soil were assessed in all the three locations and represented in the Table 1. Among the three areas surveyed maximum number of fruiting bodies (average of 13.86 m^{-2})

Table1. Details of survey

Sl.No.	Locations	Crops	Number of fruiting bodies m^{-2*}		Mean
			2017	2018	
1	Padannakkad	Cashew	15.25	12.50	13.86
2	Valiaparamba	Mango	5.75	4.50	5.13
3	Pilicode	Coconut	2.75	2.25	2.50

*Mean of four replications. In each column figures followed by same letter do not differ significantly according to DMRT

Insrtuctional farm, Padannakkad



Valiyaparamba, Nileswar

RARS, Pilicode

Plate1. Survey conducted in different locations

was found from the soil under cashew trees in the Instructional farm of College of Agriculture, Padannakkad.

4.1.2. Collection of soil samples

The soil samples from the three locations of survey were collected and analysis was done. Soil data analysis showed that the soil collected from the cashew orchard of Instructional farm of College of Agriculture, Padannakkad were rich in organic carbon (1.39%) compared to the soil from Valiaparamba and Pilicode (Table 2). Macro minerals *viz.*, potassium, calcium and micro mineral like manganese were higher in the soil of Instructional farm, Padannakkad compared to other two regions. But minerals *viz.*, phosphorus, zinc, copper were comparatively lesser than other two areas. Iron and magnesium were in medium concentrations compared to Valiaparamba and Pilicode.

4.2. ISOLATION OF *Cordyceps* sp.

For the isolation of fungus, different parts of the fungal structure like sclerotia, stipe and stroma of both fresh and dry specimens were tried in potato dextrose agar (PDA) medium. Isolation was successful from all the three parts but best results were obtained from stroma region of fresh fruiting bodies, due to the lesser chance of contamination (Plate 2). The isolate obtained after pure culturing was numbered as CD-1. The mycelia appeared initially white, later turning to creamish white to salmon colour and the underside of the plate being light brown (Plate 3). Later numerous thread like pinkish white synnemata appeared in culture representing the anamorphic stage, *Hirsutella* sp. (Plate 4). The isolate completed the full growth in 9 cm diameter Petri dish by 55 days after inoculation in PDA. The anamorph produced similar type of conidia in the culture as produced in the field.

Table2. Soil Analysis Data

Sl No.	Parameters	Locations		
		Pilicode	**Valiya paramba**	**Instructional farm, Padannakkad**
1	Phosphorus	139.42 Kg ha^{-1}	62.48 Kg ha^{-1}	42.62 Kg ha^{-1}
2	Organic carbon	1.06%	1.25%	1.39%
3	Pottassium	83.88 Kg ha^{-1}	60.82 Kg ha^{-1}	108.42 Kg ha^{-1}
4	Manganese	20.7 mg ha^{-1}	13.3 mg ha^{-1}	45.8 mg ha^{-1}
5	Zinc	4 mg ha^{-1}	7.5 mg ha^{-1}	2.4 mg ha^{-1}
6	Copper	1.87 ppm	0.99 ppm	1.06 ppm
7	Iron	22.28 ppm	24.5 ppm	25.7 ppm
8	Calcium	340ppm	360 ppm	1200 ppm
9	Magnesium	132 ppm	84 ppm	120 ppm
10	pH	4.46	4.6	4.91
11	EC	0.09	0.04	0.17

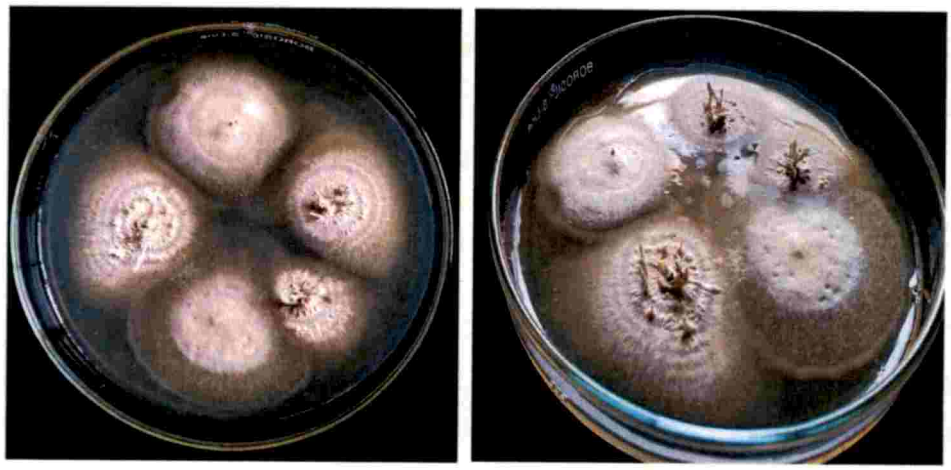


Plate 2. Typical colonies of *Cordyceps* sp. developed on isolation plate

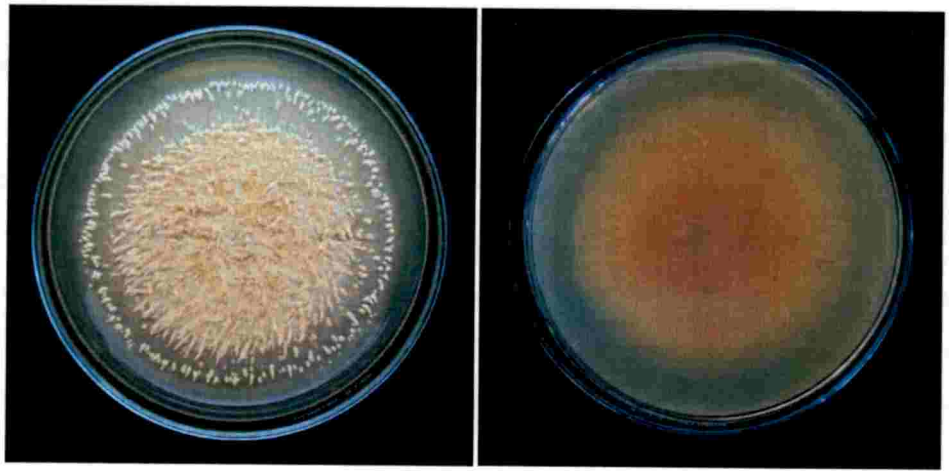


Plate 3.Culture plate of *Cordyceps* sp.

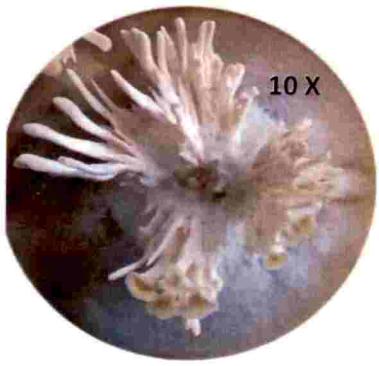


Plate 4.Synnemata formed in culture

4.3. CHARACTERIZATION OF *Cordyceps* sp.

4.3.1. Cultural characterization

The cultural characters like colour, shape, texture and the growth rate of the mycelium were studied in different media. Five different media *viz.*, potato dextrose agar (PDA), yeast potato dextrose agar (YPDA), malt extract agar (MEA), oatmeal agar (OMA) and czapek dox agar (CDA) were selected to find out the optimum medium for the growth of fungus. Growth characteristics of the fungus in different media were observed and details are given in Table 3.

In YPDA the fungus initially showed a white coloured growth with zonations then turned to creamish white colour and produced creamish to salmon coloured synnemata with honey dew like conidial mass initially at the centre later in the peripheral parts also and the colony showed a densely matted to wooly texture. Production of numerous synnemata initiated within 12 day after inoculation in this medium. In PDA the growth of fungal colony was initially white later turning to creamish white with a densely cottony texture and cream coloured synnemata were produced at the centre. The number of synnemata was less in PDA than in YPDA and the production of synnemata initiated 15 days after inoculation in PDA. In MEA the fungus showed a creamish growth with prominent zonations and densely matted, wavy and mud like texture. Very less number of synnemata were produced by 24 days after inoculation in this medium. In CDA the fungus showed an ashy white sparse growth having a cottony texture with the production of very few number of synnemata at the centre of colony by 38 days after inoculation. In OMA the fungus showed a sparse growth with a granular texture. Synnemata were produced in concentric zonations as small creamish white round protruding structures by 43 days after inoculation (Plate 5).

Observations on the colony diameter of *Cordyceps* sp. in five different media were taken at appropriate time intervals *viz.*, 35, 45, 52, 55 and 60 days after inoculation (Table 4). Among the different media tested YPDA was found to be the best medium with a highest colony diameter of 8.98 cm. The fungus completed the growth in 52nd day after inoculation, in which the growth was superior to other media. This was followed by PDA with a colony diameter of 8.73 cm in 52 days and completed the growth in 55 days. The least growth was obtained in OMA medium with a colony diameter of 6.76 cm in 52 days and completed the growth by taking 60 days. The effect of different media in the mycelial growth of the fungus was significantly different from each other (Plate 5).

4.3.2. Morphological characterization

4.3.2.1. Macroscopic characterization

Detailed macroscopic study of the fungus was done and the fruiting body of the fungus was found to be emerging from the cephalic region of the body of third instar grub of coconut root grub *Leucopholis coneophora*. The club shaped upper portion of fruiting body called as stroma, the fertile portion, in which the perithecia were present, the middle stalk like part, stipe and the mummified dead grub known as sclerotium were observed (Plate 6). The stipe and stroma portion of the fruiting bodies were found to be above the soil and infected grub was obtained at a depth of around 5 to 10 cm below the soil (Plate 7). Average length and girth of the fruiting bodies obtained from all the three locations were measured and presented in the Table 5, in which the maximum length of 11.9 cm and 1.6 cm for stipe and stroma were obtained from the soil under cashew trees. Maximum girth of 3.9 cm and 1.7 cm for stipe and stroma was also observed from the same soil (Plate 8). The longitudinal section of the fruiting body showed complete mycelial colonization of the insect body by the fungus, where the internal organs of the grub were replaced with fungal mycelium (Plate 9).

Table 3. Growth characteristics of *Cordyceps* sp. in different media

Media	Colony colour		Colony characters		No. of days for synnemata production	Production of synnemata			
	10 DAI	40 DAI	Shape	Texture		Colour	Size	Number	Position in the colony
YPDA	white	creamish white	Aerial, with zonations	Densely matted, wooly	12 days	creamish to salmon colour	Large	more	centre and periphery
PDA	white	creamish white	Aerial, zonations absent	Densely cottony	15 days	creamish colour	Large	less	centre
MEA	cream	cream	well-organized zonations with regular margin	Densely matted, wavy, mud like	24 days	creamish colour	Small	less	centre
CDA	Pure white	Pure white	Sparse, Irregular margin	cottony	38 days	White in colour	Small	less	centre
OMA	creamish white	creamish white	Sparse growth with zonations, irregular margin	granular	43 days	creamish white in concentric zonations, and as round protruding structures	Small	More	centre and periphery

Table 4. Effect of different media on mycelial growth of *Cordyceps* sp.

Sl. No.	Treatments	No. of days to complete growth	Colony diameter(cm)* DAI				
			35	45	52	55	60
1	YPDA	52	4.25 [a]	6.77 [a]	8.98[a]	9.00 [a]	9.00
2	PDA	55	4.18 [b]	6.45 [b]	8.73[b]	9.00 [a]	9.00
3	MEA	57	4.00 [c]	5.86 [c]	8.05[c]	8.87[a]	9.00
4	CDA	57	3.96 [d]	5.56 [d]	7.78[d]	8.66[b]	9.00
5	OMA	60	2.67 [e]	5.06 [e]	6.76[e]	8.42[c]	9.00
CD(0.05)			0.021	0.016	0.137	0.149	-

*Mean of four replications

In each column figures followed by same letter do not differ significantly according to DMRT

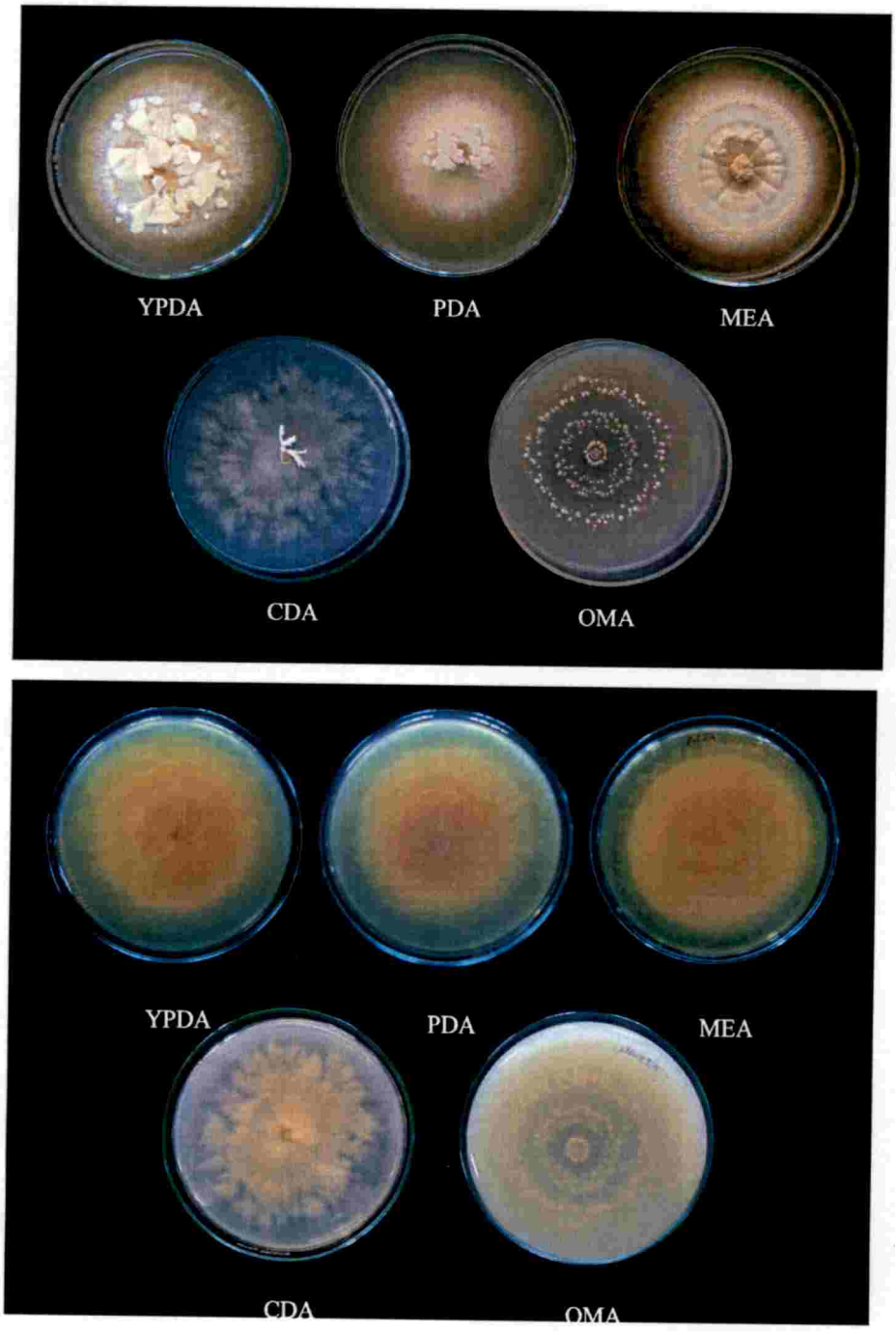


Plate 5.Effect of different medium on mycelial growth of *Cordyceps* sp.

Plate 6.Association of *Cordyceps* sp. with *Leucopholisconeophora*(Burm)

Plate 7.*Cordyceps* sp. in natural habitat

Plate 8.Variation in size of *Cordyceps* sp.

Plate 9. L. S. of *Cordyceps* sp.

Comprehensive study on the process of fruiting body emergence was done in all the three locations surveyed. From the buried infected grubs, yellowish orange coloured stalks of about 0.3-0.6 cm thickness emerged during the last week of May or first week of June and the tip of which later became bulged and turned to bright orangered colour. In the whole fruiting body the bulged tip portion was observed as stroma, the stalk like structure holding stroma as stipe and the mummified grub was found to be completely converted as the sclerotium. Stromatal heads were observed to emerge from the cephalic region of the infected third instar grubs of *L. coneophora.* After 40 days, by the second week of July the stroma region showed numerous humps which were initially orange red in colour turning to brown representing formation of perithecia. The colour turned to dark brown with the complete maturation of perithecia and the release of ascospores occurred in a period of two weeks. During August, after an interval of two weeks the stroma region became dark brown to black and numerous white colored hook like sporulating structures called synnemata were developed representing the conidial stage of the fungus *i.e.*, *Hirsutella* sp. (Plate 10). Hence a gradual change of colour and texture of stroma can be seen during different stages of development (Plate 11). Branching of stroma was noticed as double and very rarely three to five branches from a single stroma were observed (Plate 12). After one month by the second week of September, the whole fruiting body got decomposed by the action of soil dwelling saprophytes.

Various other macroscopic characters like shape of the fruiting body, colour of sclerotium, stipe and stroma along with branching pattern of the stroma collected from the three locations surveyed were also studied and the observations are shown in Table 5.

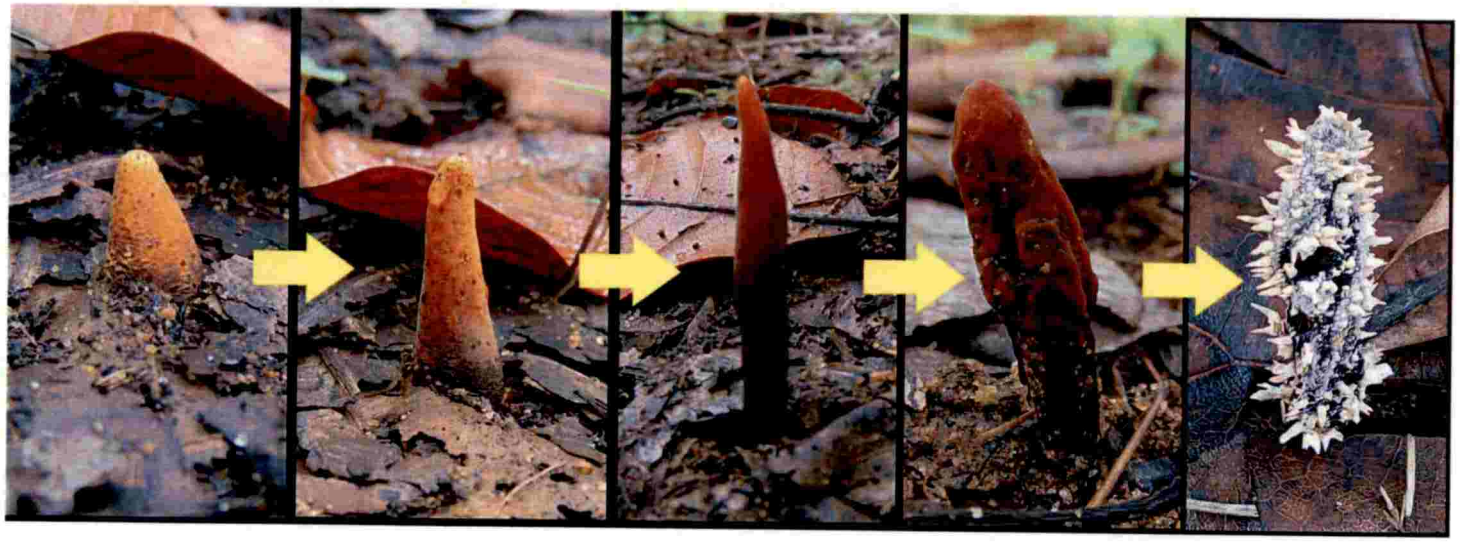


Plate 10. Different stages of fruiting body development in the field

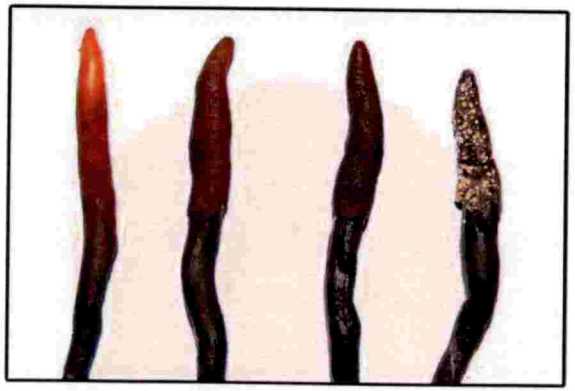


Plate 11. Changes in stroma during development

Plate 12. Branching pattern of stroma

Table 5. Macroscopic characters of *Cordyceps* sp. in different locations

Locations	Characters of fruiting body								
	Shape	Size (cm) *				Colour			Branching pattern of Stroma
		Length		Girth		Sclerotia	Stipe	Stroma	
		Stipe	Stroma	Stipe	Stroma				
Padannakkad	Spindle	11.9	3.9	1.6	1.7	Black	Dark brown	Bright orange colour turning to dark brown	Yes
Valiaparamba	Club	8.5	2.8	1.3	1.4	Dark brown	Dark brown	Light brown turning to dark brown.	No
Pilicode	Club	10.4	3.2	1.2	1.4	Dark brown	brown	Reddish brown turning to dark brown	Yes

*Mean of 15 replications

4.3.2.2. Microscopic characterization

Microscopic observations of *Cordyceps* sp. *viz.*, characters of anamorphic stage including hypha, synnemata, conidiophores and conidia and teleomorphic stage including perithecia, asci and ascospores were taken.

Hyphae of *Cordyceps* sp. were hyaline septate and branched with a width of 1.1 to 1.52 µm (Plate 13). The length and width of synnemata from which the conidiophores arising was found to be 2136.42 to 2350.50 µm and 990.56 to 1100.05 µm respectively (Plate 14). Synnemata was found to have a cylindrical shape with numerous conidiophores all over the surface. In natural habitat the synnemata started to form initially as white cottony cushion later turning to light pinkish white hook like structures on the stroma having a length of 0.2 - 1.3 cm long (Plate 15).

The conidia were formed either singly or in chains on long conidiophores measuring 5.64 to 8.78µm. The conidiophores can be single or branched. The conidia were dimorphic and hyaline among which one type of the conidia was spherical with a diameter of 1.9 to 5.7µm (Plate 17) and the other was spindle shaped with a length of 2.5 to 6.3µm and width of 0.53 to 2.41µm (Plate 19). The spherical conidia were arranged singly (Plate 16) and spindle conidia were arranged in chains on the conidiophores (Plate18) (Table 6).

Microscopic examination of thin cross sections of stroma showed numerous perithecia towards the peripheral regions. Perithecia were peripheral to slightly immersed globular to oval in shape, narrow towards the tip. Openings of perithecia, the ostioles through which release of ascospores to the outside occurs were found at the tip (Plates 20 and 21). The length and width of perithecia was found to be 82.6 to 396.1 µm and 93.6 to 171.27 µm respectively. The thickness of the perithecial wall ranged from 16.6 to 21.65µm. On examination of perithecia numerous elongated asci were observed inside the perithecium. Crozier formation stage during the ascus

development was observed in immature perithecium (Plate 22). On maturation, asci were observed as a bundle inside the perithecium (Plate 23). The ascus was elongated with length ranging from 115.4 to 170.11 µm and width of 5.2 to 10 tip µm (Plate 24). The tip of ascus had a prominent cap or operculam, the lid like structure, through which the ascospores were released out of ascus (Plate 25). The ascus containined eight filiform ascospores which were four-partite with 105.3 to 135.7µm length and 2.51 to 2.73 width. The ascospores usually split into four part spores in which the middle two spores were cylindrical and the terminal spores were pointed at the tip, having 25.58 to 44.59 µm length and 2.52-2.74µm width (Plate 26) (Table 7). The life cycle of the fungus in association with the insect was drawn after the detailed morphological characterization and given in the figure 1.

4.3.3. Molecular characterization of *Cordyceps* sp.

The molecular characterization of the fungus was carried out at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram by ITS sequencing to identify the fungus at species level. PCR amplification was carried out followed by sequencing. The PCR profile of amplified region is given in the Plate 27. Sequence analysis to find out the nucleotide homology of the fungus was performed using the BLASTn programme of National Center for Biotechnology Information [NCBI (http:// ncbi.nlm.nhm.gov/blast)]. The results of BLAST analysis showing the distribution of 107 blast hits on the query sequence of the isolate CD-1 was given in the figure 2. Analysis showed homology with *Ophiocordyceps neovolkiana* srain KC1 having 98 per cent identity. Hence the isolate was identified as *Ophiocordyceps neovolkiana* (Kobayasi).

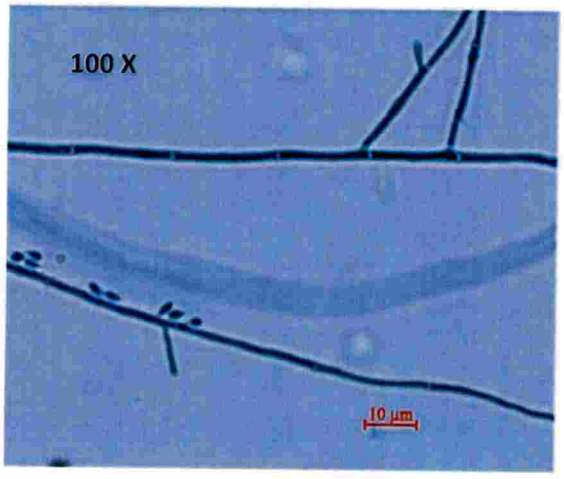


Plate 13.Hypha of *Hirsutella* sp.

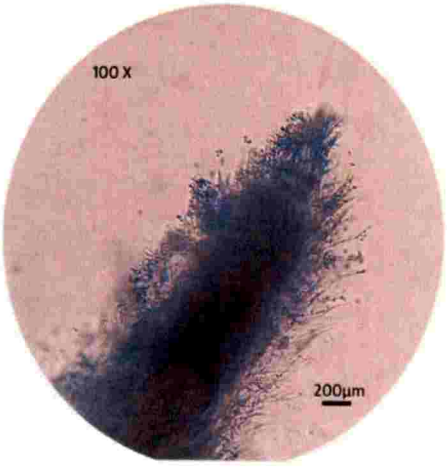


Plate 14.Synnemata

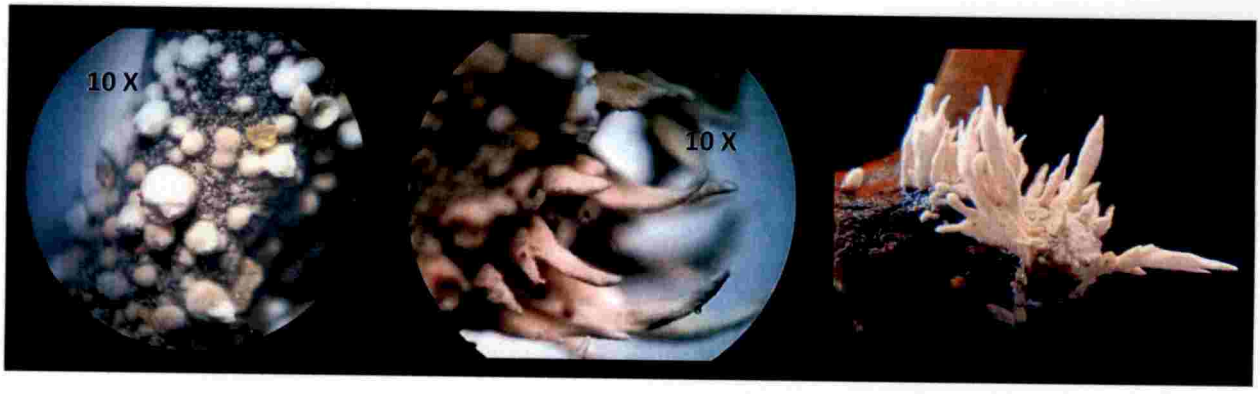


Plate 15.Different stages of development of synnemata on the fruiting body in natural habitat

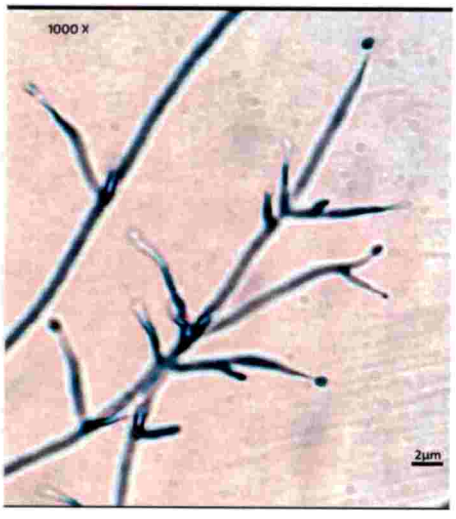


Plate 16. Single spherical conidia on conidiophore

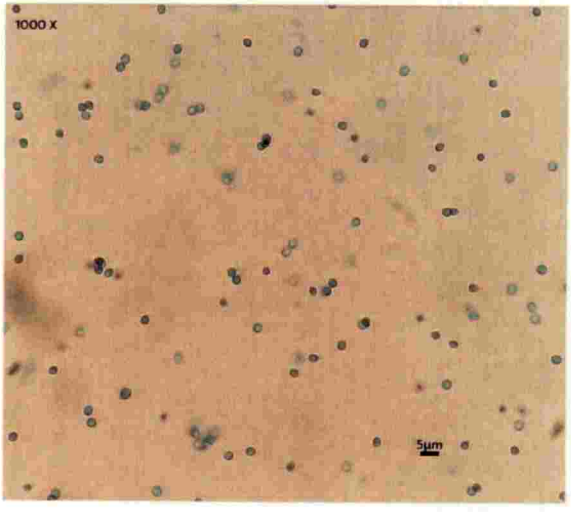


Plate 17. Spherical conidia

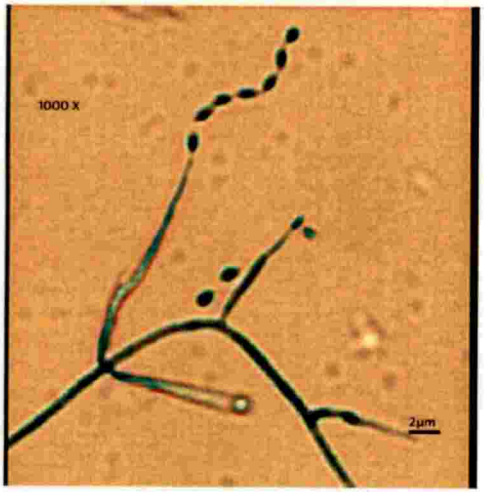


Plate 18. Spindle conidia in chain on conidiophore

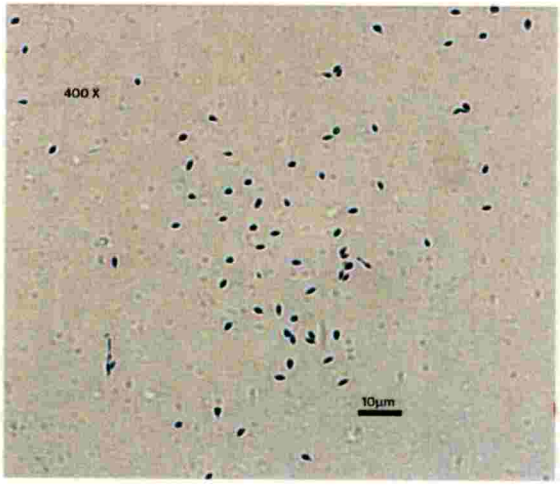


Plate 19. Spindle conidia

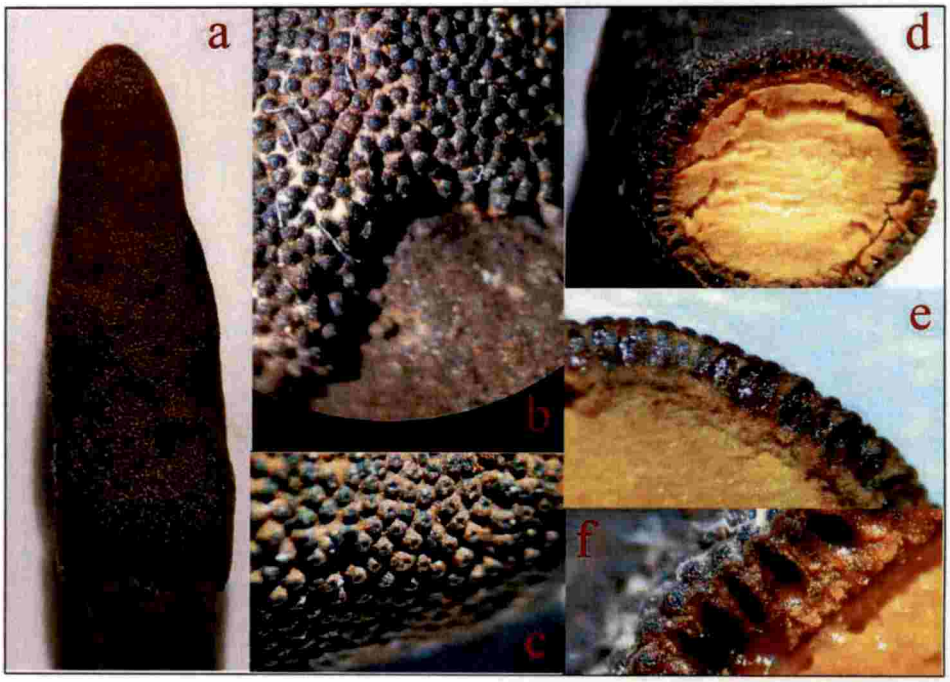


Plate 20. Characters of stroma: a.Stroma; b. Perithecia; c. Ostiole; d. C. S. of stroma; e. Peripheral arrangement of perithecia; f. Globular to oval perithecia

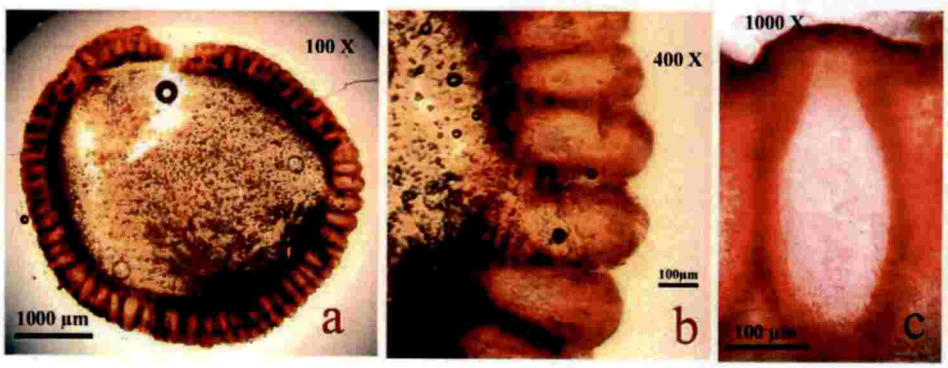


Plate 21. Microscopic characters of stroma

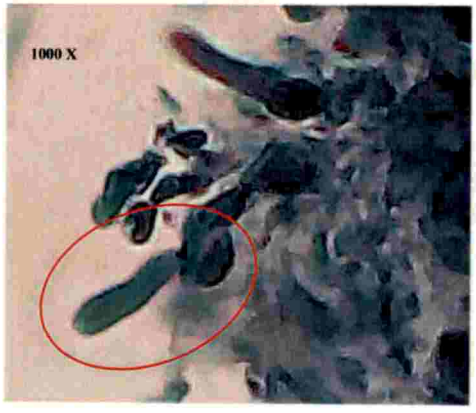


Plate 22. Crozier formation during ascus development

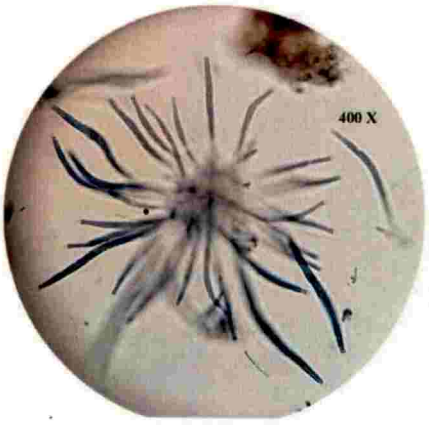


Plate 23. Mass of mature asci

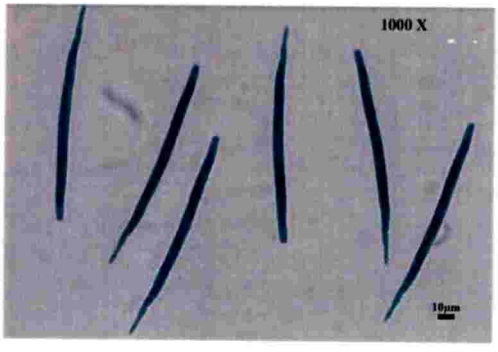


Plate 24.Asci

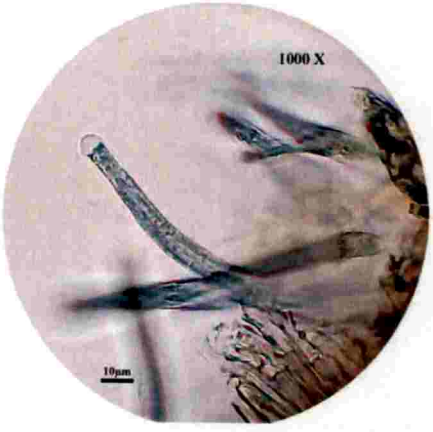


Plate 25.Ascus with operculam

a b c

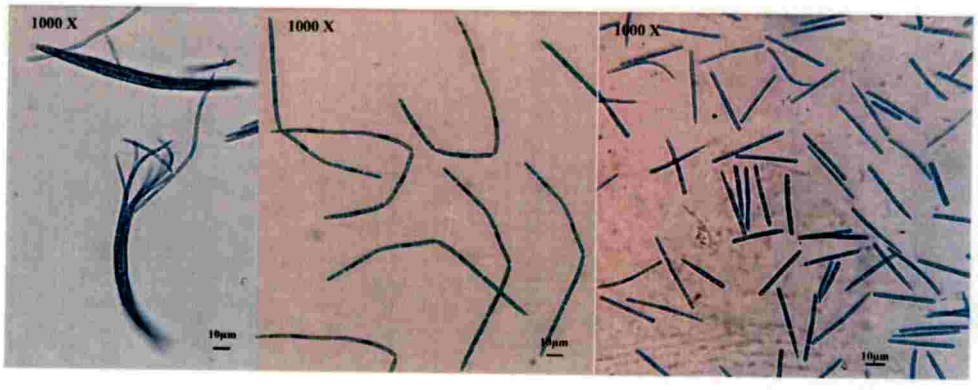


Plate 26. Microscopic characters of ascospore: a. Eight ascospores in an ascus; b. Ascospores; c.Partspores of ascospore

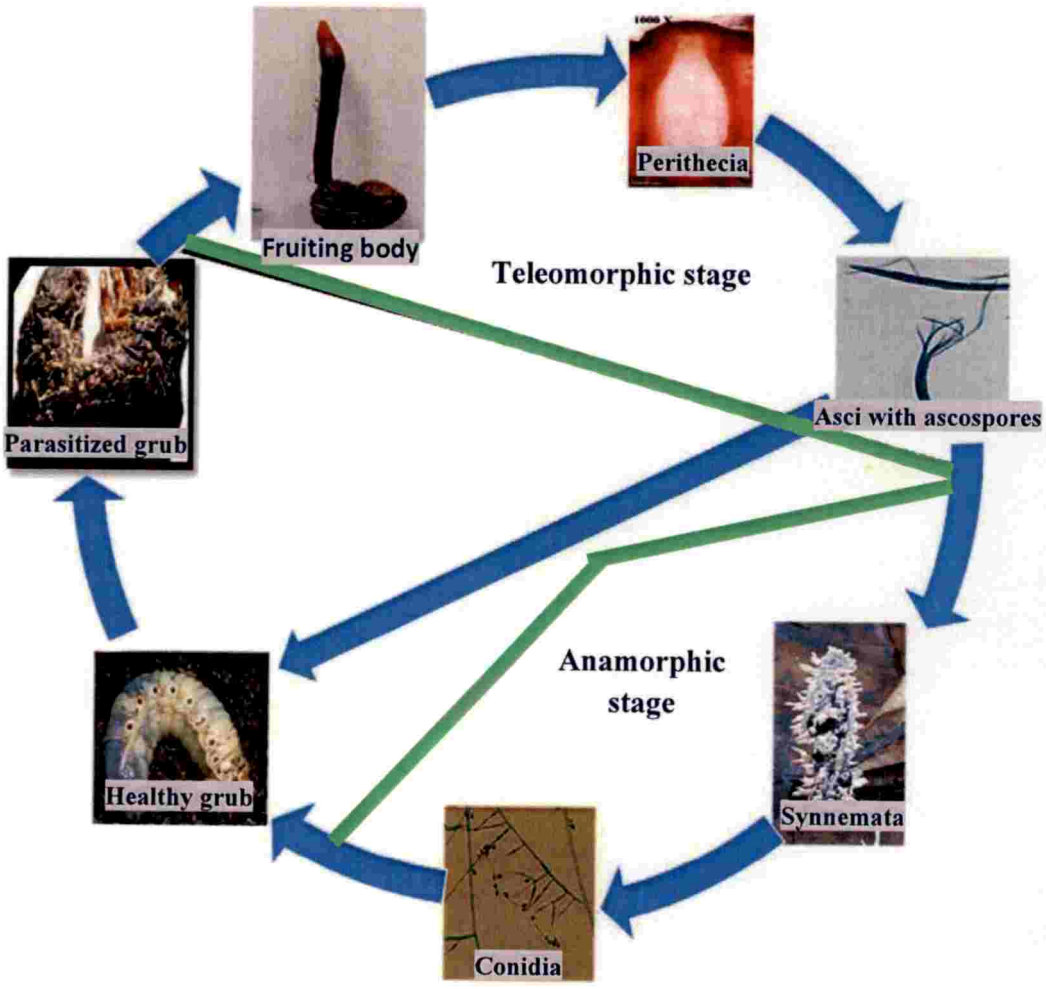


Figure 1.Life cycle of *Cordyceps* sp.

Plate 27. PCR amplification profile of the isolate CD1 with the universal primers of ITS 1 and ITS 4

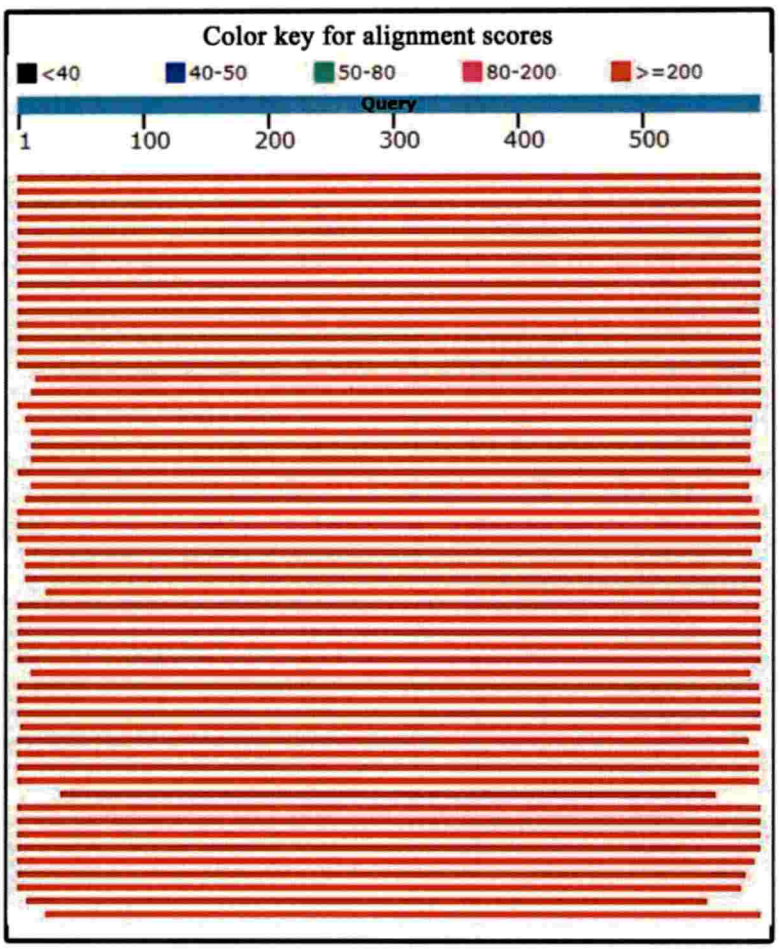


Figure 2. Chromatogram showing distribution of 107 blast hits on the query sequence of the isolate CD1

Table 6. Microscopic characters of anamorphic stage.

Microscopic Characters	Description	Dimension (µm)
Hypha	Hyaline, septate and branched	1.1- 1.52
Synnemata	Cylidrical with numerous conidiophores	2136.42-2350.50 × 990.56-1100.05
Conidiophore	Elongated, single or branched	5.64-8.78
Spherical conidia	Born singly on conidiophore	1.9-5.7
Spindle conidiia	Born in chains on conidiophore	2.5-6.3 × 0.53-2.41

Table 7. Microscopic characters of teleomorphic stage.

Microscopic Characters	Description	Diamension (µm)*
Perithecia	Globular -oval with ostiole	82.6-396.1 × 93.6-171.27
Perithecial Wall	Thick	16.6-21.65
Ascus	Elongated	115.4-170.11 × 5.2-10
Acsospore	Filiform and four partite	105.3-135.7 × 2.51-2.73
Ascospore part spore	Terminal spore pointed at the tip and middle spores are cylindrical	25.58-44.59 × 2.52-2.74

The ITS sequence of the fungus is as follows

AGGGATCATTACCGAGTTTACAACTCCCAAACCCCAGTGTGAACCTGTACCTCAT
CGTTGCCTCGGCGGGATCGCCCCGGCGCGCCCTCTCCAGGGCGTCCGGATCACAC
GGCGCCCGCCGCAGGACCCCCCAAACTCTTTTGCATCTCCGGCGTCGTTTTCTTCT
GAGTAGATACTTTTTCCCCGCGAGAGCGGGGTGAAAATGAATCAAAACTTTCAA
CAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGT
AATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCC
GCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCGACCCTCAGGCCCCG
TAGGGGACTGGTGTTGGGGGACGGCACCGCTGGGGAGGAAGGGAGGCTTCGTCT
CCCCCTCCGCCCGCCGCCGCCCCCGAAATGCAGTGGCGGCCTCGCCGCAGCGAC
CTCCGCGTAGTAGCATAAACCTCGCGGCCTGGGAGCTGGGCGCGGCCACGCCGT
AAAACAACCCCCACGCGAAAACTCTTCGCACCAAGAGTTGACCTCGG

The sequence of the fungus was deposited at the Gen Bank of NCBI and the accession number obtained was MH 668282 and the culture of the fungus was deposited at National Fungal Culture Collection of India, a national facility by MACS Agharkar Research Institute, Pune and the accession number obtained was NFCCI 4331.

A dendrogram was constructed using Mega 7 software (Kumar et al., 2016) for the isolate CD-1 with *Ophiocordyceps neovolkiana* strain KC1 and 21 related species showing maximum similarity to the isolate along with the out group of *Ophiocordyceps neovolkiana i.e.*, *Cosmospora coccinea* to infer the evolutionary history, using ITS - 5.8S rDNA sequences of these species obtained from the Gen Bank of NCBI. The evolutionary history was inferred using Neighbor - Joining method and the evolutionary distances were computed using Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Figure3)

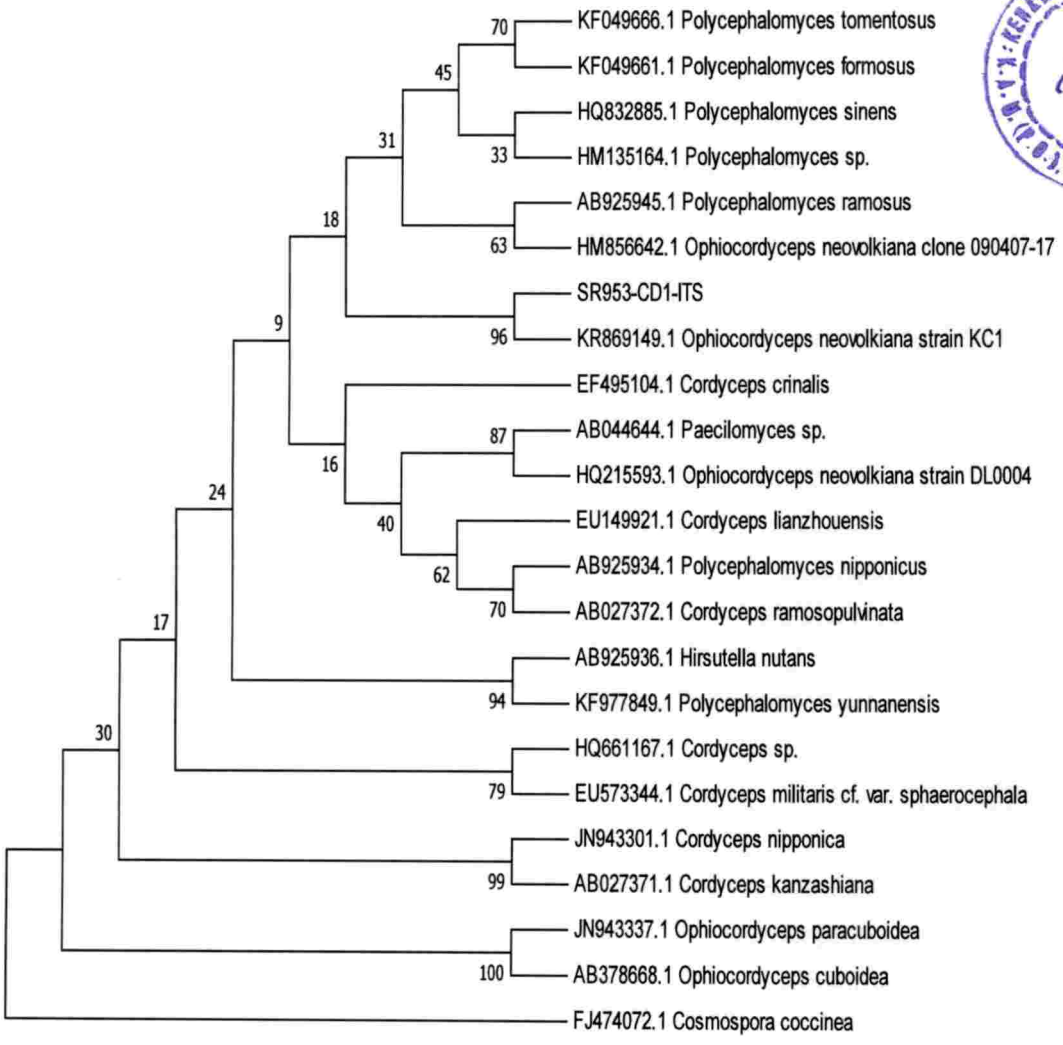


Figure 3. Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method (Saitou *et al.,* 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, 2004) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). Numbers before the name of species are the accession numbers of published sequences.

Based on the above studies it was inferred that the systematic position of the fungus is Domain: Eukarya, Kingdom: Fungi, Division: Ascomycota, Class: Sordariomycetes, Order: Hypocreales, Family: Ophiocordycipitaceae, Genus: *Ophiocordyceps* and Species: *neovolkiana*.

4.3.4. PHYSIOLOGICAL STUDIES OF *Ophiocordyceps neovolkiana*

4.3.4.1. Effect of temperature on the mycelial growth of O. neovolkiana

Eight different temperature conditions *viz,* 5°C, 10°C, 15°C, 20°C, 25°C, room temperature(26 ± 2°C), 30°C and 35°C were evaluated to find out the best temperature for the growth of *O. neovolkiana* and the observations are given in Table 8. The highest growth of 8.98 cm was observed in Petri dish kept under 30°C, in which the growth was completed within 38 days after inoculation. The growth was absolutely absent at 5°C and 10°C. A gradual increase in growth was observed with increase in temperature from 15°C onwards. But there was a decrease in growth at 35°C, which proved that 30°C was the optimum temperature for growth of *O. neovolkiana*. The growth at temperature 30°C was significantly different from all other temperature conditions (Plate 28).

4.3.4.2. Effect of pH on the mycelial growth of O. neovolkiana

To evaluate the effect of pH on the mycelial growth of *O. neovolkiana*, thirteen different H^+ ion concentrations (pH) *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 and 10 were tested. Among these pH levels, pH seven was found to be optimum for mycelial growth of the fungus in which the highest colony diameter of 8.93 cm was observed and growth was completed within 35 days after inoculation, which was significantly different from all other pH conditions. Mycelial growth was found to be gradually reducing in pH levels above 7 and below 7. The least growth was observed at pH 4 (3.9 cm). The pH 8 and 9, 6.5 and 9.5, 10 and 6, 5.5 and 5 were having on par effect on growth of *O. neovolkiana* (Table 9) (Plate 29).

Table 8. Effect of temperature on mycelial growth of *O. neovolkiana*

Sl. No.	Treatments	Colony diameter(cm)* DAI			
		10	20	30	38
1	5°C	0[g]	0[g]	0[g]	0[g]
2	10°C	0[g]	0[g]	0[g]	0[g]
3	15°C	0.875[e]	2.15[e]	4.25[e]	4.925[e]
4	20°C	1.5[d]	3.35[d]	5.525[d]	7.885[d]
5	25°C	2.4[c]	5.375[c]	6.45[c]	8.215[c]
6	26±2°C (RT)	2.6[b]	5.8[b]	7.525[b]	8.585[b]
7	30°C	3.375[a]	6.575[a]	8.425[a]	8.975[a]
8	35°C	0.575[f]	0.875[f]	2.65[f]	3.275[f]
CD(0.05)		0.168	0.230	0.428	0.127

*Mean of four replications. In each column figures followed by same letter do not differ significantly according to DMRT

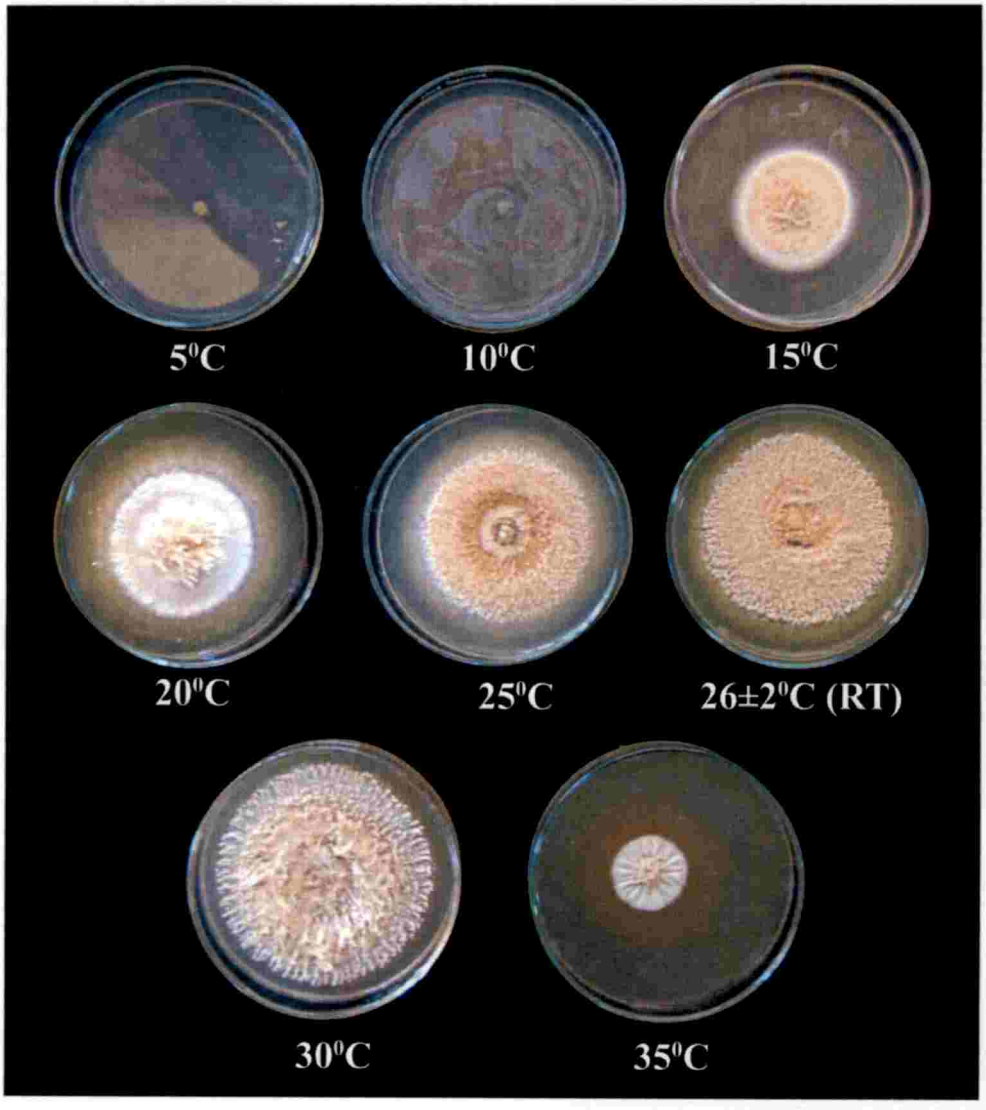


Plate 28 . Effect of temperature on mycelial growth of *O. neovolkiana*

Table 9. Effect of pH on mycelial growth of *O. neovolkiana*

Sl. No.	Treatments	Colony diameter(cm)* DAI			
		10	20	30	35
1	4	1.40^{i}	2.40^{h}	3.43^{j}	3.90^{i}
2	4.5	1.60^{i}	2.50^{h}	3.65^{j}	4.43^{h}
3	5	2.02^{h}	3.38^{g}	4.40^{i}	5.43^{g}
4	5.5	2.60^{g}	4.40^{f}	4.88^{h}	5.53^{g}
5	6	3.25^{f}	5.43^{de}	5.45^{f}	5.98^{f}
6	6.5	4.15^{d}	6.25^{c}	5.63^{f}	6.33^{e}
7	7	5.30^{a}	7.35^{a}	8.28^{a}	8.93^{a}
8	7.5	4.98^{b}	6.95^{b}	7.63^{b}	8.43^{b}
9	8	4.48^{c}	6.45^{c}	7.20^{c}	8.20^{c}
10	8.5	4.20^{d}	6.28^{c}	7.10^{c}	8.10^{c}
11	9	3.83^{e}	5.73^{d}	6.50^{d}	7.15^{d}
12	9.5	3.28^{f}	5.25^{e}	5.95^{e}	6.30^{e}
13	10	2.75^{g}	4.65^{f}	5.15^{g}	6.08^{f}
	CD(0.05)	0.246	0.31	0.241	0.167

*Mean of four replications. In each column figures followed by same letter do not differ significantly according to DMRT

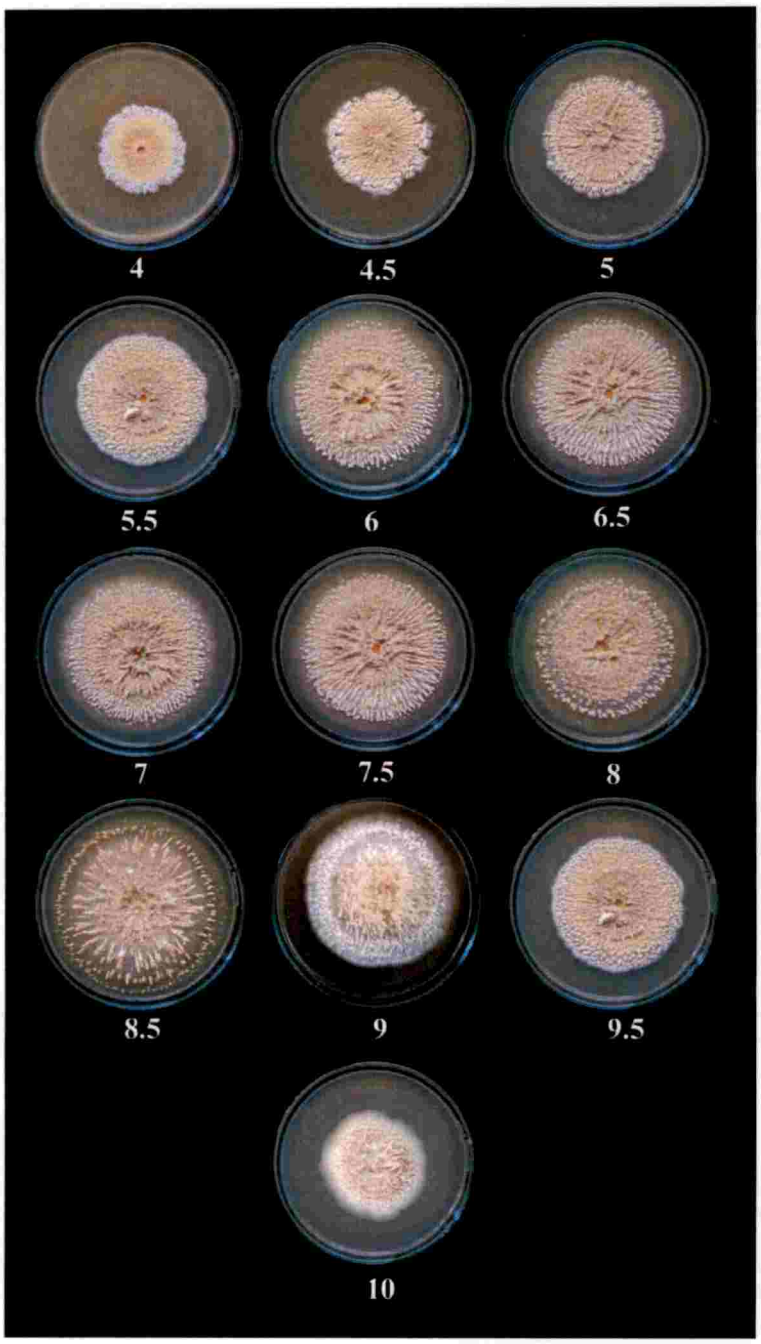


Plate 29. Effect of pH on mycelial growth of *O. neovolkiana*

4.3.4.3. Effect of light and darkness on the mycelial growth of O. neovolkiana

Four different conditions of light and darkness for growth of *O. neovolkiana* was tested *viz*., 24 hrs of light (500 lux under incubator at 30^0C), 24 hrs of light (500 lux under room temperature: 27 ± 2^0C), 24 hrs of darkness (under incubator at 30^0C) and 24 hrs of darkness (under room temperature: 27 ± 2^0C). Among the different conditions, 24 h of darkness under incubator at 30^0C was preferred for growth by *O. neovolkiana* with a colony diameter of 8.97 cm, which was significantly higher than the growth in all other conditions and the growth was completed within 35 days after inoculation, followed by 24 h of darkness at room temperature (8.57 cm in 35 days), which was on par with light under incubator (8.45 cm in 35 days). The least growth was obtained in light under room temperature (7.45 cm in 35 days) (Table 10) (Plate 30).

4.3.4.4. Effect of carbon sources on the mycelial growth of O. neovolkiana

To study the effect of different carbon sources on the mycelial growth of *O. neovolkiana*, four sources of carbon were used *viz,* dextrose, sucrose, fructose and maltose. Results showed that fructose was the best source of carbon in which maximum mycelial growth of 8.98 cm was obtained within 36 days after inoculation. The second best carbon source was sucrose (8.7 cm in 36 days) followed by maltose and dextrose. The effect of all the carbon sources on the mycelial growth of the fungus was significantly different from each other (Table 11) (Plate 31).

4.3.4.5. Effect of nitrogen sources on the mycelial growth of O. neovolkiana

To study the effect of different nitrogen sources on the mycelial growth of *O. neovolkiana* four sources of nitrogen were used *viz.,* yeast extract, beef extract, potassium nitrate and ammonium nitrate. Results showed that yeast extract was the best source of nitrogen in which the highest mycelial growth of 8.95 cm was

Table 10.Effect of light and darkness on mycelial growth of *O. neovolkiana*

Sl. No.	Treatments	Colony diameter(cm)* 35 DAI			
		10	20	30	35
1	24 hrs of light (500 lux under room temperature: 27 ± 2°C)	2.50[c]	4.48[c]	6.48[c]	7.45[c]
2	24 hrs of light (500 lux under incubator)	3.33[b]	5.45[b]	7.30[b]	8.30[b]
3	24 hrs of darkness (under room temperature: 27 ± 2°C)	3.83[b]	5.88[b]	7.60[b]	8.43[b]
4	24 hrs of darkness (under incubator)	5.18[a]	6.63[a]	8.40[a]	8.97[a]
	CD(0.05)	0.326	0.314	0.266	0.280

*Mean of four replications. In each column figures followed by same letter do not differ significantly according to DMRT

Table 11. Effect of carbon sources on mycelial growth of *O. neovolkiana*

Sl. No.	Treatments	Colony diameter(cm)* DAI			
		10	20	30	36
1	Dextrose	2.45[c]	4.40[d]	7.05[d]	7.60[d]
2	Sucrose	3.65[b]	5.18[c]	8.13[b]	8.70[b]
3	Fructose	5.20[a]	6.38[a]	8.55[a]	8.98[a]
4	Maltose	3.63[b]	5.58[b]	7.65[c]	8.55[c]
	CD(0.05)	0.410	0.383	0.217	0.138

*Mean of four replications. In each column figures followed by same letter do not differ significantly according to DMRT

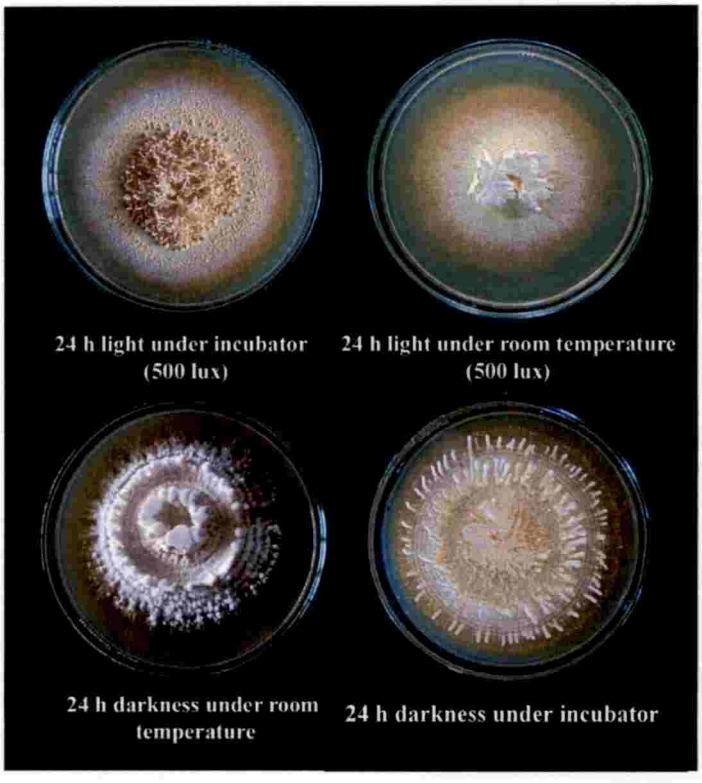


Plate 30. Effect of light and darkness on mycelial growth of *O. neovolkiana*

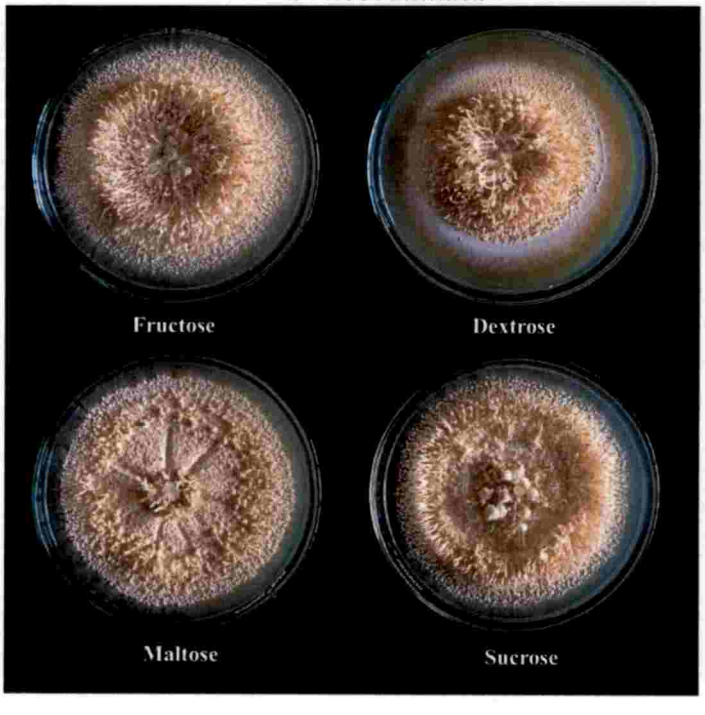


Plate 31. Effect of carbon sources on mycelial growth of *O. neovolkiana*

Table 12. Effect of nitrogen sources on mycelial growth of *O. neovolkiana*

Sl. No.	Treatments	Colony diameter(cm)* DAI			
		10	20	30	35
1	Yeast extract	5.28 [a]	6.63 [a]	8.55 [a]	8.95[a]
2	Beef extract	4.13 [b]	5.33 [b]	8.13 [b]	8.48[b]
3	Potassium nitrate	3.65 [c]	4.35 [c]	7.28 [c]	7.98[c]
4	Ammonium nitrate	2.45 [d]	4.58 [c]	6.95 [d]	7.38[d]
	CD(0.05)	0.424	0.352	0.232	0.245

*Mean of four replications. In each column figures followed by same letter do not differ significantly according to DMRT

observed within 35 days after inoculation. The second best nitrogen source was beef extract (8.48 cm in 35 days) followed by potassium nitrate (7.98 cm) and ammonium nitrate (7.38 cm). The effect of all the nitrogen sources on the mycelial growth of the fungus were significantly different from each other (Table 12) (Plate 32)

.4.3.4.6. Effect of macro minerals on the mycelial growth of O. neovolkiana

To study the effect of macro minerals on the mycelial growth of *O. neovolkiana* four sources of macro minerals were used *viz,* $CaCl_2$, KH_2PO_4, K_2HPO_4 and NaCl. The results showed that KH_2PO_4 was the best source of macro mineral for the growth of *O. neovolkina* in which the fungus completed the growth within 38 days after inoculation with a highest colony diameter of 8.93 cm, which was significantly higher than the growth obtained from all other macro mineral sources. The second best source of macomineral was $CaCl_2$ (8.8 cm) and the least growth was obtained in NaCl (8.6 cm). The growth in K_2HPO_4 was on par with the growth in both $CaCl_2$ and NaCl at 38 days after inoculation (Table 13) (Plate 33).

4.3.4.7. Effect of micro minerals on the mycelial growth of O. neovolkiana

To study the effect of different sources of micro minerals on the mycelial growth of *O. neovolkiana*, four sources of micro minerals were used *viz,* $ZnCl_2$, $MnCl_2$, $CuSO_4$ and $FeSO_4$. The highest growth of 8.98 cm was observed in $ZnCl_2$, in which the fungus completed the growth within 35 days after inoculation. The second best micro mineral source was $MnCl_2$ (8.7 cm in 35 days) followed by $FeSO_4$ (8.13cm) and $CuSO_4$ (5.1cm). Effect of all the micro mineral sources on the mycelial growth was significantly different from each other (Table 14) (Plate 34).

Table 13. Effect of macro mineral sources on mycelial growth of *O. neovolkiana*

Sl. No	Treatments	Colony diameter(cm)* DAI			
		10	20	30	38
1	K_2HPO_4	3.63^{c}	4.43^{c}	7.83^{b}	8.70^{bc}
2	KH_2PO_4	5.50^{a}	6.63^{a}	8.60^{a}	8.93^{a}
3	$CaCl_2$	4.10^{b}	5.35^{b}	8.13^{b}	8.80^{b}
4	NaCl	2.45^{d}	4.40^{c}	7.93^{b}	8.60^{c}
	CD(0.05)	0.361	0.279	0.342	0.115

*Mean of four replications. In each column figures followed by same letter do not differ significantly according to DMRT

Table 14. Effect of micro mineral sources on mycelial growth of *O. neovolkiana*

Sl. No	Treatments	Colony diameter(cm)* DAI			
		10	20	30	35
1	$CuSO_4$	2.63^{c}	3.40^{c}	4.78^{d}	5.10^{d}
2	$FeSO_4$	3.93^{b}	4.43^{b}	7.35^{c}	8.13^{c}
3	$ZnCl_2$	5.95^{a}	7.05^{a}	8.55^{a}	8.98^{a}
4	$MnCl_2$	4.13^{b}	6.80^{a}	8.13^{b}	8.70^{b}
	CD(0.05)	0.260	0.308	0.305	0.130

*Mean of four replications. In each column figures followed by same letter do not differ significantly according to DMRT

Plate 32. Effect of nitrogen sources on mycelial growth of *O. neovolkiana*

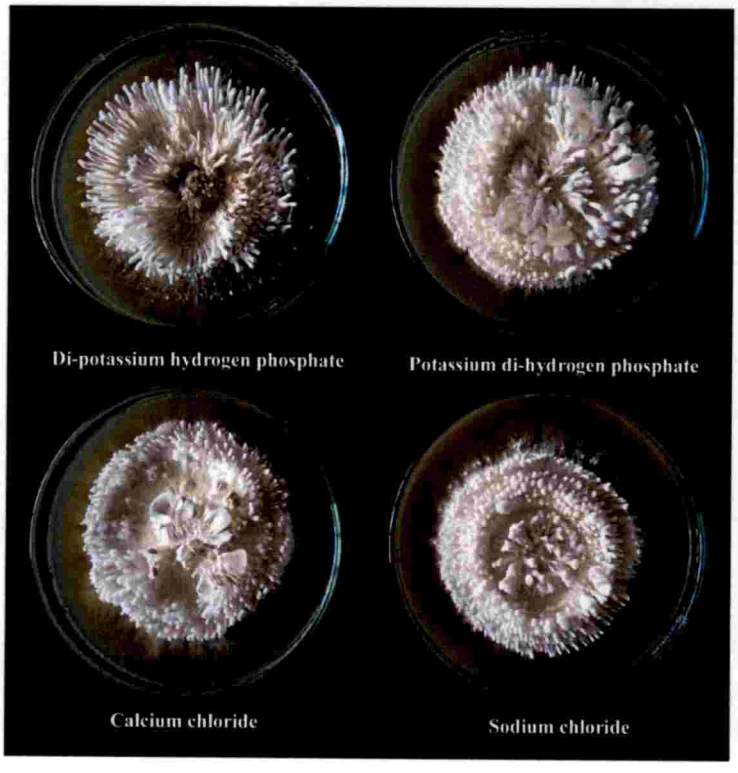


Plate 33. Effect of macro mineral sources on mycelial growth of *O. neovolkiana*

4.3.4.8. Effect of vitamins on the mycelial growth of O. neovolkian

To study the effect of different sources of vitamins on the mycelial growth of *O. neovolkina* six sources of the vitamins were used *viz.*, folic acid, nicotinic acid, riboflavin, thiamine, biotin and pyridoxine. Results showed that folic acid was the best source of vitamin for the growth of *O. neovolkiana* with a colony diameter of 8.93 cm, in which the fungus completed the growth within 37 days after inoculation. The second best vitamin source was biotin (8.65 cm in 37 days) and the least growth was observed in riboflavin (7.8 cm in 37 days). Effect of all the vitamin sources on the mycelial growth of the fungus was significantly different from each other (Table 15) (Plate 35).

From the observations of the above experiments, one optimum media for the growth of *O. neovolkiana* was formulated. The optimum media constituted 300 g of potato, 20 g of fructose, 5 g of yeast extract, one gram of KH_2PO_4, 500 mg of $ZnCl_2$, 10 mg of folic acid and 20 g of agar in one litre of distilled water and the medium was named as yeast extract potato fructose agar (YEPFA).

4.3.5. Comparison of YEPFA with conventional media

The performance of the medium YEPFA formulated for the growth of *O. neovolkiana* was evaluated with PDA and YPDA both in solid and liquid form.

4.3.5.1. Growth rate of O. neovolkiana

The results showed the highest radial mycelial growth of *O. neovolkiana* was observed in YEPFA *i.e.*, 8.95 cm in 30.85 days after inoculation, which was significantly higher than the growth in YPDA (8.35 cm) and PDA (6.49 cm). The number of days taken for complete growth of the fungus was 30.85 days in YEPFA which was superior to the other two media which were 50.71 days after inoculation in YPDA and 55.15 days after inoculation in PDA (Plate 36).

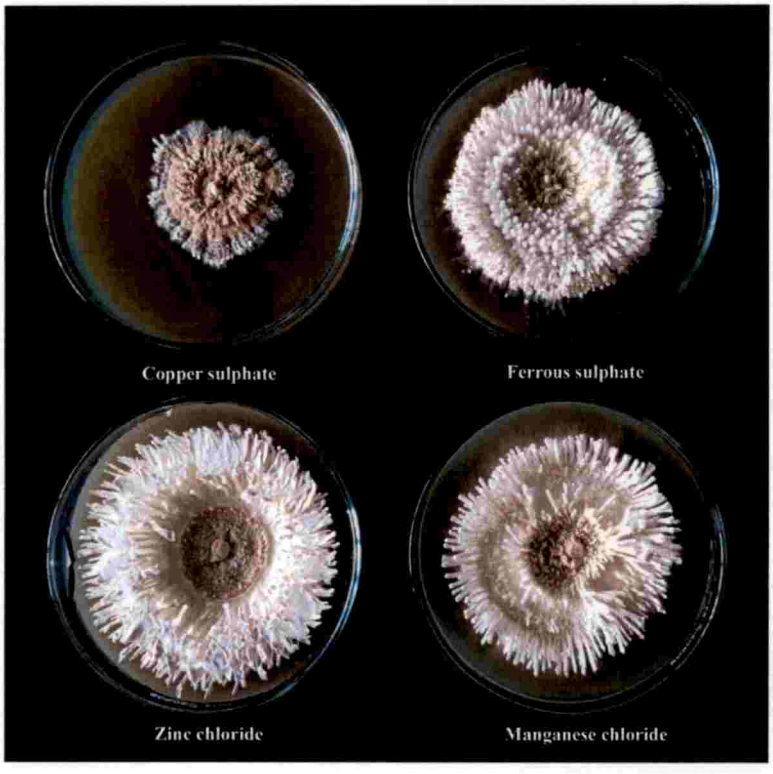


Plate 34. Effect of micro mineral sources on mycelial growth of *O. neovolkiana*

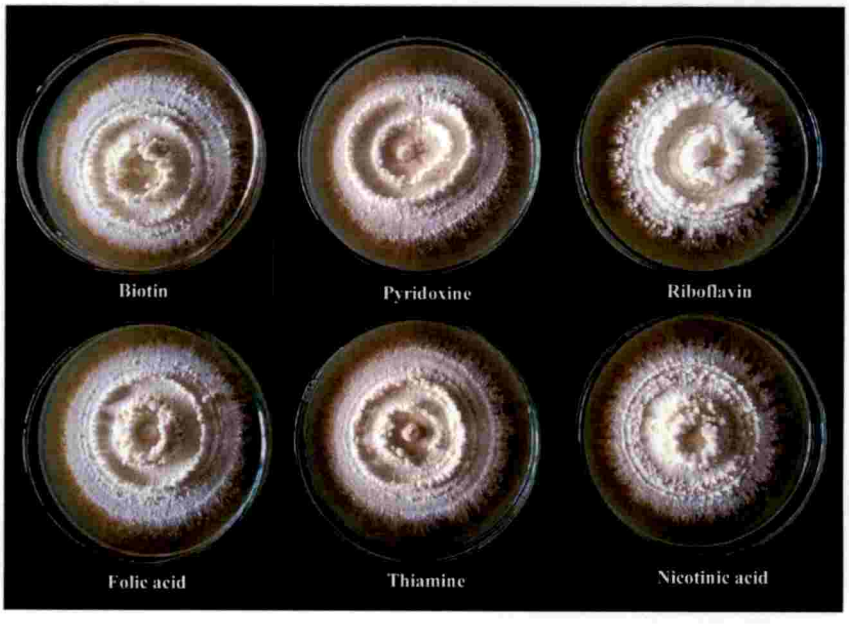


Plate 35. Effect of vitamins on mycelial growth of *O. neovolkiana*

Table 15. Effect of Vitamins on mycelial growth of *O. neovolkiana*

Sl. No	Treatments	Colony diameter(cm)* DAI			
		10	**20**	**30**	**37**
1	Riboflavin	3.60^{c}	5.55^{d}	6.85^{d}	7.80^{f}
2	Thiamine	4.08^{c}	6.28^{c}	7.23^{c}	8.40^{c}
3	Folic acid	5.63^{a}	7.23^{a}	8.13^{a}	8.93^{a}
4	Pyridoxine	3.85^{c}	6.05^{c}	7.30^{c}	8.20^{d}
5	Nicotinic acid	5.08^{b}	6.03^{c}	7.03^{d}	8.05^{e}
6	Biotin	3.88^{c}	6.60^{b}	7.60^{b}	8.65^{b}
	CD(0.05)	0.496	0.316	0.213	0.118

*Mean of four replications. In each column figures followed by same letter do not differ significantly according to DMRT

4.3.5.2. Mycelial weight of O. neovolkiana

Mycelial weight of the fungus in the broth of the three media (yeast extract potato fructose broth: YEPFB, yeast potato dextrose broth: YPDB and potato dextrose broth: PDB) were observed and the results showed the highest mycelial weight of *O. neovolkiana* was observed in YEPFA *i.e.*, of 23.61 mg in 32.57 days after inoculation, which was significantly higher than the other two media (17.90 mg in YPDB and 15.51 mg in PDB). The number of days taken for complete growth of the fungus was 32.57 days in YEPFB which was superior to the other two media which were 54.85 days after inoculation in YPDB and 58.15 days after inoculation in PDB (Plate 37)

The above results benefitted in reducing the growing time of *O. neovolkiana* upto 24.3 and 25.58 days in the newly formulated solid and liquid media respectively, under *in vitro* culturing (Table 16).

4.4. ARTIFICIAL CULTURING OF *Ophiocordyceps* sp.

Different cereal grains *viz.*, rice, wheat and sorghum were tried for artificial culturing of *Ophiocordyceps* sp. fungus under *in vitro* conditions. It was found out that sorghum grains was the best substrate for mycelial growth followed by wheat and rice. The number of days taken for coverage of the mycelium in 50g of grains was 96.43 days for sorghum which was significantly different from rice (115.15 days) and wheat (142.15 days) (Table 17) (Plate 38).

All the above findings showed that the experiments for the artificial cultivation of *O. neovolkiana* are noteworthy and it is a requisite for getting more valuable products

Table 16. Comparison of solid and liquid media for mycelial growth of *O. neovolkiana*

Sl. No	Treatments (solid medium)	Colony diameter(cm)* 31 DAI	No. of days to complete growth	Treatments (Liquid medium)	Weight of mycelium (g)* 32 DAI	No. of days to complete growth
1	YEPFA	8.95[a]	30.85[a]	YEPFB	23.61[a]	32.57[a]
2	YPDA	8.35[b]	50.71[b]	YPDB	17.90[b]	54.85[b]
3	PDA	6.49[c]	55.15[c]	PDB	15.51[c]	58.15[c]
CD(0.05)	-	0.205	1.698	-	1.313	2.386

*Mean of seven replications. In each column figures followed by same letter do not differ significantly according to DMRT

Table 17. Effect of different substrates on mycelial growth of *O. neovolkiana*

Sl. No	Treatments	No of days to complete mycelial growth*
1	Wheat grains	142.15[c]
2	Rice grains	115.15[b]
3	Sorghum grains	96.43[a]
	CD(0.05)	3.901

*Mean of seven replications. In each column figures fillowed by same letter do not differ significantly according to DMRT.

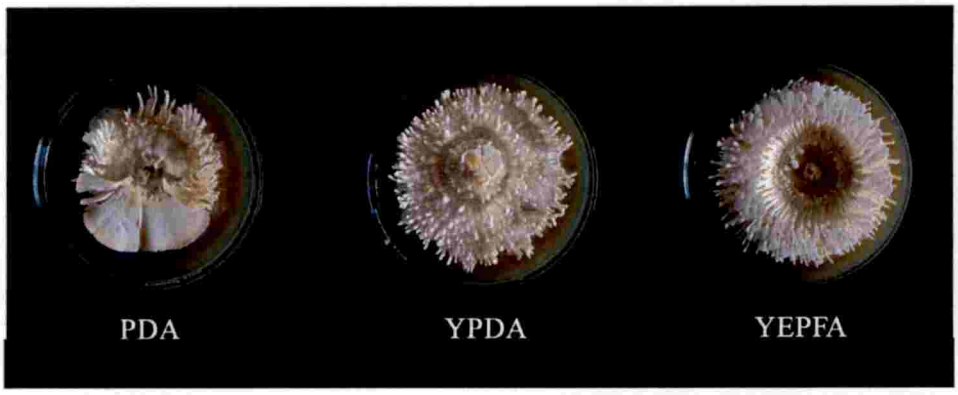


Plate 36. Comparison of growth of *O. neovolkiana* in YEPFA, PDA and YPDA

Plate 37. Comparison of growth of *O. neovolkiana* in YEPFB, PDB and YPDB

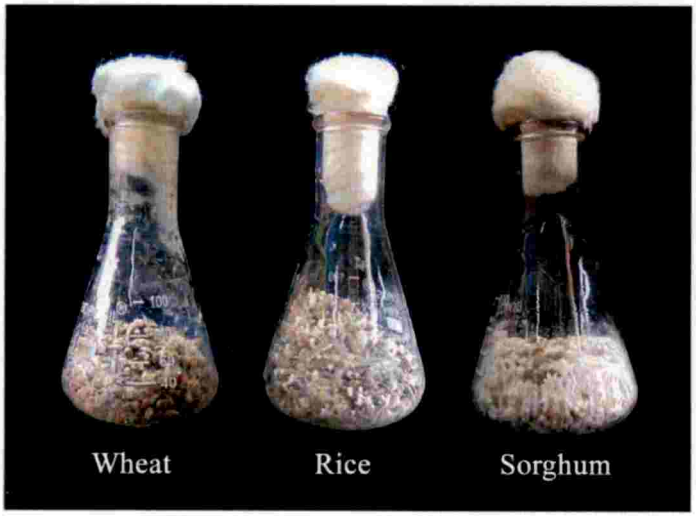


Plate 38. Effect of different substrates on mycelial growth of *O. neovolkiana*

4.5. PROXIMATE ANALYSIS OF *Ophiocordyceps* sp.

Analysis of proximate constituents like carbohydrate, protein, fibre content, total minerals, moisture, ash content and vitamin C were compared using the collected fruiting body and mycelial growth obtained on YEPFB. The carbohydrate content was found to be 5 per cent and 9.7 per cent in fruiting body and mycelium respectively. Protein content was estimated using Lowry's method and found to be 2.9 per cent and 1.75 per cent in fruiting body and mycelium respectively. Crude fibre was more in mycelium (25%) than fruiting body (16.8%). Moisture content was 76.8 per cent in fruiting body and 27.6 per cent in mycelium which was very less than the moisture content of fruiting body. There was not much variation in the ash content which was found to be 1.96 per cent and 2.1 per cent in fruiting body and respectively. The ascorbic acid content was found to be 3.27 per cent in fruiting body and 2.5 per cent in mycelium (Table 18).

Analysis of proximate constituents in the collected fruiting body and mycelial growth obtained under *in vitro* culture showed that most of the parameters were comparable between the fruiting body and mycelium and the less moisture content in the mycelium increase the shelf life also. Therefore the mycelia obtained under *in vitro* culture may be used as an alternative to the fruiting body since the population of *O. neovolkiana* is decreasing in the natural habitat and also it is very difficult to produce the fruiting body under *in vitro* conditions

Table 18. Proximate analysis of Fruiting bodies in the field and cultivated mycelial mat of *O. neovolkiana*

Sl. No.	Parameter	* Value in per cent	
		Fruiting body	Mycelium
1	Carbohydrate	5	9.7
2	Protein	2.9	1.75
3	Crude Fibre	16.8	25
4	Moisture	76.8	27.6
5	Ash content(Total minerals)	1.96	2.1
6	Ascorbic Acid(Vitamin C)	3.27	2.5

*Mean of four replications

Discussion

5. DISCUSSION

Cordyceps sinensis, a well-known and valued traditional Chinese medicinal mushroom, also called Dong Chong Xia Cao (winter worm summer grass) is a parasitic complex of fungus and caterpillar of *Hepialis armoricanus,* family Hepialidae, which has been used for medicinal purposes for centuries in China, Japan and other Asian countries. It is also called as Himalayan gold or Himalayan viagra. The coconut root grub (*Leucopholis coneophora* Burm.) is a serious pest of coconut found in sandy loam tracts of northern districts of Kerala. The natural incidence of *Cordyceps* has been found on the root grubs during June to September period in these regions and no attempts were taken to identify, and document the morphological, cultural, physiological, phylogenetic and nutritional characters of this fungus due to the constraints in the isolation process where contamination of a large number of incorrect fungi occurs. Therefore this work of 'Characterization of medicinal mushroom, *Cordyceps* sp. from Kasargod district' is a first attempt to isolate, identify, characterize and analyse the nutritional status of this fungus found in Kerala. The genera *Cordyceps* which attack Coleoptera group of insects were transferred to the genus *Ophiocordyceps* by Sung *et al.* in 2007, and therefore in the present study the genus of the target fungus is renamed as *Ophiocordyceps*. The studies were carried out as per the technical programme during the period 2016-2018 at the Department of Plant Pathology, College of Agriculture, Padannakkad, Kasargod and College of Horticulture, Vellanikkara, Thrissur, Kerala and the results are discussed in this chapter.

The fungus *Ophiocordyceps* was observed in coastal sandy tracts of Kasargod district during June to September months. Purposive sampling surveys were conducted in three selected locations *viz.*, Instructional Farm, College of Agriculture, Padannakkad, Valiyaparamba area of Nileshwar and Regional Agricultural Research Station, Pilicode of Kasargod district during 2017 and 2018.

The period of occurrence of this fungus have similarity with reports of other scientists in different countries. It was reported that the best time of collection of *Cordyceps sphecocephala* specimens were from July to September in Japan (Kobayasi and Shimizu, 1980) and in Korea (Nam *et al.*, 2006).

The specimens were collected from three locations and the population of fruiting bodies in one square metre area was assessed. The survey revealed that maximum number of fruiting bodies were found in the Instructional Farm, College of Agriculture, Padannakkad under cashew trees (average of 13.86 m^{-2}) (Table 1). The organic matter content was higher (1.39%) in this area compared to other two areas. Due to high organic matter content, the water holding capacity of soil in this area will be higher, favouring the spore germination and survival of the fungus. Along with this the shaded condition under the cashew trees might also favour the presence of higher number of fruiting bodies in this area. (Table 2, Plate 1)

The identity of *Ophiocordyceps* at species level was already proved by many workers. Cock *et al.* (2013) reported that *O. barnesii* infects third instar larvae of sugarcane root grub (*Brachylepis werneri*) in Southwestern Somalia and Sanghdee *et al.* (2013) observed that *Ophiocordyceps longissima* infects cicada larva in Northeast Thailand. Surveys conducted by Kumar and Aparna (2014) in coastal sandy areas of Kasargod district, reported the presence of *Cordyceps* sp. attacking the coconut root grubs (*Leucopholis coneophora* Burm.) during June to September months, which was a similar observation as in the present study.

The low population density in Valiaparamba and Pilicode areas might be due to the lesser quantity of organic matter along with low calcium, magnesium, iron and potassium (Table 2). The justification behind this situation was the disturbances in the upper layer of soil by the use of machineries, and intense cultivation practices. These observations showed that forest type of ecosystem with undisturbed soil having high organic matter is necessary for emergence of this fungus favouring the

germination and survival of spores. These results were also in line with the findings of Sridhar *et al.* (2017) where the occurrence of *O. nutans* associated with the stinkbug *Halyomorpha halys* of *Cassine glauca* was found in high altitude forests of Western Ghats.

The fungus was isolated on PDA medium from the fruiting bodies collected during survey and the mycelia appeared initially as white, later turning to creamish white to salmon colour and the underside of the plate being light brown representing the anamorphic stage, *Hirsutella* sp. In later stages thread like pinkish synnemata was emerged in the culture (Plate 3 and 4). This showed that the Potato Dextrose Agar, which is the most widely used medium for fungal isolation can be used for isolation of *Ophiocordyceps.* Isolation in PDA was already reported by many scientists like Sung *et al.* (2011), Sangdee and Sangdee (2013) and Ko *et al.* (2017).

The cultural characters like colour, shape, texture and the growth rate of the mycelium were studied in five different media. Among which, yeast potato dextrose agar media (YPDA) was found to be the best medium followed by potato dextrose agar medium (Table 3, Plate 5). In yeast potato dextrose agar, the fungus showed initially a white coloured growth with zonations then turned to creamish white colour and produced creamish to salmon coloured synnemata with honey dew like conidial mass at first at the centre later in the peripheral parts also. In PDA the fungus initially grow as a white colony later turning to creamish white with creamish synnemata at the centre. Similar observations were obtained by Holliday and Cleaver (2004) where the colour of the mycelium of *C. sinensis* was initially white and later on, densely matted and appeared as orange-brown to tan in colour. The above results are also in accordance with the observations of Arora *et al.*, (2013), where he reported that colony of *C. sinensis* on sabouraud's dextrose agar with yeast extract (SDAY) was initially cream with lined depressions, later dark orange and the reverse side of the culture showed dark tan in colour. Reports by Sangdee and Sangdee

(2013) in Thailand also showed the growth of *Ophiocordyceps longissima* as cottony white cream colonies.

Yeast potato dextrose agar medium (YPDA) was found to be the best medium for the growth *Cordyceps* sp. followed by potato dextrose agar media (PDA). The preference of yeast extract by *Cordyceps* sp. may be attributed to the presence of mixture of amino acids, peptides, vitamins and carbohydrates. (Sorensen and Sondergaard, 2014)

The fungus completed the growth in 52 days in 9 cm Petri plates in YPDA medium and 55 days in PDA medium. Least growth was obtained in OMA medium by taking 60 days for the completion of growth (Table 4) (Figure 4). Result of present study is analogous to the observations of Pathania *et al.* (2015) and Sehgal *et al.* (2006), where among the different solid media tried for the growth of *Cordyceps militaris,* yeast potato dextrose agar was found to be the best solid medium. Other researchers like Arora *et al.* (2013) observed that SDAY was the best medium with maximum mycelial growth. Regarding the growth rate of fungus in Petri dishes, Nam *et al.* (2006) obtained 32 mm growth on potato dextrose agar (PDA) within 30 days under 24 ± 1^{0}C for *C. sphecocephala*. Amin *et al.* (2008) also found that *C. sinensis* completed its full growth in PDA medium within 47 days.

In the morphological studies of the fruiting bodies of *Ophiocordyceps* sp. the bulged tip portion was observed as stroma and stalk like structure as stipe. Sclerotium was the mummified grub which was completely filled with mycelia of the fungus. The tips of perithecia were found as numerous humps which are initially orange red in colour turning to dark brown (Plates 10 and 11). These findings were having similarity with observations of other researchers. The morphological characters of *O. sinensis* was described by Shrestha *et al.* (2010) as it consists of two parts, a fungal endosclerotium (caterpillar) and stroma which was initially yellow in

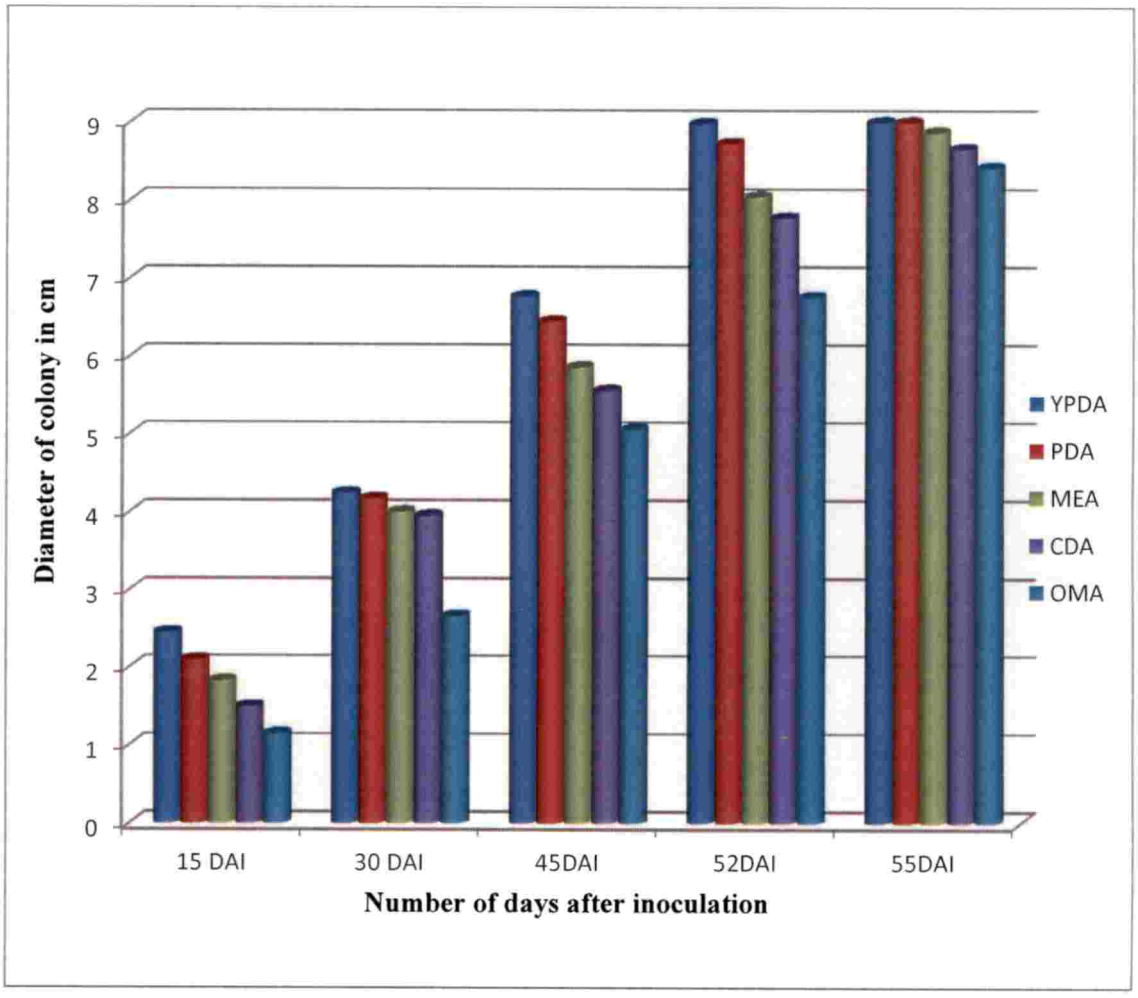


Figure 4. Effect of different medium on mycelial growth of *Cordyceps sp.*

colour later turning to dark brown or black colour, which was longer than the caterpillar itself, usually 4 –10 cm. Sung *et al.* (2010) also reported the morphological characters of the fruiting body of *O. sinensis.* They noted that fruiting body grew singly from the larval head, and was clavate, sub-lanceolate or fusiform and distinct from the stipe, which was slender, glabrous, and longitudinally furrowed or ridged. Lo *et al.* (2013) also reported similar observations that *O. sinensis* had dark pigments and tough to pliant stromata.

The microscopic studies of the asexual stage of *Cordyceps* were conducted in detail in the present study. Observations on hyphae, conidiophores and conidia were taken. The hyphae of *Cordyceps* were hyaline, septate and branched with a width of 1.1 - 1.52 µm (Table 6, Plate 13). Chen *et al.* (2006) observed similar characters for the hyphae of *C. sinensis.* They noted hyphae as ramose, septate and hyaline. Arora *et al.* (2013) also reported that the hyphae of *Cordyceps sinensis* as aerial, cottony white to creamish or yellowish, septate, branched and fast growing. Pathania *et al.* (2015) reported that in *Cordyceps militaris* the hyphae was thin walled, branched around 14.8 - 16.1 µm broad.

In the present study, the conidia of the anamorph were formed either singly or in chains on conidiophores. The conidiophores were observed as single or branched arising from synnemata. The conidia were hyaline, dimorphic having two shapes spherical and spindle shaped measuring 1.9 to 5.7 µm diameter and 2.5 to 6.3 µm length and width of 0.53 to 2.41µm (Plates 17 and 19). Shin *et al.* (2004) also got matching observations that the imperfect forms of *Ophiocordyceps* produced spherical or spindle shaped conidia on phialides. Shreshta *et al.* (2005) reported that in *Cordyceps militaris*, conidia produced directly from the tips of germinating hyphae of part-spores within 36 hours after ascospore discharge, exhibiting microcyclic conidiation. The shape of the first formed conidia was cylindrical or clavate followed by globose and ellipsoidal ones. The characteristic features of asexual stage of

C. militaris mentioned by them were microcyclic conidiation, undifferentiated conidiogenous hyphae (phialides) which were solitary or opposite to whorled type in arrangement and polymorphic conidia. Erect, hyaline, glabrate, conidiophores having one or two ramification and simplicial or acrogenous phialides were the observations of Chen *et al.* (2006) in *Coryceps sinensis*. According to the study by Pathania *et al.* (2015) *Cordyceps militaris* had barrel shaped smooth to velvety conidia on sub cylindrical conidiophores with flask shaped phialides and the hyphae was thin walled, branched around 14.8-16.1 µm broad.

Microscopic examination of thin cross sections of stroma in the present study showed numerous perithecia towards the peripheral regions (Plates20 and 21). Perithecia were peripheral to slightly immersed globular to oval in shape, narrow towards the top and having ostioles at the tip. The length of perithecia was 82.6-396.1 µm and the width was 93.6-171.27 µm. Numerous elongated asci containing filiform ascospores which were four-partite and eight in numbers were observed within the perithecia (Plates 26). The size of ascus was about 115.4-170.11 × 5.2-10 µm and the ascospore was 105.3-135.7 × 2.51-2.73 µm. Characters of the perithecium in *Ophiocordyceps* reported by many scientists are in accordance with the above observations. Gwangpo (2000) noted the perithecium of *C. sinensis* as either oval - shaped or egg - shaped, consists of numerous numbers of thin, long ascus and ascospores. Shin *et al.* (2004) also observed that the apex of the stroma contains numerous perithecia with several ascospores inside asci. The ascospores were characterized by a thread like structure in the middle with non disarticulating part spores attached on both sides. Sung *et al.* (2007), observed perithecia of *Ophiocordyceps* as superficial to completely immersed, ordinal or oblique in arrangement. Asci were hyaline, cylindrical and ascospores were cylindrical, multiseptate, disarticulating into part-spores or non-disarticulating. Observations of Xie *et al.* and Zhong *et al.* (2010) were also similar to the above study, where they found thread like ascospores in ascus. Microscopic observations

on by Sung *et al.* (2010) in *Cordyceps cardinalis* observed that the perithecia were ovoid in shape and semi-immersed on the stroma and the ascospores were unevenly septate but do not disarticulate into part-spores. According to Shrestha *et al.* (2010) the characters of perithecium, ascus and ascospores in the fruiting bodies were the key identification characteristics of *Ophiocordyceps.* They also got similar findings *viz.*, the perithecia were ovoid and the asci were cylindrical or slightly tapering at both ends, straight or curved, with a capitate and hemispheroid apex and two to four spored. Similarly, ascospores were hyaline, filiform, multiseptate having a length of 5 - 12 µm and sub - attenuated on both sides. Analogous observations were reported by Arora *et al.* (2013) as the perithecia of *C. sinensis* were oval - shaped or egg shaped filled with numerous elongated, unitunicate, capitate, cylindrical and hyaline ascus.

Molecular identification of the isolate was done from Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum by comparison of ITS sequences to identify at species level. Sequence analysis of the culture showed homology with *Ophiocordyceps neovolkiana* strain KC1 having 98 per cent identity. *O. neovolkiana* strain KC1 was reported from Tamil Nadu by Sangeetha and Krishnamoorthi in 2017 (NCBI Gen Bank: http:// ncbi.nlm.nhm.gov/blast). Hence the present study was the first report of *O. neovolkiana* from Kerala. Regarding the molecular characterization of *Cordyceps* sp. efficient and reliable studies were undertaken by various scientists. Identification of *Cordyceps sinensis* by PCR-Single-Stranded Conformation Polymorphism and phylogenetic relationship was done by Kuo *et al.* (2005). But as per the classification done by Sung *et al.*(2007) where the mega genus *Cordyceps* was divided into four genera *viz.*, *Cordyceps*, *Metacordyceps*, *Ophiocordyceps* and *Elaphocordyceps.* The entomopathgen obtained from coconut root grub in the present study can be included in the genera *Ophiocordyceps i.e.*, *Ophiocordyceps neovolkiana (*Kobayasi). For the phylogenetic tree analysis in the present study, findings of Wang *et al.* in 2012 were reviewed.

Different physiological characters of *Ophiocordyceps* sp. were also studied under *in vitro* conditions. Evaluation of favourable conditions for mycelial growth of *Ophiocordyceps* was conducted with eight different temperature conditions *viz.*,5°C, 10°C, 15°C, 20°C, 25°C, room temperature (26 ± 2°C), 30°C and 35°C in yeast potato dextrose agar. It was observed that 30°C was found to be the optimum temperature in which the growth was completed within 38 days after inoculation. At 5°C and 10°C, mycelial growth was absent. From 15°C onwards growth was found increasing and a decrease in growth at 35°C, which proved that 30°C is the optimum temperature for growth of *O. neovolkiana* in YPDA medium (Figure 5). Since, most of the *Ophiocordyceps* sp. are growing in temperate climate, 25°C is the preferred temperature for their growth. The tropical condition in Kerala may be the reason for the temperature preference of 30°C by the *Ophiocordyceps* sp. attacking the root grubs in the present study. The soil temperature at 5-7cm depth and the body temperature of the root grub were measured and they were in a range of 29-32 °C. These factors also confirmed the temperature preference for the growth of *O. neovolkiana.*

For the evaluation of the effect of pH on the mycelial growth of *Ophiocordyceps neovolkiana* thirteen different pH levels were tried *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 and 10 in YPDA medium. The pH seven was found to be optimum for mycelial growth where, the number of days taken for the growth was 35 after inoculation. There was gradual reduction in growth for pH levels above 7 and below 7 (Figure 6). The pH of the hemocoel of insect body is usually 6–7.5 (Buck, 1953). This may be the reason for the pH preference of the *Ophiocordyceps neovolkiana* as seven. The optimum pH level for fungal growth of *Cordyceps nutans* was pH 7.0 (Kirk *et al.*, 2001). Sasaki *et al.* (2005) observed the mycelial growth of three strains of *Cordyceps nutans* Pat. and found that growth was significantly more in neutral to weak alkaline pH levels (pH 7.0 - 9.0) compared with acidic pH levels. Mycelial growth at a temperature of 25°C and a pH of 7 in

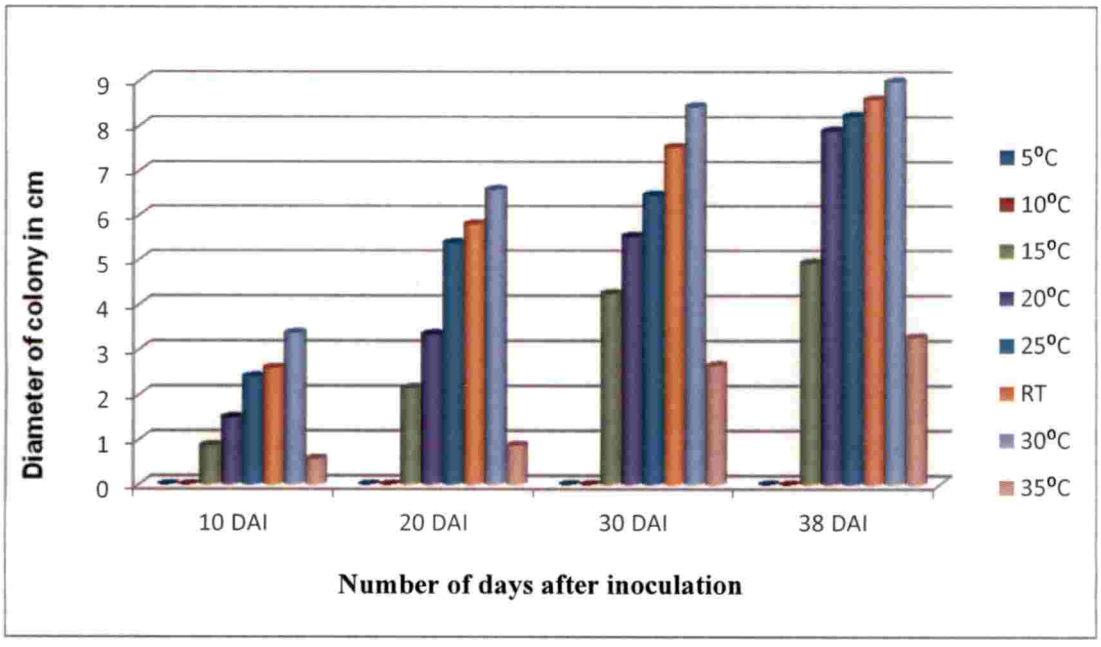


Figure 5. Effect of temperature on mycelial growth of *O. neovolkiana*

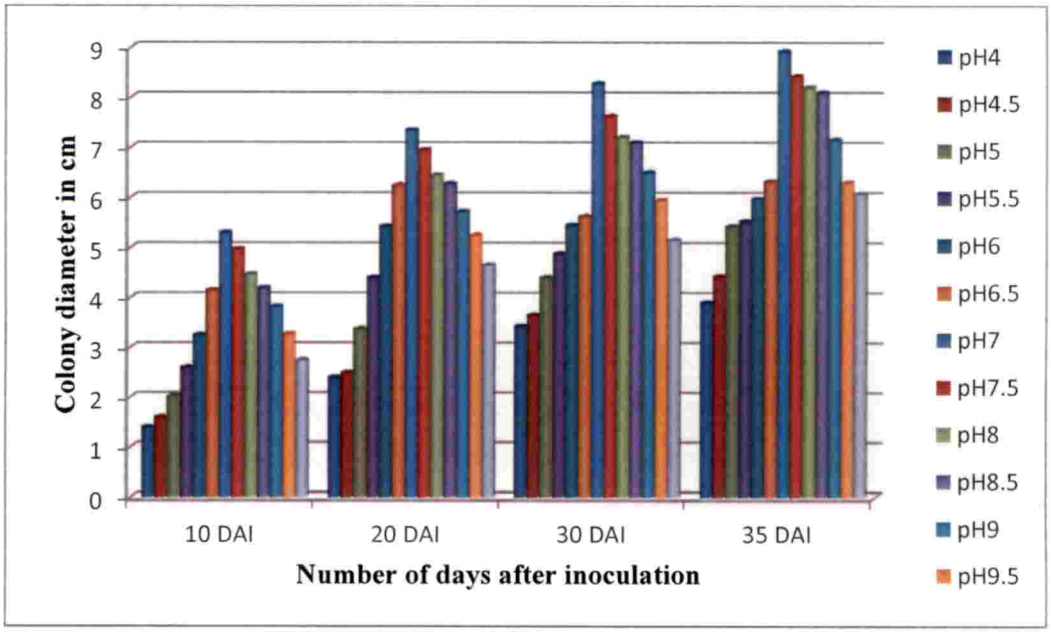


Figure 6. Effect of pH on mycelial growth of *O. neovolkiana*

in *C. cardinalis* was observed by Sung *et al.* (2010). Sung *et al.* (2011) also got similar observation that for the growth of *Ophiocordyceps heteropoda* from Korea, where mycelial growth was best at 25°C. Mei *et al.* (2013) reported that for the growth of *Ophiocordyceps sinensis,* the ideal temperature range is 4 - 25°C. The best mycelial growth of *C.militaris* was observed at 25°C in yeastal potato dextrose agar medium with optimum level of pH 7.5 (Pathania *et al.* 2015; Sehgal *et al.*2006). 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 in *Cordyceps militaris* (Adnan *et al.,* 2017).

To evaluate the effect of light and darkness on growth of *O. neovolkiana*, four different conditions of light and darkness was tested *viz.*, 24 h of light under room temperature (27 ± 2°C), 24 h of light under incubator (30°C), 24 h of darkness under incubator (30°C) and 24 h of darkness under room temperature (27 ± 2°C). Among these situations, 24 h of darkness under incubator was preferred by *O. neovolkiana* followed by 24 h of darkness under open condition. Under darkness in incubator the fungus completed the growth within 35 days after inoculation (Figure 7). Preference of darkness for the growth was recorded by many researchers. Sehgal *et al.* (2006) got maximum mycelial growth in yeast potato dextrose agar by *C.militaris* under darkness in comparison to light. Sung *et al.* (2011) also got identical observation that for the growth of *Ophiocordyceps heteropoda* from Korea, the growth rate was faster in the dark than the light, but mycelial density was less compact in the dark. Pathania *et al.* (2015) also noted similar results that the mycelial growth of *C.militaris* was found to give better growth under darkness in comparison with light. Situation is entirely opposite in nature, where light is necessary for the development of fruiting body showing a positive phototropism. This character is evident in the movement of coconut grub, when it moves upwards from the inner layers of soil after parasitization by the fungus and also from the elongation of fruiting body toward the direction of light. According to Zhang *et al.* (2017), light is a

necessary environmental factor for production of conidia, pigment and formation and development of stroma in *Cordyceps militaris.*

Four different carbon sources were used to study the effect on growth of mycelium of *Ophiocordyceps neovolkiana viz,* dextrose, sucrose, fructose and maltose. Results showed that fructose was the best source of carbon in which maximum mycelial growth of *Ophiocordyceps* sp. was obtained within 36 days after inoculation. The second best carbon source was sucrose followed by maltose and dextrose (Figure 8). While reviewing the various carbon sources required for the growth of *Ophiocordyceps* sp. effect of fructose was observed by some of the scientists.Sung *et al.* (2011) observed that for the growth of *Ophiocordyceps heteropoda* from Korea, different carbon sources such as dextrin, saccharose, starch, lactose, maltose, fructose, and dextrose resulted in better growth.

But the second best carbon source in the present study, sucrose was reported by many researchers as a good promoter of growth in many *Cordyceps* sp. Dong and Yao (2005) in *Cordyceps sinensis* and Sehgal *et al.* (2006) and Pathania *et al.* (2015) in *C.militaris* obtained maximum growth of mycelium in sucrose. Singh *et al.* (2014) also found that mycelium yield under *in vitro* culture of *Ophiocordyceps sinensis* was significantly higher on sucrose.

The effect of different nitrogen sources on the mycelial growth of *Ophiocordyceps neovolkiana* was studied and results showed that yeast extract was the best source of nitrogen in which the fungus completed growth within 35 days after inoculation. The second best nitrogen source was beef extract followed by potassium nitrate and ammonium nitrate (Figure 9). Shin *et al.* (2004) observed that *Cordyceps pruinosa* showed maximum mycelial growth in the medium with yeast

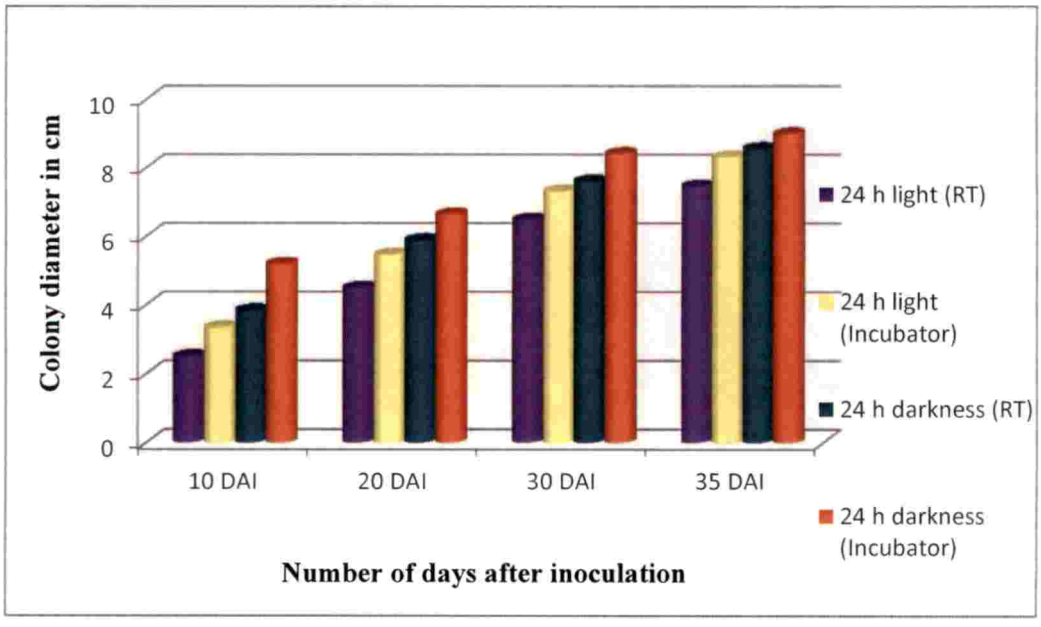


Figure 7. Effect of light and darkness on mycelial growth of *O. neovolkiana*

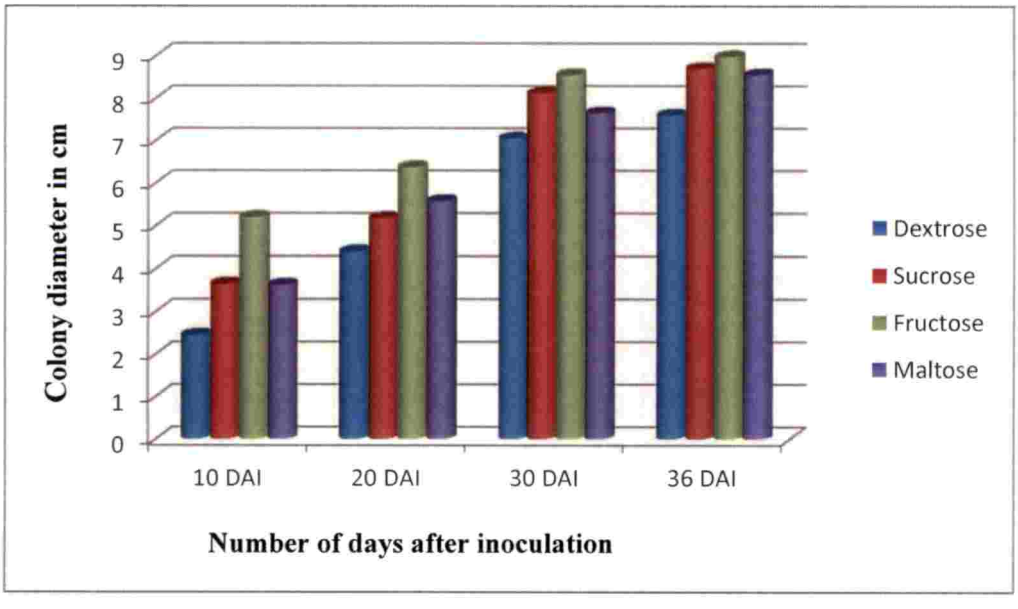


Figure 8. Effect of carbon sources on mycelial growth of *O. neovolkiana*

extract. Dong and Yao (2005) attained similar result in *Cordyceps sinensis* with yeast extract at 3 g /L. For the growth of *Ophiocordyceps heteropoda* from Korea Sung *et al.* (2011) also got similar observation that yeast extract and peptone were best sources for the most effective growth. Singh *et al.* (2014) observed that maximum growth of *Ophiocordyceps sinensis* was obtained in the medium with yeast extract.

Different sources of macro minerals were tested to find effect of on the mycelial growth of *O. neovolkiana* and results showed that KH_2PO_4 was the best source of macro mineral in which the fungus completed growth within 38 days after inoculation. The second best macro mineral source was $CaCl_2$ followed by K_2HPO_4 and $NaCl_2$ (Figure 10). These results were in accordance with the observations of He *et al.* (2016) where the yield of *Ophiocordyceps sinensis* was maximum in medium containing glucose (40 g/L), yeast powder (65 g/L), KH_2PO_4 (1.5 g/L).

The study conducted to find out the effect of different sources of micro minerals on the mycelial growth of *O. neovolkiana* showed that $ZnCl_2$ was the best source of micro mineral for the growth of this fungus, in which it completed the growth within 35 days after inoculation. The second best micro mineral source was $MnCl_2$ followed by $FeSO_4$ and $CuSO_4$ (Figure 11). In all micro nutrients and macro nutrients tested for the growth of *Ophiocordyceps sinensis*, Singh *et al.* (2014) found out that the growth obtained in calcium chloride and zinc chloride respectively were significantly higher than other variables used.

The effect of different sources of vitamins on the mycelial growth of *O. neovolkiana* was studied and it was found that among six sources of the vitamins used, folic acid was the best source of vitamin for the growth, in which the fungus completed the growth within 37 days after inoculation. The second best micro mineral source was biotin followed by thiamine, pyridoxine, nicotinic acid and riboflavin (Figure 12). Among the vitamin sources tested by Singh *et al.* (2014) yield of *Ophiocordyceps sinensis* obtained with folic acid was significantly higher than the

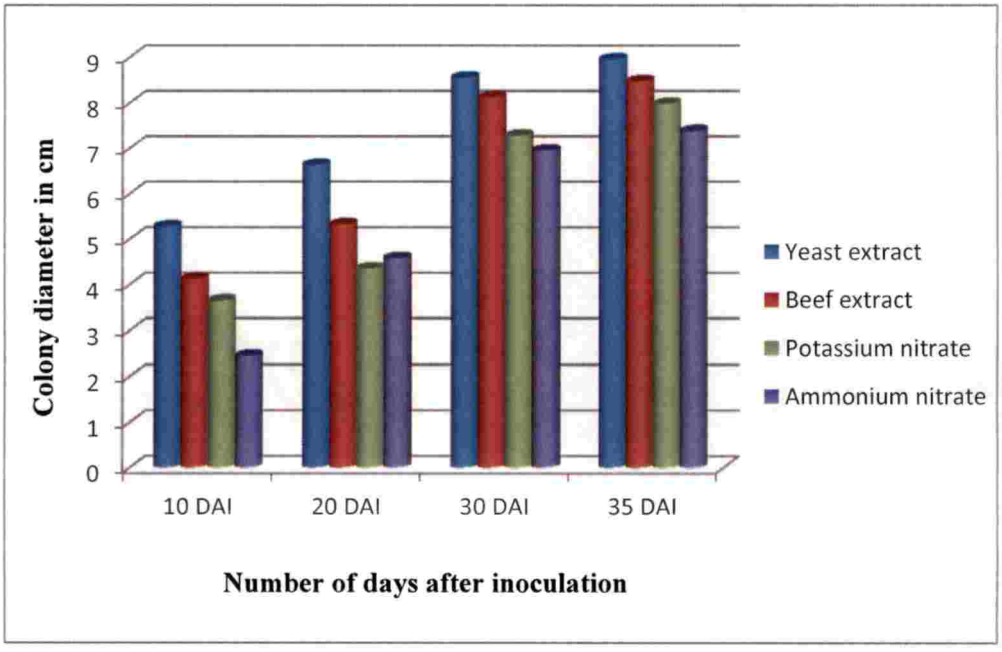


Figure 9. Effect of nitrogen sources on mycelial growth of *O. neovolkiana*

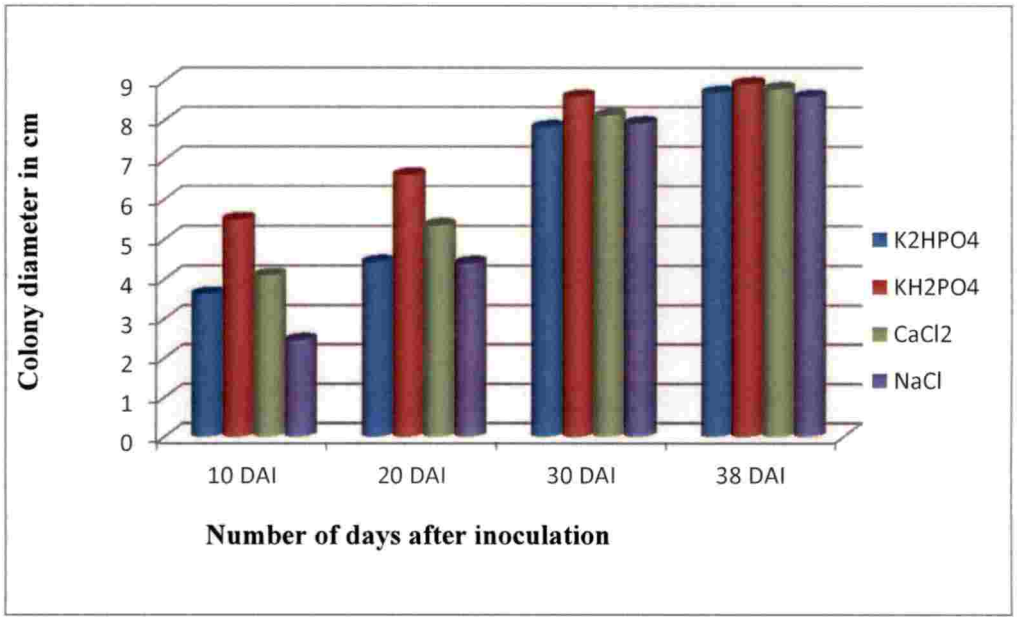


Figure 10. Effect of macro mineral sources on mycelial growth of *O. neovolkiana*

Other vitamins *i.e.*, zinc chloride and folic acid produced the maximum mycelial yield of *Cordyceps militaris*.

After completing all the above experiments, one optimum medium for the growth of *Ophiocordyceps* sp. was standardized. This standardized medium was named as yeast extract potato fructose agar (YEPFA) medium. Which contains 300g of potato, 20 g of fructose, 5 g of yeast extract, one gram of KH_2PO_4, 500 mg of $ZnCl_2$, 10 mg of folic acid and 20g of agar in one litre of distilled water.

By analyzing the soil test data, considerably highest values of organic carbon, potassium, manganese and calcium were observed in the soil under cashew trees of Instructional farm, Padannakkad compared to Valiaparamba and Pilicode. This may be reason for incidence of higher population of fruiting bodies in the soil of cashew plantation, which is rich in organic matter, remain undisturbed and having shaded conditions. The *in vitro* experiments conducted above showed the preference of this fungus to potassium, manganese and calcium. The higher rate of potassium, manganese and calcium in the soil under cashew plantations may favour the population of *Ophiocordyceps* because these conditions may favour the spore germination and survival.

The growth of *Ophiocordyceps neovolkiana* in the basic medium PDA, the medium obtained in the selection of best media *i.e.*, YPDA and the optimum medium formulated, YEPFA both in solid and liquid media were compared. The results showed that the growth of *Ophiocordyceps* in YEPFA medium was significantly higher than YPDA and PDA where the fungus completed the growth in 30.85 days in 9 cm Petri dish. The standardized media, YEPFA significantly reduced the time taken for growth of *Ophiocordyceps* from 55.15 days to 30.85 days, which is a noteworthy achievement of this project (Figure 13). The mycelial weight of the fungus obtained from the broth of the optimum medium (YEPFB) was higher than the other two media (YPDB and PDB) which were 23.61 mg, 17.90 mg and 15.51 mg respectively (Figure 14). From this It was confirmed that YEPFA is the best medium for

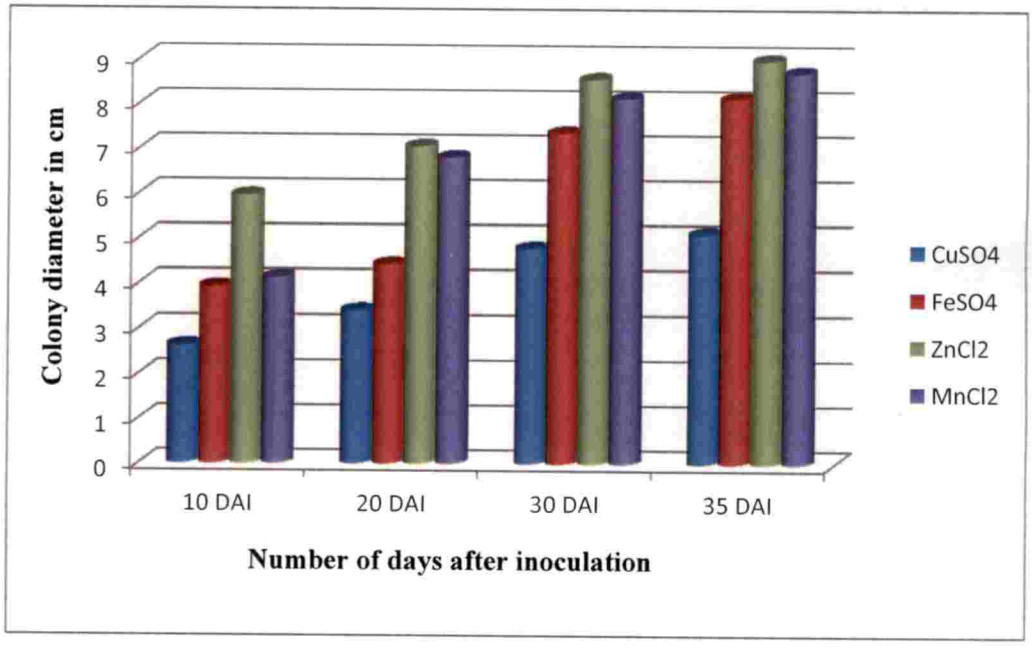


Figure 11. Effect of micro mineral sources on mycelial growth of *O. neovolkiana*

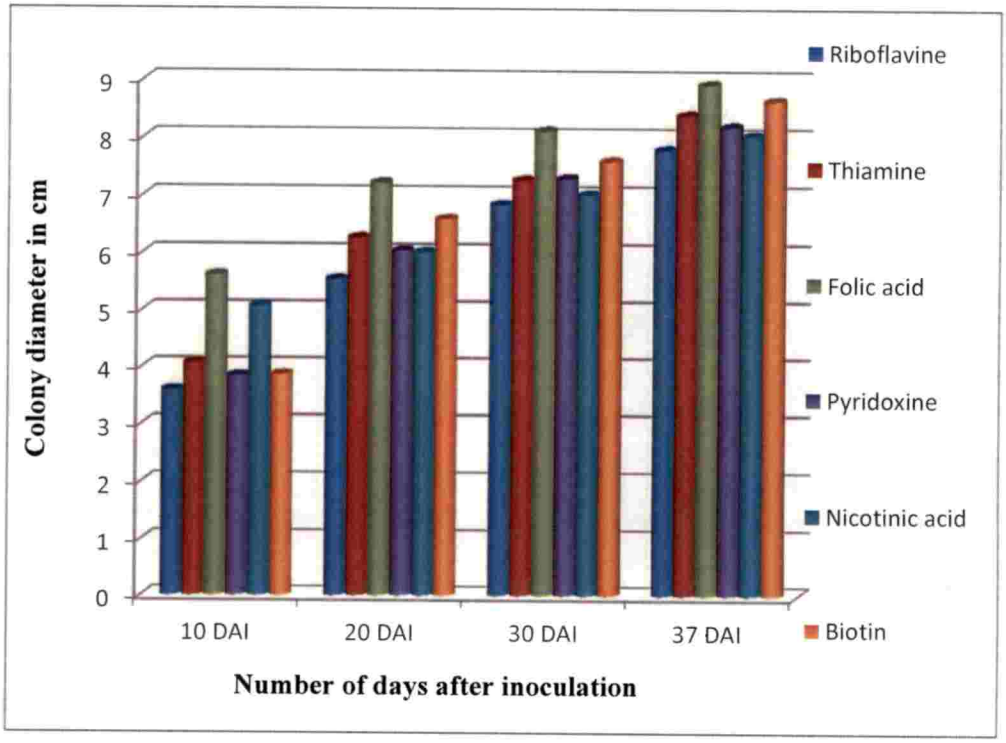


Figure 12. Effect of vitamins on mycelial growth of *O. neovolkiana*

mycelial growth of *Ophiocordyceps* sp. Since the laboratory-grown *Ophiocordyceps* mycelia have similar clinical efficacy as those grown in the wild and less associated toxicity as per the observations of Liang (2007), the higher mycelial weight obtained in YEPFA medium in the experiment is also a remarkable success in the field of research in modern medicine.

Different cereal grains *viz.*, rice, wheat and sorghum were tried for artificial culturing of *O. neovolkiana* under *in vitro* conditions. It was found out that sorghum grains was the best substrate for mycelial growth followed by wheat and rice. The number of days taken for coverage of mycelium in 50 g of grains was 96.43 days for sorghum, which was significantly different from rice (115.15 day) and wheat (142.15 days) (Figure 15). Kim *et al* (2010) observed that 50 - 60 g of brown rice and 10 - 20 g of silk worm pupa mixed with 50 - 60 ml of water with liquid inoculums in 1,000 ml polypropylene (PP) bottle incubated at 25°C temperature under continuous light was found to be optimum conditions for fruiting body production of *Cordyceps cardinalis*.

Comparison of pxoximate analysis of sporocarps and mycelium of *O. neovolkiana* was done. Analysis of constituents like carbohydrate, protein, fibre content, total minerals, moisture, ash content and vitamin C were conducted. The carbohydrate content was more in mycelium than in fruiting body. (9.7% and 5% respectively). Crude fibre was more in mycelium (25%) than fruiting body (16.8%) (Figure 16). Similar observations were obtained by Chan *et al.* (2015) for *Cordyceps militaris* in which the protein content was (59.8%) and (39.5%) in the fruiting body and mycelium respectively and the mycelium was distinguished by its carbohydrate content (39.6%), which was higher than that of the fruiting body (29.1%). They also studied the vitamin composition and the most abundant vitamins were found to be vitamin A, vitamin B_3, and vitamin E. Rakhee *et al.* in 2016

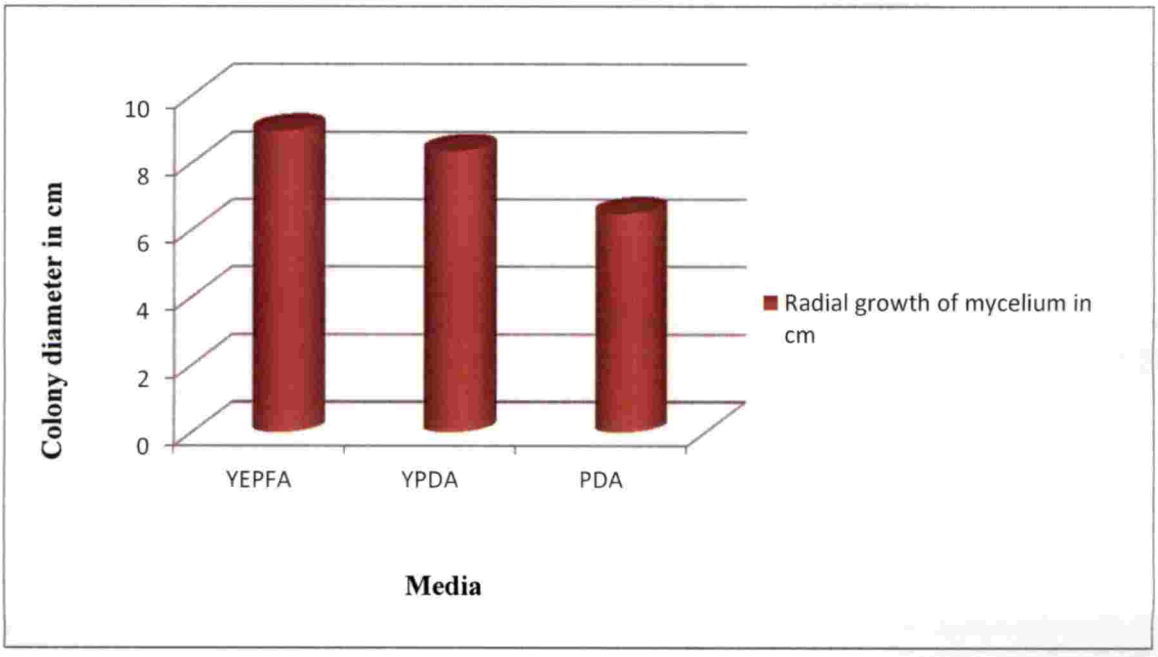


Figure 13. Comparison of solid media for mycelial growth of *O. neovolkiana*

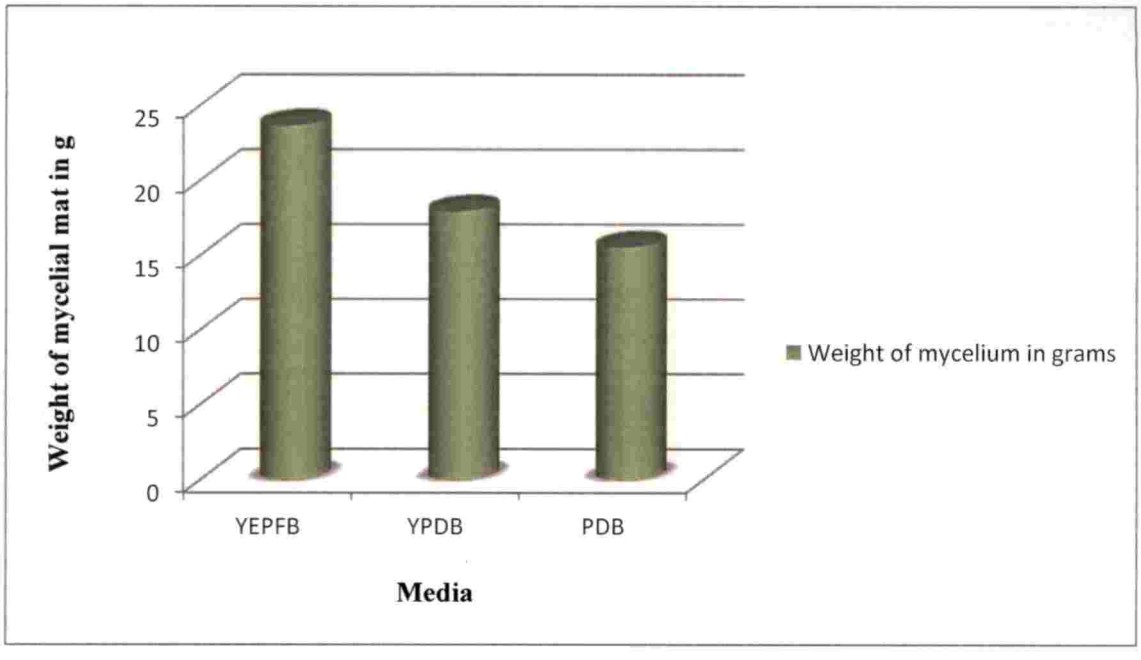


Figure 14. Comparison of liquid media for mycelial growth of *O. neovolkiana*

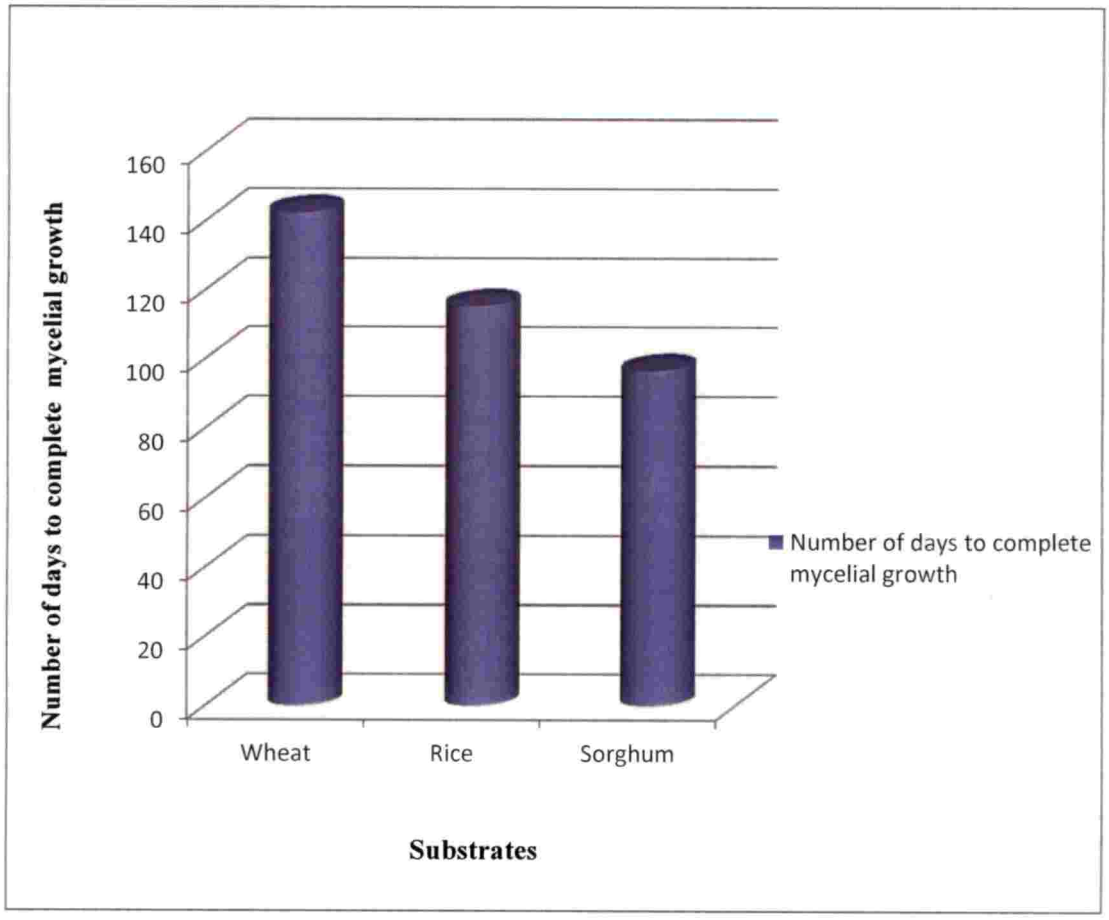


Figure 15. Effect of different substrates on mycelial growth of *O. neovolkiana*

evaluated chemical composition of the Indian Himalayan medicinal mushroom *C. sinensis* and they reported that the carbohydrate content was 55.68 per cent,the crude fiber content was found to be 6.40 per cent, fat 1.80 per cent, moisture content 7.18 per cent, protein 21.46 per cent and total ash content was 7.48 per cent. But in the present study, protein content was more in fruiting body than mycelium (2.9% and 1.75% respectively). Above figures shows that artificially cultured mycelium of *O. neovolkiana* are more nutritious with regard to carbohydrate and crude fibre. Since the moisture content was 76.8 per cent in fruiting body and 27.6 per cent in mycelium which was very less, the mycelium are more suitable for effective preservation without spoilage. There was not much variation in the ash content which was found to be 1.96 per cent and 2.1 per cent in fruiting body and mycelium respectively. The ascorbic acid content was found to be 3.27 per cent in fruiting body and 2.5 per cent in mycelium. Observations of this experiment showed that the problem of scarcity of fruiting bodies can be solved by the process of artificial cultivation of *O. neovolkiana* and more scientific study should be undertaken in this field. Artificial cultivation will also protect the natural habitat of *Ophiocordyceps* in terms of conservation and prevent from extinction. A review of Chinese *Cordyceps* with special reference to Nepal, focusing on conservation done by Baral *et al.* (2015) showed that there should be more work on its cultural and physiological characteristics. Over exploitation of the resource of this fungus may create a population crash. Therefore it is of paramount importance to develop science - based resource management plans for conservation of *Ophiocordyceps.*

Although more precise research is going on with this mysterious fungus recently, information about the mycology of the fungus and the mode of attack remain inexplicable to the scientific world. Debate is continuing among scientists, whether the genus *Cordyceps* is in fact single organism or if they are symbiotic colonies of more than one organism as a fungal - bacterial symbiosis. Extensive research on metabolites from *Ophiocordyceps* is still awaited and more mechanism-

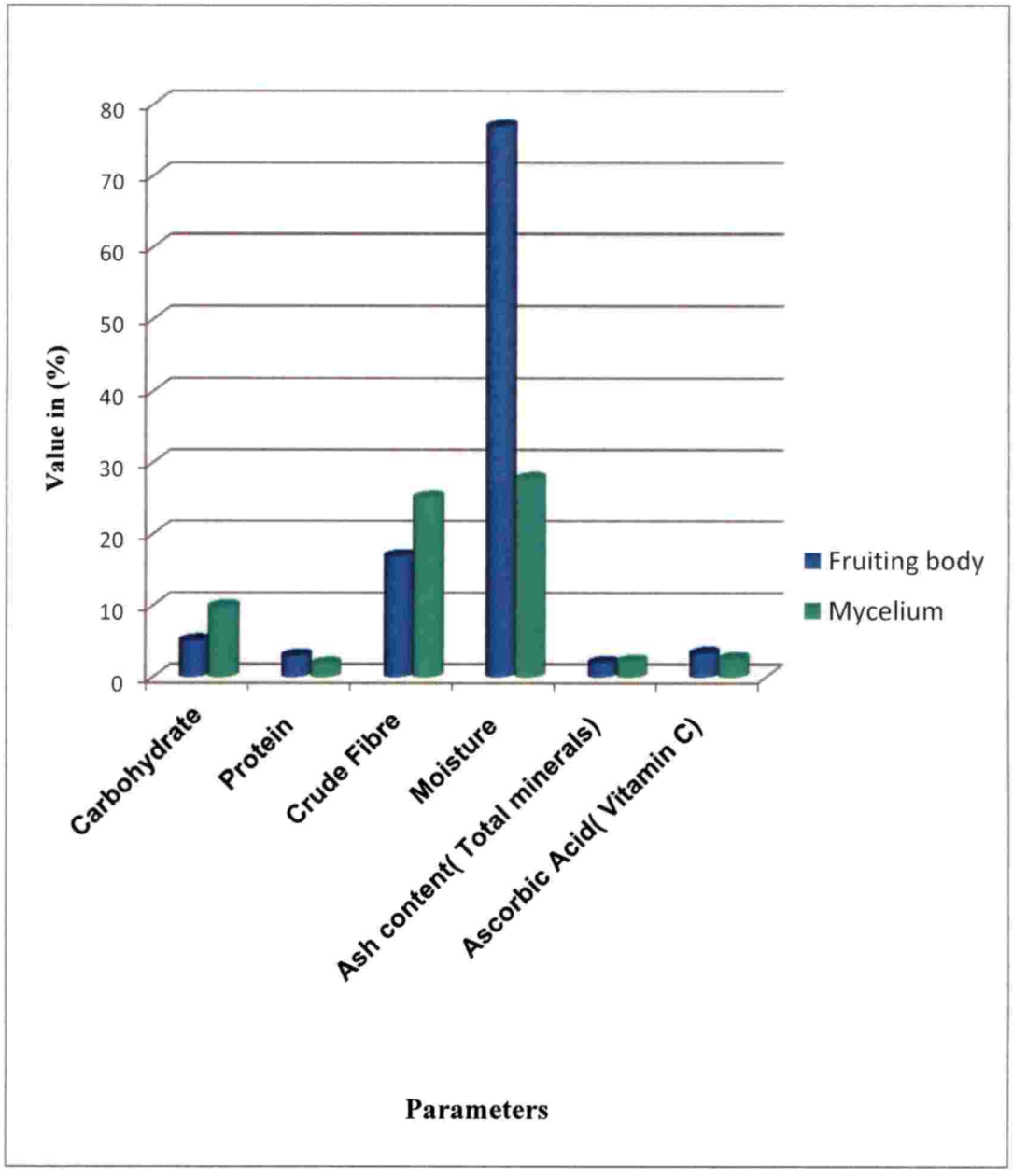


Figure 16. Proximate composition of *O. neovolkiana*

based and disease - oriented pharmacological studies are the need of the hour for its pharmacokinetics, pharmacodynamics, and toxicities in humans. There are various interesting school of thoughts regarding the insect-fungal relationship. DNA sequencing had proven that as the DNA sequence tends to change with time, as if the fungus were incorporating some of the insect DNA into its own DNA code for the initiation of its fruiting body, then losing the insect DNA when it goes back into its mycelial form (Yin and Tang, 1995). Another interpretation is that *C. sinensis* actually has a symbiotic relationship with the host, and that the connection is mutually beneficial, rather than parasitic. The insect perhaps gains an energy boost or other benefit from the *Cordyceps* living in its body, as it may be the death of the insect host that is the stressor triggering the *Cordyceps* to produce its fruit body. Once the host insect dies, the *Cordyceps* would have to go into a reproduce - or - die mode. It is most common in the fungal kingdom that fruit body formation does not happen unless and until some severe stress occurs, forcing this defensive reproductive - phase response. In nature, these stressors are usually heat and cold, fire and flood, or the complete consumption of the food source and the resulting nutrient deficiency. In the laboratory it is very difficult to trigger *Cordyceps* to fruit, but when fruiting does occur, it is always in connection with one or more of these types of stress (Holliday and Cleaver, 2008).

Very few toxic side effects have been demonstrated with *Cordyceps* use, although a very small number of people may experience dry mouth, nausea, or diarrhea (Zhou *et al.*, 1998). There is little published data on the use of *Cordyceps* in pregnant or lactating women or in very young children, and appropriate precautions should be taken with these types of patients. Animal models failed to find an LD_{50} (median lethal dose) injected i.p. in mice at up to 80 g/kg per day, with no fatalities after 7 days (Holliday and Cleaver, 2008).

Study by Chinnusamy and Krishnamoorthy (2017) in *Ophiocordyceps neovolkiana* from Tamil Nadu reported that NMR spectral analysis of cordycepin produced by both, *O. sinensis* and *O.neovolkiana* (CFC filtrate condensate and mycelial mat extract) showed anomeric carbon peaks at 3.4 ppm, which were comparable with proton NMR spectrum of standard cordycepin already done. This study leads to a conclusion that *Ophiocordyceps neovolkiana* has to be studied in detail for further information about various bioactive compounds in it and also the medicinal properties of this valuable fungus.

Summary

6. SUMMARY

Cordyceps sp. is an entomopathogenic fungus on arthropods which on germination, kills and mummifies their larva, and then grows from the body of the host. Most of the bioactive compounds from *Cordyceps* sp. have been exploited for use in traditional and modern ethnomedicine, for treatment of various diseases like cancer, diarrhoea, head ache and muscle pain. The distribution of this fungus is cosmopolitan, but mostly confined to high Himalayan Mountains in China, Tibet, Nepal and India. *Cordyceps* sp. which is now renamed as *Ophiocordyceps* sp. attacks coconut root grubs *Leucopholis coneophora* (Burm). Studies on its ecological niches, mycological perspectives should be performed for effective utilization and conservation of this fungus. In this context, the present study, 'Characterization of medicinal mushroom, *Cordyceps* sp. from Kasargod district' was conducted to identify and characterize the fungus *Cordyceps* sp. from the Kasargod district of Kerala and to assess the its nutritional status.

1. Survey for the collection of *Cordyceps* sp

- Maximum number of fruiting bodies of *Cordyceps* were obtained from the soil under cashew trees in the Instructional Farm, College of Agriculture, Padannakkad (Average of 13.86 m^{-2}) due to the rich in organic carbon (1.39%), and shaded condition compared to Valiaparamba and Pilicode. Macro minerals *viz.*, potassium, calcium and micro minerals *viz.*, manganese were in higher quantity under soil of cashew trees compared to other two regions, which may be helping in the survival and germination of spores.

2. The fungus was isolated on PDA medium from the stroma portion of the fruiting body and the isolate obtained after pure culturing was numbered as CD-1.

- The mycelia appeared initially white, later turning to creamish white to salmon colour and the underside of the plate being light brown in the PDA medium. Later numerous thread like pinkish synnemata appeared in culture representing the anamorphic stage *Hirsutella* sp.

- The isolate completed the full growth by 55 days after inoculation in PDA medium. The anamorph produced similar type of conidia as produced in the field.

3. Characterization of *Cordyceps* sp. based on cultural, morphological, molecular and physiological characters.

- The cultural characters like colour, shape, texture and the growth rate of the mycelium were studied in different media. Among the different media tested yeast potato dextrose agar medium (YPDA) was found to be the best medium followed by potato dextrose agar medium (PDA), which were significantly superior from other media.

- Various macroscopic characters like shape, size and colour of sclerotia, stipe and stroma and branching pattern of the stroma in three locations were observed. Average length and girth of the fruiting bodies obtained from all the three locations were measured, in which the maximum length of 11.9cm and 1.6cm for stipe and stroma were obtained from soils of cashew trees. Maximum girth of 3.9cm and 1.7cm for stipe and stroma was also from the same soil.
- Microscopic studies of the *Cordyceps* sp. *viz.*, characters of anamorph including hyphae, synnemata, conidiophores and conidia and teleomorph including perithecia, asci, and ascospores were conducted.
- The molecular characterization of the fungus was carried out at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram by ITS sequencing to identify at species level. Sequence analysis of the culture showed homology

with *Ophiocordyceps neovolkiana* (Kobayasi) strain KC1 having 98 per cent identity.

- Physiological characters *viz.*, effect of temperature, pH, light and darkness, carbon and nitrogen source, macro and micro mineral sources, vitamin sources were evaluated.
- Among eight different temperatures, 30°C was found to be the optimum temperature in which the growth was completed within 38 days after inoculation in YPDA medium.
- The neutral pH seven was found to be optimum for mycelial growth. In this pH the mycelial growth was completed within 35 days after inoculation in YPDA at 30°C.
- Among the different conditions of light and darkness tested, darkness under incubator was preferred by *O. neovolkiana* followed by darkness under room temperature and light under incubator (onpar) and light under room temperature was the least preferred for mycelial growth.
- Results showed that among the different carbon sources tested, fructose is the best source of carbon in which maximum mycelial growth of *O. neovolkiana* was obtained within 36 days after inoculation.
- The best source of nitrogen was yeast extract in which maximum mycelial growth of *O. neovolkiana* was obtained within 35 days after inoculation.
- KH_2PO_4 was the best source of macro mineral for the growth of *O. neovolkiana* in which the fungus completed the growth within 38 days after inoculation.
- Among micro minerals, $ZnCl_2$ is the best source for the growth of *O. neovolkiana* in which the fungus completed the growth within 35 days after inoculation.
- Folic acid was the best source of vitamin for the growth of *O. neovolkiana* in which the fungus completed the growth within 37 days after inoculation.

- One optimum medium for the growth of *O. neovolkiana* was formulated. The optimum media constituted 300 g of potato, 20 g of fructose, 5 g of yeast extract, one gram of KH_2PO_4, 500 mg of $ZnCl_2$, 10 mg of folic acid and 20 g of agar in one litre of distilled water and the medium was named as yeast extract potato fructose agar (YEPFA).
- The growth of *O. neovolkiana* in the YEPFA medium was significantly higher than the best media YPDA and PDA, so that the time required for the mycelial growth could be considerably reduced from 55.15 days to 30.85 days. The mycelial dry weight was also significantly higher in YEPFB medium than YPDB and PDB and growth completed within 32.57 days after inoculation.

4. Different cereal grains *viz.*, rice, wheat and sorghum were tried for artificial culturing of fungus under *in vitro* conditions and sorghum grains was the best substrate for mycelial growth followed by wheat and rice. The number of days taken for coverage of the mycelium in 50g of grains was 96.43 days for sorghum which was significantly different from rice (115.15 days) and wheat (142.15 days).

5. Analysis of proximate constituents like carbohydrate, protein, fibre content, total minerals, moisture, ash content and vitamin C were compared using the collected sporocarps from the field and mycelial growth obtained on the broth of YEPF medium.

- The carbohydrate content was found to be more in mycelium (9.7%) than in fruiting body (5%).
- Protein content was found to be 2.9 per cent and 1.75 per cent in fruiting body and mycelium respectively.
- Crude fibre was more in mycelium (25%) than fruiting body (16.8%).

- Moisture content in fruiting body was high (76.8%) and in mycelium was (27.6 %) low which is a preferred character for long shelf life.
- There was not much variation in the ash content which was found to be 1.96 per cent and 2.1 per cent in fruiting body and mycelium respectively.
- The ascorbic acid content was found to be 3.27 per cent in fruiting body and 2.5 per cent in mycelium.
- The proximate analysis proved that cultivated mycelium is a best alternative for the natural fruiting body.

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Appendices

APPENDIX- I

Composition of media used

1. **Potato dextrose agar**
   - Potato - 200 g
   - Dextrose - 20 g
   - Agar - 20 g
   - Distilled water - 1000 ml
2. **Yeast potato dextrose agar**
   - Potato - 300 g
   - Yeast extract - 5 g
   - Dextrose - 20 g
   - Agar - 20 g
   - Distilled water - 1000 ml
3. **Malt extract agar**
   - Malt extract - 20 g
   - Agar - 20 g
   - Distilled water - 1000 ml
4. **Czapek dox agar**
   - $NaNO_3$ - 2 g
   - K_2HPO_4 - 1 g
   - KCl - 0.5 g
   - $MgSO_4.7H2O$ - 0.5 g
   - $FeSO_4$ - 0.01 g
   - Sucrose - 30 g
   - Agar - 20 g
   - Distilled water - 1000 ml
5. **Oatmeal agar**
   - Oatmeal - 60 g
   - Agar - 20 g
   - Distilled water - 1000 ml

APPENDIX-II

Composition of stain used

1. Lactophenol- cotton blue

Anhydrous lactophenol	- 67.0 ml
Distilled water	- 20.0 ml
Cotton blue	- 0.1 g

CHARACTERIZATION OF MEDICINAL MUSHROOM, *Cordyceps* sp. FROM KASARGOD DISTRICT

By
LAYA P.K
(2016-11-024)

ABSTRACT

Submitted in partial fulfillment of the requirement for the degree of

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(PLANT PATHOLOGY)
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ABSTRACT

Cordyceps which is an entomo-pathogenic fungus got the name from two Latin words, *cord* and *ceps*, meaning 'club'and 'head'. This mysterious fungus has a long reputation of being the single-most expensive raw material used in traditional Chinese medicine due to its dearth in availability. As a result of its high efficacy and potency in curing various diseases, this fungus has been recommended by medicinal practioners as a 'panacea of all ills'. Distribution of *Cordyceps* sp. is cosmopolitan, but mostly confined to high Himalayan Mountains in China, Tibet, Nepal and India, at an altitude of 3000 to 5000 metres or in Asian high altitude grass land ecosystems. *Cordyceps* sp. which is renamed as *Ophiocordyceps* sp. attacks on the coconut root grub (*Leucopholis coneophora* Burm.) in coastal sandy areas of Kasargod district of Kerala. In this context, in the present study, 'Characterization of medicinal mushroom, *Cordyceps* sp. from Kasargod district' an attempt was made to untangle many of the mysteries of *Cordyceps* sp. by isolation, identification, characterization, cultivation and assessment of the proximate composition with special attention to *Cordyceps* sp.

Purposive sampling surveys were conducted in three locations of coastal sandy tracts of Kasargod district during June to September months of 2017 and 2018. The fruiting bodies of *Cordyceps* sp. were collected from three locations *viz.*, Instructional farm of College of Agriculture, Padannakkad, Valiyaparamba area of Nileshwar and Regional Agricultural Research Station, Pilicode. Maximum number of fruiting bodies were obtained from the soil under cashew trees in the Instructional farm of College of Agriculture, Padannakkad (Average of 13.86/m^2) due to the richness in organic carbon (1.39%), and shaded condition in the soil under cashew orchard compared to Valiaparamba and Pilicode. Macro minerals *viz.*, potassium, calcium and micro mineral like, manganese were in higher quantity under soil of cashew trees compared to other two regions, which may be helping in the survival and germination of spores.

The fungus was isolated on PDA medium from the stroma portion of the fruiting body and the isolate obtained after pure culturing was numbered as CD-1. The isolate completed the full growth in a 9 cm Petri dish by 55 days after inoculation in PDA medium. The anamorph produced similar type of conidia as produced in the field.

Characterization of *Cordyceps* sp. based on cultural, morphological and physiological characters were carried out. The cultural characters like colour, shape, texture and the growth rate of the mycelium were studied in different media. Among the different media tested yeast potato dextrose agar medium (YPDA) was found to be the best medium followed by potato dextrose agar media (PDA), which were significantly superior from other medium.

Various macroscopic characters like shape, size and colour of sclerotia, stipe and stroma and branching pattern of the stroma in three locations were observed. Microscopic studies of both anamorph (hyphae, synnemata, conidiophores and conidia) and teleomorph (perithetia, asci and ascospores) were conducted.

The molecular characterization of the fungus was carried out at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram and showed homology with *Ophiocordyceps neovolkiana* (Kobayasi) strain KC1 having 98 per cent identity. The sequence of the fungus was deposited at the Gen Bank of NCBI and the accession number obtained was MH 668282. The culture was deposited in National Fungal Culture Collection of India at MACS Agharkar Institute, Pune with culture no. NFCCI 4331.

Physiological characterization of *O. neovolkiana* was carried out. Among eight different temperatures tested, 30°C was found to be the optimum temperature in which the growth was completed within 38 days after inoculation. The neutral pH seven was found to be optimum for mycelial growth. Among the different light conditions, 24 hrs of darkness under incubator (30°C) was preferred by this fungus. Fructose was the best source of carbon in which maximum mycelial growth was

obtained within 36 days after inoculation. The best source of nitrogen was yeast extract in which maximum mycelial growth was obtained within 35 days after inoculation. KH_2PO_4 was the best source of macro mineral for the growth, in which the fungus completed the growth within 38 days after inoculation. Among micro minerals, $ZnCl_2$ was the best for the growth in which the fungus completed the growth within 35 days after inoculation. Folic acid was the best source of vitamin for the growth in which the fungus completed the growth within 37 days after inoculation.

One optimum media for the growth of *O. neovolkiana* was standardized as Yeast Potato Fructose medium supplemented with one gram of KH_2PO_4, 500 mg of $ZnCl_2$, 10 mg of folic acid in one litre of distilled water which was named as Yeast Extract Potato Fructose Agar (YEPFA).

The growth of *O. neovolkiana* in the YEPFA medium was significantly higher than the best media YPDA and PDA, so that the time required for the mycelial growth could be considerably reduced from 55.15 days to 30.85 days. The mycelial dry weight was also significantly higher in YEPFB medium than YPDB and PDB and growth completed within 32.57 days after inoculation.

Different cereal grains *viz.*, rice, wheat and sorghum were tried for artificial culturing of *O. neovolkiana* under *in vitro* conditions. The number of days taken for coverage of the mycelium in 50g of grains was 96.4 days for sorghum which was significantly different from rice (115.2 days) and wheat (142.2 days). Hence sorghum was selected as the best substrate for artificial culturing.

Analysis of proximate constituents was estimated using the collected sporocarps from the field and mycelial growth obtained on the broth of YEPF medium. The carbohydrate was more in artificially cultivated mycelia than fruiting bodies (5 and 9.7% respectively). But protein, (2.9 and 1.75%) and vitamin C (3.27 and 2.5) were more in fruiting bodies than mycelia. The crude fibre was more in

mycelia than the fruiting bodies (16.8 and 25%). The total minerals content was almost similar in fruiting bodies and mycelia.

Present work is the first attempt in India, which resulted in a detailed systematic study on *O. neovolkiana* parasitizing on coconut root grub. Future line of work should be concentrated on mass production of this fungus, the estimation of its anti-cancerous components and the antifungal and antibacterial effect of this fungus on pathogens in plant disease management and its potential as a biocontrol agent in pest management.