IN-VITRO CULTURE OF *OPHIOCORDYCEPS SINENSIS* (YARSAGUMBA) AND THEIR ASSOCIATED ENDOPHYTIC FUNGI OF NEPAL HIMALAYA

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Abstract: An array of *Ophiocordyceps sinensis* and its associated endophytic fungi residing in the high Nepalese meadows were isolated and studied for their association and symbiotic effectiveness with the host plant. Fungi are noted to be quite common in nature and some of them have been shown to have adverse effects against insects, nematodes and plant pathogens. Out of the few isolates, different types of associated fungi with *Ophiocordyceps sinensis* were screened viz. *Acremonium strictum, Altenaria solani, Aspergillus flavus*, *Trichoderma virens*, *Colletotrichum gloeosporiode, Curvularia lunata, Thielaviopsis* sp. and *Verticillium* sp. The growth rate of the *Ophiocordyceps sinensis* was found to be very slow as compared to other associated fungi with the diameter of only 1 cm in 2 weeks interval. Fast growing endophytic fungi was found to be *Acremonium strictum* followed by *Altenaria solani.* However, the maintenance of the pure culture of the *Ophiocordyceps* fungi is rather difficult and was soon covered up with other associated fungi. Besides, it was rather difficult to sporulate the *O. sinensis* in the laboratory conditions despite using specific medias.

Keywords: Caterpillar mushroom; Endophytic fungi; Insect-fungi combination; *In-vitro* culture; Sporulation.

INTRODUCTION

Ophiocordyceps sinensis (syn. *Cordyceps sinensis*) (Berk.) G. H. Sung, J. M. Sung, Hywel-Jones & Spatafora, a caterpillar fungus (Winkler, 2008a) is an entomopathogenic fungi (Hajek and Leger, 1994; Kim *et al*., 2003) belonging to the order Hypocreales of the family Clavicipitaceae (Sung *et al*., 2001) infecting and eventually killing the mummified Lepidopteran larvae (Hajek and Leger, 1994) usually that of the Himalayan Bat Moth, *Hepialus armonicanus* (Holliday *et al.*, 2005). *Cordyceps* are insect parasitic fungi, exhibiting a high degree of host specificity (Paterson, 2008). A high degree of genetic variation within *Cordyceps sinensis* exist because of the Lepidopterean host that do not represent a monophyletic group, finally creating difficulties in verifying samples. In nature, it is found only at high alps at an altitude ranging from 4,600 – 5,000m asl (Garbyal *et al*., 2004; Winkler, 2008a), appearing annually on the Himalayan Plateau of Nepal, Sikkim, Bhutan, Arunchal Pradesh (Negi *et al.*, 2006), and Tibetan plateau (Garbyal *et al*., 2004), and because of being very small and their growth restricted to a small area (Kim *et al*., 2003), they are thus very difficult to harvest (Holliday *et al*., 2005). The fungus is endemic in the alpine shrub-meadow zone of high mountains or highlands of Northern Nepal (Zang and Kinjo, 1998). About 400 species of *Cordyceps* are known so far (Shimizu, 1994), and they are classified by color and shape of fruiting body or spore, shape of the ascus, and kind of the infecting host insect (Shimizu, 1994; Ito & Hirano, 1997).The caterpillar, being 3.5–4 cm long, is usually yellowish when cleaned while the stroma, usually 4–10 cm, is dark brown or black. *Cordyceps* species are mainly found in the Dolpa district of Nepal and the other important reported districts are Darchula, Bajhang, Mugu, Humla and Rasuwa. The main harvesting period ranges from May-July.

Kobayashi (1941) listed 137 species in the genus *Cordyceps* and, of these, 125 were parasitic on insects (with one or two growing on subterrarean insects), Dube (1983) identified 200 species (Negi *et al*., 2006) while 400 species of *Cordyceps* are known so far till date (Shimizu, 1994). The identification of the host insects by larvae stage is rather difficult and identification of the exact host species awaits future determination (Kinjo & Zang, 2001). The fungus has been noted for its great potential medicinal uses (Winkler, 2008b) and hence extensive pharmaceutical studies have been made (Kinjo *et al*., 1996).

Ophiocordyceps contain a broad range of bioactive compounds (Kneifel *et al*., 1977; Furuya *et al*., 1983), which are considered nutritional (Hobbs, 1995; Holliday and Cleaver, 2004), such as Cordycepin and Cordycepic acid (Chen *et al*., 1996) with their diverse structural architecture. Others components found included are various saccharides and polysaccharides, including cyclofurans, beta-glucans, beta-mannans, complex polysaccharides etc (Kiho *et al*., 1996; Bok *et al*., 1999), anti-bacterial and anti-tumor adenosine derivatives, Ophiocordin, an anti-fungal agent and L-

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Fungal strains	Growth size (cm) of colony at different days interval*				
	4	7	10	13	15
Acremonium strictum	1.2±0.21	1.8±0.29	2.5±0.67	3.1±0.83	3.9±0.71
Altenaria solani	0.9±0.12	1.6±0.19	1.9±0.68	2.6±0.51	3.6±0.77
Aspergillus flavus	0.8±0.63	1.4±0.12	1.4±0.42	1.9±0.77	2.9±0.39
Trichoderma virens	3.8±0.22	5.1±0.16	6.7±0.31	10.3±0.29	13.9±0.74
Colletotrichum gloeosporiode	0.9±0.6	1.2±0.14	1.6±0.23	2.1±0.3	2.6±0.14
Cordyceps sp.	0.2±0.16	0.3±0.07	0.7±0.31	0.9±0.37	1.0±0.41
Curvularia lunata	0.9±0.46	1.6±0.63	1.7±0.33	2.5±0.91	2.7±0.83
Thielaviopsis sp.	0.5±0.27	1.1±0.46	1.6±0.83	1.6±0.54	2.1±0.76
Verticillium sp.	1.1±0.23	1.3±0.36	1.6±0.19	1.7±0.36	2.2±0.10

* $\bar{x} \pm SD$ *(n = 4)*

Tryptophan (Paterson, 2008). The caterpillar fungus has a hypoglycemic effect and may be beneficial for people with insulin resistance (Kiho *et al*., 1993, 1996; Zhao *et al.*, 2002; Lo *et al*., 2004; Li *et al*., 2006).

Mycelium of *O. sinensis* has similar clinical efficacy as the natural one and also possess less associated toxicity (Bensky *et al.*, 2004). Optimization of the necessary applicable protocol for laboratory production of the mycelium of *O. sinensis* may prove successful in the preparation of various products from the dried mycelium, which has numerous potential therapeutic applications, as used in the traditional folk medicinal systems. Infact, *O. sinensis* is an intimate relationship of a complex structure of plant and the animal. It is believed that the fungus incorporates some of the insect DNA into its DNA structure creating this type of unparalleled medicinal mushroom. For the formation of the different bioactive compounds, the endophytes residing within the plants have a pivotal role. The aim of the present study was first to culture the *Cordyceps* specimens in different cultural media and isolating the different associated fungus residing with and within the *Cordyceps* species.

Different strains of *Cordyceps* isolated from different places may produce variable cultural characteristics. The extracts of *Cordyceps* sp. possess extensive pharmacological properties, such as anti-inflammatory, anti-tumor growth, anti-fibrotic and anti-oxidant activities. The size, shape and the color of the colony may differ from the different strains when observed on the artificial solid media and do not change with age. Because of the rarity and peculiarity, fruiting bodies are difficult to form, raising the question of the culture mycelium of being the truly anamorphs of *Cordyceps* sp.

MATERIALS AND METHODS

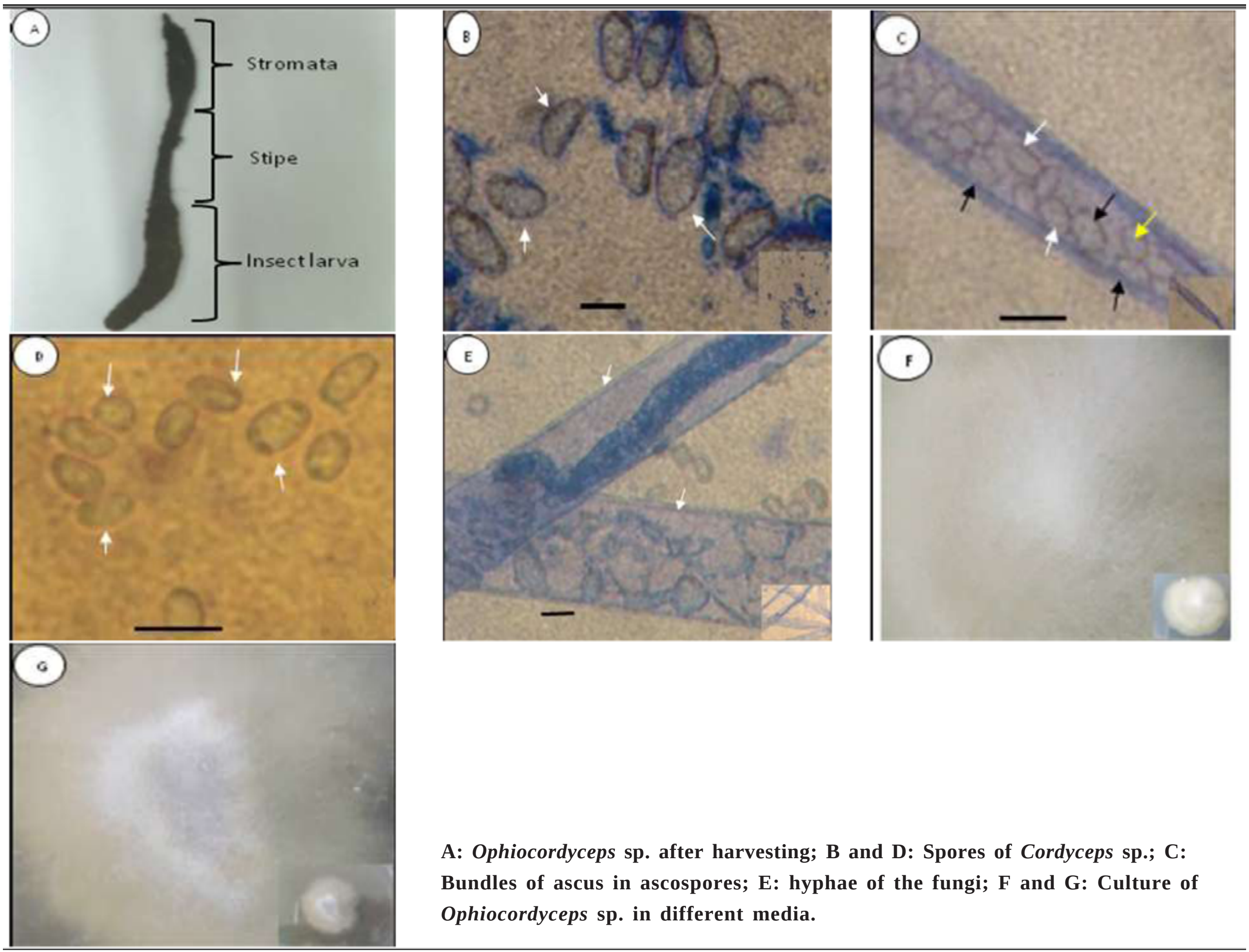
***Investigated locality*:** The samples were harvested with the necessary precautions from the various localities, viz. Manaslu Conservation Area, Manang district and Dolpa district.

Culture Media: All culture medias used viz., Potato Dextrose agar (PDA), Corn Meal agar (CDA), Malt extract agar (MEA), Oat meal agar (OAT), Water agar (WA) were obtained from the HiMedia Pvt. Ltd, India.

Isolation of the fungus: The fungus along with the insect were sealed and harvested from the uprooted site and brought with care to the NAST laboratory. The fungal part was washed carefully with the sterile double distilled deionized water and the head of the stroma was dissected longitudinally. The bundled asci were picked up on the tip of a fine needle and transferred and spread to the surface of a Sabouraud glucose agar plate (SGA; Kinjo & Zang, 2001). Primary isolation was made after confirmation of germination with the aid of microscope. Germinating single ascospore was picked up and transferred to a fresh SGA slant. The cultures were maintained at 18-20°C on the SGA slants at every three days interval starting from four days of and were grown for few weeks for recording its growth requirements.

Morphological Observations: The morphology of the colonies was observed on the different medias (as far as possible selective media, Sabouraud glucose agar, SGA, was used). Petri dishes of 9 cm diameter containing 20 ml of media were inoculated with 6mm disk of mycelia taken from the rim of 30 days old cultures. Plates were kept wrapped in Parafilm to avoid dehydration of the culture media and maintained in the dark, at 24 ± 1ÚC. The growth of the fungal colonies was recorded every three days along two preset diametrical lines. The different stages of the obtained fungus were taken for the observation of its morphological characters. The change in the color of the fungus (if any) was recorded at every alternate day along with their growth rate.

***Isolation of the associated fungus*:** The different fungi in association with the *Ophiocordyceps* sp. were isolated in the various media viz., Potato Dextrose agar (PDA), Corn Meal agar (CDA), Malt extract agar (MEA), Oat meal agar (OAT), Water agar (WA) etc. The obtained fungi were then isolated by their reconstitution in the respective media till the emergence of pure culture. They were then cover slip cultured and was left to grow on the media for the additional



A: *Ophiocordyceps* sp. after harvesting; B and D: Spores of *Cordyceps* sp.; C: Bundles of ascus in ascospores; E: hyphae of the fungi; F and G: Culture of *Ophiocordyceps* sp. in different media.

few days till the full growth of the colony. They were followed by mounting on the slides through cellophane technique and staining by cotton blue. The isolates were identified by their colony characters, spores size and shape observing under the compound microscope. The hyphal growths were measured in the peripheral growth zone of the mycelium (Iotti *et al*., 2005).

Results

The growth of the *O. sinensis* mycelium was very less as compared to the other associated fungi which was approximately one cm in two weeks interval time.

Habitat of O. sinensis: The collecting sites were high mountain areas, over 5,000m asl, with plains of alpine meadow subdominant with graminea grasses, smart weeds and other herbaceous plants. When the fungus is growing up from the ground after snow melts, it is easy to find the growing stipe protruding from the fruiting body. Below the growing fungus, a small hole approximately 16 cm deep by 3 cm wide was usually found. These pores (small holes) were considered the remains of tunnels through which worms which may serve as fungal hosts attacked the roots or rhizomes of several herbaceous plants. These species usually grow on the open high places at the alpine zone where the environmental condition is frigid cold and the oxygen concentration is extremely low. In such harsh conditions where other species fail to grow, *Cordyceps* is found growing luxuriantly.

***Isolation of the associated fungus*:** The associated fungus along with the *O. sinensis* were *Colletotrichum gloeosporiode*, *Verticillium* sp., *Aspergillus flavus*, *Altenaria solani*, *Acremonium strictum*, *Trichoderma virens*, *Curvularia lunata* and *Thielaviopsis* sp.

***Morphological and growth characteristics*:** The morphological and the growth characteristics of the different associated fungus along with the *O. sinensis* were recorded.

Morphological and microscopic characteristics of the associated fungi:

Colletotrichum gloeosporiode: The fungus produces hyaline, one-celled, ovoid to oblong, slightly curved or dumbbell shaped conidia. Conidia in aggregates seem to be pink in color. Conidiophores simple, elongate; conidia hyaline, 1-celled, ovoid or oblong and parasitic.

***Verticillium* sp.**: Conidiophores are usually well differentiated and erect slender, branched, at least some of the branches verticillate; conidia ovoid to ellipsoid, hyaline, 1-celled, borne singly or in small clusters apically; parasitic on other fungi, or growing saprophytically, fast growing colonies, pale yellow in color, becoming pinkish brown, with a colorless, verticillately branched over most of their length, conidia are hyaline or brightly colored, mostly one-celled.

Aspergillus flavus: Conidiophores upright, simple, terminating in a globose or clavate swelling, bearing phialides at the apex or radiating from the entire surface; conidia 1-celled, globose, often variously colored in mass ranging from dark black to greenish brown, catenulate, produced basipetally. A large genus containing many species saprophytic on a wide variety of substrata and a few parasitic species.

Altenaria solani: Conidiophores dark, simple, rather short or elongate, typically bearing a simple or branched chain of conidia; conidia dark, typically with both cross and longitudinal septa; variously shaped, obclavate to elliptical or ovoid, frequently born in long chains often more than 4 conidia, less often borne singly and having an apical simple or branched appendage. Conidia pale brown to light brown, ellipsoid, short beak at the tip with surface smooth; produced in chains.

***Thielaviopsis* sp.:** Chlamydospores are produced in chains (two to six spores per chain). The dark, thick walled chlamydospores separate at maturity and each cell is capable of germination. The spores are variable in size with slightly rounded ends. Mycelium is light brown to light black. The septate hyphae are hyaline at first and become pigmented with age.

***Curvularia lunata**:* Hyphae septate; brown, conidia are transversely septate from edge to edge of the conidial wall and are slightly curved.

Acremonium strictum: Slow growing; hyphae are delicate, fine and hyaline; conidia globose to cylindrical and are mostly one-celled.

Trichoderma virens: Cultures were typically fast growing. Colonies were white on potato dextrose agar (PDA). Myceliums were not typically obvious. A yellow pigment may be secreted into the agar, especially on PDA. Conidiophores are highly branched and thus difficult to define or measure, loosely or compactly tufted, often formed in distinct concentric rings or borne along the scant aerial hyphae.

DISCUSSION AND CONCLUSION

O. sinensis is a fungus with a peculiar habitat from the alpine regions of the Tibetan plateau. Due to the herb's rarity, high efficacy, potency in curing various diseases and high prices associated with the wild collected variety, different attempts have long been made to cultivate *Ophiocordyceps*. These have been made come true by the application of the modern biotechnological – based cultivation methods. *Cordyceps* species are known to be parasitic on many kinds of insects, and host selectivity is very severe. It is possible that a new classification of *Cordyceps* species could be constructed according to accumulated phylogenetic information obtained from rDNA sequences (Ito & Hirano, 1997).

Both *O. sinensis* and its host insects are endemic species on the Himalaya; and the geography and climate may have played an important role in their evolution and coevolution. The differences in the micro-climatic conditions and the life cycle of fungus that parasitizes the insect may also have impacted on the diversification of *O. sinensis*. The caterpillar fungus is more valuable when young, that is, before it sporulates or early during sporulation. In the final stages of sporulation, the host larva becomes soft and undesirable, and the upper part of the mushroom sometimes split. The price of a specimen depends mostly upon the size of the larval host (Winkler, 2008a). The native type specimen of the Nepalese *Cordyceps* specimens is not available in the national herbarium, questioning about its existence. Many collections of *O. sinensis* by different people are often immature or lack the ascostromatal parts. Therefore, scientific studies concerning this fungus have frequently been confronted with taxonomical problems (Kinjo & Zang, 2001). There is also a need to evaluate the effectiveness of the fungi, whether the high medicinal properties of the *O. sinensis* is due to its own effect or due to the secretion of the secondary metabolites by the associated endophytic fungi. A question therefore arises, does the fungus itself possess the medicinal properties or are the bioactive compounds being secreted by the combined action of endophytes residing within their cells?

Endemic nature of the fungus along with mass collection attributes from the wild puts it in the category of threatened plants. There is also a need for scientific exploration for the presence of the fungus in different parts of the Himalayan Region, document the occurrence and status, of the wild population and formulate a strategy for conservation as well as sustainable harvesting of the fungus. Hence, it may be concluded that there are many associated species of fungus with the *O. sinensis* which contributes mutually through the secretion of secondary metabolites that possesses high medicinal properties creating this unique unparalled fungi.

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