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Study of *Ophiocordyceps sinensis* (Berk.) growth in artificial media and virulence test against different stages of silkworm in Nepal

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Abstract
Ophiocordyceps sinensis (*Cordyceps sinensis*), *Yarsagumba* in Nepali, is a special type of entomo-pathogenic creature. It is an endoparasitic fungus that normally grows on *Thitarodes armoricanus* (Oberthür) larvae and is available in the Himalayan regions (3300–5000 meters above sea level (Masl) of Nepal. In this research, yarsagumba were collected from the Kanda Rural Municipality of Bajhanag for conducting growth studies on Sabouraud dextrose agar (SDA) and potato dextrose agar (PDA) media and determining their incidence on silkworm larvae in laboratory conditions at the Nepal Academy of Science and Technology. The *Ophiocordyceps* fungus was isolated and cultured, and its growth was compared using two different artificial media—SDA and PDA—at 27 °C in the laboratory. To conduct a mortality test, the solutions of *Ophiocordyceps* fungus at different concentration (1, 10, 100 and 1000 ppm) were prepared and sprayed on the second and third instar silkworm larvae. It was observed that the growth of the *Ophiocordyceps* fungus occurred on both media, but faster growth was observed on the SDA compared with the PDA. Sclerosis was observed in the mycelium that was obtained from both types of culture media, and this could be seen clearly when placed under a compound microscope. The results revealed that the mortality caused by different concentrations of *Ophiocordyceps* against silkworm larvae was significantly higher compared with the control at 5, 10, 15 and 20 Days after inoculation (DAI). The higher the concentration of fungus, the higher the infection of both instars of silkworm larvae was recorded in all the observed cases. The results showed the further possibility of artificial mass culture of *O. sinensis* under laboratory conditions.

Keywords: Endoparasitic, Infection, *Ophiocordyceps sinensis*, PDA and SDA

1. Introduction
1.1 Background
Nowadays, collection of *Ophiocordyceps sinensis* (Berk.) (=*Cordyceps sinensis*) *Yarsagumba*, found in mountainous regions of Nepal, has been the source of income of many rural livelihoods [17]. *Yarsagumba* is a special type of entomo-patho creature otherwise known as summer grass–winter insect: *Ophiocordyceps* spp. From endoparasitic fungi grow on insect larvae during monsoons. The parasitic fungus *Ophiocordyceps* associates with the caterpillar larvae of *Thitarodes armoricanus* (Oberthür), of family Hepialidae, and forms avaluable structure known as *Yarsagumba* in Nepal. Over a dozen species of Hepialid moths (including eight species of *Thitarodes*, in which the *Ophiocordyceps* grow) have been found in Nepal [22, 2]. Robinson reported that the distribution and prevalence of this creature is mainly confined to China, India, Bhutan, Russia (Far East), Japan, Myanmar, Taiwan and Nepal, as well as otherSouth Asian countries. In Nepal, twenty districts possess the appropriate environmental conditions for the creature's occurrence: alpine and sub-alpine mountainous pastureland ranging from 3000 to 5000 metres above sea level (masl) [18]. There are about 100 identified species of *Ophiocordyceps* fungus. Out of these, 31 species are found in Korea, 21 in China, 30 in Japan, 12 in the UK [13], 7 in India [7] and 3 in Nepal [2]. Among these species of fungi, 3 have proven to be economically important: *C. militaris*, *C. Barnesii* and *C. sinensis*. Nepal has also reported 3 species, such as *Ophiocordyceps sinensis* (Berk.), *O. nutans* Patolii. And *O. nepalensis* Kinjo. *O. sinensis* in particular possesses a high medicinal value [1, 16].
Ophiocordyceps spores disperse from mid-May to the last week of June, when there is favourable weather for their development on insect bodies. The host insect of this fungus has been reported to be *Thitarode* ssp. [3].

The fungus multiplies rapidly and, within a few weeks, larvae are converted into Yarsagumba. Yarsagumba has been widely used in traditional Chinese medicine. It is mostly used for medicinal purposes like improving lung function, energy levels and sex drive (including sexual impotency), immune modulation, cholesterol reduction, anti-oxidant effects and, potentially, stamina and libido [8, 10, 28, 29]. The fungus has also been identified as a precious source of antibiotics for human beings [19], marketed and used as such by large numbers of people every year [23]. The objectives of this study are to evaluate the growth of *O. sinensis* in different artificial media, and to test the virulence of *O. sinensis* against silkworm larvae under laboratory condition in Nepal.

2. Materials and Methods

2.1 Collection of *O. sinensis* samples

Sample larvae of Yarsagumba were collected from the Kanda Rural Municipality of the Bajhang District (29° 23′ 0″ to 30° 03′ 15″ N and 80° 44′ 30″ to 81° 34′ 00″ E, 3500 masl to 4500 masl average 4300 masl), a popular area for Yarsagumba collection. For transportation and preservation, the samples were kept in an ice box (Model no; AIDVC 24, marketed by Apex international). Further study of the samples was conducted at the Nepal Academy of Science and Technology (NAST), in Khumaltar, Lalitpur.

2.2 Isolation and inoculation of *O. sinensis* from sample larvae

Fungus was isolated from the collected samples in the laboratory, and their growth studies were observed in two different media: Sabauroud Dextrose Agar (SDA), adopted from Strasser et al., and Potato Dextrose Agar (PDA). For the PDA medium, 39 grams of PDA powder (from Merck, Germany), and for SDA medium, 65 grams of SDA powder were mixed separately with one litre of distilled water and autoclaved at 121 ℃ for 15 minutes. The composition of SDA and PDA is given below (Table 1).

Table 1: Composition of SDA and PDA

Ingredients (gm/l)	SDA	PDA
Dextrose (Glucose)	20gm	20gm
Peptone	10gm	-
Agar	18gm	15gm
Distilled water	1000 ml	1000ml
Potatoes (sliced washed unpeeled)	-	200gm

The fungus inoculation was cultured in media using point inoculation technique with the help of a loop, and then the fungus was sub-cultured for 19 times using staking method to obtain a pure culture of Ophiocordyceps sample under lab conditions. Both the media were maintained in Petridish. After the inoculation, the growth of mycelium was analysed by keeping inoculated Petridish having media in the incubator at 27 ℃. The growth of mycelium was observed under a compound microscope (Olympus CX21i), and the colony diameter of the developed fungus was compared between two media.

2.3 Silkworms

Eggs of the mulberry silkworm (Bombyx mori Linnaeus) were collected from the Sericulture Development Division, Khopasi, Kavrepalanchok, Nepal, and were kept at laboratory conditions (25 ℃, 80% RH and natural light conditions). After hatching, tiny larvae were provided with tender mulberry leaves sterilized with 5% sodium hypochlorite (NaOCl) and washed with pure water. Excess water present in the mulberry leaves was allowed to drain for 15 minutes. Healthy second and third instar larvae were used in this study.

2.4 Inoculation of fungus in silkworm larvae

For the larval mortality test, stock solutions of Ophiocordyceps fungus were prepared by adding 1gram of mycelium grown on SDA in 1000 ml of water (1000 PPM). Other concentrations (100 PPM, 10 PPM and 1PPM) were prepared form the stock solution by using a dilution factor of 10. These prepared concentrations were sprayed through atomizer on second and third instar silkworm larvae. Pure water was sprayed in the control treatment. The number of silkworms in each treatment was 10, and the experiment was replicated four times using Randomized Complete Block Design (RCBD) in the laboratory.

3. Results and Discussion

3.1 Growth of Ophiocordyceps fungus in artificial media

Observations were taken for the mycelium spread on the two different media. During the experiment, it was noted that growth of Ophiocordyceps occurred in both media, but growth developed more rapidly in SDA compared to PDA. The growth trend in both the media was similar from 2DAI to 14DAI. Growth of fungus in SDA was significantly higher than in PDA for all observed cases (Table 2). Sclerosis was observed in the mycelium obtained on both types of culture media, which could be clearly seen under the compound microscope.

Table 2: Colony growth of *O. sinensis* in Sabauroud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) media

Treatment	Growth of *O. sinensis* colony (Ø in cm)						
	2 DAI	4 DAI	6 DAI	8 DAI	10 DAI	12 DAI	14 DAI
SDA	1.45[a]	2.11 a	2.86 a	4.08 a	5.56 a	7.43 a	9.24 a
PDA	0.79[b]	1.45 b	1.60 b	2.50 b	3.36 b	4.20 b	5.17 b
Mean	1.12	1.78	2.23	3.29	4.46	5.82	7.21
SeM	0.00026	0.0001	0.0002	0.00058	0.005	0.0220	0.0067
LSD	0.017	0.013	0.0094	0.026	0.081	0.162	0.089
CV	1.44	0.71	0.38	0.73	1.65	2.55	1.14

Mean in the same column with same letter are not significant at a significance level of 5%. DAI: Days after inoculation

3.2 Inoculation of *O. sinensis* fungus on silkworm larvae

The results revealed that different concentrations of Ophiocordyceps against the second and third instar silkworm larvae caused significant mortality. At 5 DAI, the mortality of both the second (df=12, F=30.207, p=0.001) and third instar larvae (df=12, F=22.86, p=0.001) was significantly higher compared with the control. At 10 DAI, as compared with pure water treatment, the mortality caused by different concentrations of test fungus against second (df=12, F=30.20, p=0.001) and third (df=12, F=22.26, p=0.001) instar silkworm larvae was significantly higher. Similarly, with a higher concentration of Ophiocordyceps, a higher mortality of the both second (df=12, F=37.54, p=0.001) and third instars (df=12, F=40.44, p=0.001) was achieved at 15 DAI. At 20 DAI, significantly higher mortality over the control was recorded at all four concentrations in the second (df=12, F=95.335, p=0.001) and third instar silkworm larvae (df=12, F=93.85, p=0.001; Table 3). The fungus isolated from the dead larvae of silkworm and Ophiocordyceps was confirmed by microscopic examination, as described by Baral and Perlin and Baral.

Table 3: Effect of different concentration of *O. sinensis* on mortality of second and third instar silkworm larvae at different DAI

Larval stages of silkworm	Observation time	Treatments					Mean	SEM	CV	LSD
		1000 PPM	100 PPM	10 PPM	1 PPM	Control (water)				
Second	5 DAI	26.19^{a}	18.43^{b}	6.49c	0.90^{c}	0.90^{c}	10.58	16.86	8.78	6.32***
	10 DAI	42.11^{a}	27.85^{b}	24.53^{b}	5.28^{c}	0.90^{c}	20.13	11.54	13.04	7.15***
	15 DAI	55.28^{a}	42.11^{b}	37.72^{b}	24.53^{c}	9.670^{d}	33.86	18.34	10.79	8.76***
	20 DAI	89.09^{a}	63.43^{b}	49.32^{c}	33.21^{d}	11.70^{e}	49.35	19.24	12.19	9.27***
Third	5 DAI	22.50^{a}	14.05^{ab}	0.90^{c}	0.90^{c}	0.90^{c}	9.13	3.08	7.84	10.11**
	10 DAI	36.22^{a}	24.53^{b}	14.05^{c}	0.90^{d}	0.90^{d}	15.32	9.99	14.641	7.42***
	15 DAI	53.77^{a}	40.67^{b}	29.88^{c}	18.435^{d}	5.28^{e}	32.1	11.04	13.98	7.05***
	20 DAI	84.71^{a}	58.60^{b}	43.55^{c}	29.88^{d}	5.28^{e}	44.41	17.2	12.79	10.04***

In vitro culture of fungi has been increasingly employed [25]. Solid substrate cultivation practices of *O. sinensis* common in the United States and Japan, where mycelia are grown in plastic or glass jars containing sterilised medium with ingredient of cereal grains like rice, wheat and rye. This method is easy and requires low capital investment for growers [15, 24, 26, 27]. Dong and Yao used various solid media to determine the growth of *O. sinensis* in various temperature ranges, and they found that *O. sinensis* grows slowly at low temperature.

Both *O. sinensis* and its host insects are endemic species in the Himalayan region, and geography and climate may have played an important role in their evolution and co-evolution [15]. The natural rearing of Cordyceps spp. is an extremely difficult job, so this fungus and its products are mostly derived from mycelial cultures of the asexual forms that are commercially available [12]. Aramwit et al reported that some other species, such as Cordyceps militaris, grow on the dead larvae of B. mori. Some studies show Ophiocordyceps species are known to be parasitic on many kinds of insects, and host selectivity is severe [14].

4. Conclusion

From the growth study on SDA and PDA at 27°C, it can be concluded that the fungus could be further tested in other media, and artificial mass production could be achieved. This fungus invades silkworm larvae and causes significant mortality indicates that the study could take the further step of concentrating on the relationship between silkworm larvae and *O. sinensis*. Therefore, there is good scope for further studies on the growth and development of this fungus on silkworm larvae.

Data availability

The data used to support the findings of this study are available from the corresponding author upon written request.

Conflicts of interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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6. References

1. Adhikari B. Household characteristics and common property forest use: complementarities and contradictions. Journal of forest and livelihood, 2002.
2. Adhikari MK. The diversity of Ophiocordyceps fungi (Ascomycota: Clacivicipitales) reported from Nepal. Department of Plant Resource Bulletin. 2008; 30:1-9.
3. Amayata G. Trade and socioeconomic attribution of Ophiocordyceps sinensis (Berk) Saccardo in Darchula district, Nepal. In: P.K. Jha, S.B. Karmacharya, M.K. Chhetri, C.B. Thapa, and B.B. Shrestha (eds.) Medicinal Plants in Nepal: An Anthology of Contemporary Research. Ecological Society (ECOS), Kathmandu, Nepal. 2008; 194-202.
4. Aramwit P, Bang N, Ratanavaraporn J, Nakpheng T, Srichana T. An anti-cancer cordycepin produced by Cordyceps militaris growing on the dead larva of Bombyx mori silkworm. Journal of Agricultural Science. 2014; 6(6):41.
5. Baral B. Entomopathogenicity and biological attributes of himalayan treasured fungus Ophiocordyceps sinensis (Yarsagumba). Journal of Fungi, 2017; 3(1):4.
6. Baral B, Maharjan J. *In-vitro* culture of Ophiocordyceps sinensis (Yarsagumba) and their associated endophytic fungi of Nepal Himalaya. Nepal Academy of Science and Technology, Khumaltar, Lalitpur, Nepal, 2012.
7. Bilgrami K, Jamaliddin S, Rizwi MA. Fungi of India part-I. Today and tomorrow printers and publishers, New Dehli, PP- 467. Also included in folliculous fungi from bahraich U.P. (India) by Ajaya Kumar and T. P. Mall in 2012. Post graduate department of botany Kisan PG College Bahraich U.P., India, 1979.
8. Canney S. Ophiocordyceps Sinensis- Animal, vegetable or both? Journal of Chinese Medicine. 2006; 80:43-49.
9. DADO. Bajhang. Bajhang Dairy. District agriculture Development Office, Chainpur, Bajhang, Nepal, 2016.
10. Devkota S. Yarsagumba (*Ophiocordyceps sinensis*): Traditional utilization in Dolpa district, western Nepal. Our Nature, 2006, 48-52.
11. Dong CH, Yao YJ. On the reliability of fungal materials used in studies on Ophiocordyceps Sinensis. Journal of industrial microbiology & biotechnology. 2011; 38(8):1027-1035.
12. Hamburger M. Comment on comparison of protective effects between cultured Ophiocordyceps militaris and natural Ophiocordyceps sinensis against oxidative damage. Journal of Agriculture and Food Chemistry. 2007; 55:7213-7214.
13. Hawksmorth GM. Vegetative patterns, disturbances and forest health in eastern Oregon and Washington. Pacific Northwest research station, Interior Columbia basin ecosystem management project, Portland, 1983.
14. Ito Y, Hirano T. The determination of the partial 18S ribosomal DNA sequences of Ophiocordyceps species. Letter of Applied Microbiology. 1997; 25:239-242.
15. Kiho T, Yamane A, Hui J, Usui S, Ukai S. Polysaccharides in fungi, Hypoglycemic activity of a

polysaccharide (CS-F30) from the cultural mycelium of Ophiocordyceps Sinensis And its effect on glucose metabolism in mouse liver. Biological and Pharmaceutical Bulletin. 1996; 19:294-296.
16. Kinjo N, Zang M. Morphological and phylogenetic studies on Ophiocordyceps Sinensis Distribution in Southwestern China. Mycoscience, 2001; 42:567-574.
17. MoAD. Ministry of agriculture and Development. Statistical Report. Simhadurbar, Kathmandu, Nepal, 2016.
18. Neupane FP. Yarsagumba: Bionomics. In: P. K. Jha, F. P. Neupane, M. L. Shrestha and I. P. Khanal (eds.) Biological Diversity and Conservation Nepalpedia Series No. 2. Nepal Academy of Science and Technology (NAST), Kathmandu, Nepal, 201, 597-606.
19. Nielsen ES, Robinson GS, Wagner DL. Ghost moths of the world: A global inventory and bibliography of the Exoporia (Mnesarchaeoidea and Hepialoidea) (Lepidoptera). Journal of Natural History. 2000; 34(6):823-878.
20. Robinson GS. A note on adult flight time of Thitarodes. In: T. Haruta (ed.) Moths of Nepal Part 6, Tinea Vol. 16 (Supplement 1). Japan Heterocerist's Society, c/o Natural History Division, Nepal Science Museum, Hyakunincho 3, Shinjuku, Tokyo, Japan, 2000, 91.
21. Strasser H, Forer A, Schinner F. Development of media for the selective isolation and maintenance of virulence of Beauveria brongniartii. In: T.A. Jacksonand T.R. Glare, (eds) Proceeding of 3rd International Workshop onMicrobial Control of Soil Dwelling Pests. Agresearch Lincoln, New Zealand, 1997, 125-130.
22. Thapa VK. An inventory of Nepal's insects Vol. 2. IUCN, Kathmandu, Nepal, 1998.
23. Ueda K. Hepialidae of Nepal. In: T. Haruta (ed.) Moths of Nepal Part 6, Tinea Vol 16 (Supplement 1). Japan Heterocerist's Society, c/o Natural History Division, Nepal Science Museum, Hyakunincho 3, Shinjuku, Tokyo, Japan, 2000, 70-93.
24. Yamaguchi Y, Kagota S, Nakamura K, Shinozuba K, Kunitomo M. Inhibitory effects of water extracts from fruiting bodies of cultured Ophiocordycepssinensison raised serum lipid peroxide levels and aortic cholesterol deposition in atherosclerotic mice. Phytomer Research. 2000; 14(8):650-652.
25. Yang J, Zhang W, Shi P, Chen J, Han X, Wang Y *et al*. Effects of exopolysaccharide fraction (EPSF) from a cultivated Ophiocordyceps sinensis fungus on c-Myc, c-Fos, and VEGF expression in B16 melanoma-bearing mice. Pathol. Res. Pract. 2005; 201:745-750.
26. Zhao CS, Yin WT, Wang JY, Zhang Y, Yu H, Cooper R *et al*. Cordy Max Cs-4 improves glucose metabolism and increases insulin sensitivity in normal rats. Journal of Alternative and Complementary Medicine. 2002; 8(3):309-314.
27. Zhou LT, Yang YZ, Xu YM, Zhu QY, Zhu YR, Ge XY *et al*. Short-term curative effect of cultured Ophiocordyceps Sinensis (Berk) Sacc. mycelia in chronic hepatitis B. Journal of Chinese Materia Medica1. 1990; 5(1):53-55.
28. Zhu JS, Halpern GM, Jones K. The scientific rediscovery of an ancient Chinese herbal medicine: Ophiocordyceps Sinensis Part 1. Journal of Alternative and Complementary Medicine. 1998; 4:289-303.
29. Holliday J, Cleaver M, Wasser SP. Ophiocordyceps. In: Encyclopedia of dietary supplements. Dekker Encyclopedias. Taylor and Francis Publishing, London, 2005, 1-13.