# STUDY ON CULTURING METHODS TO INCREASE THE QUANTITIES OF OPHIOCORDYCEPS FOUND IN THE AREA OF DOI INTHANON NATIONAL PARK, CHIANG MAI PROVINCE, THAILAND

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**Abstract:** The investigation was carried out by surveying and field collecting, the entomopathogenic fungi in the genus *Ophiocordyceps* (Hypocreales: Ophiocordycipitaceae) in Doi Inthanon National Park, Chiang Mai Province, found that the majority specimens were identified as *Ophiocordyceps unilateralis*, *O. sobolifera* and *O. sphecocephala* respectively; a few specimens were identified as O. *irangiensis* and three specimens collected i.e. *Ophiocordyceps* sp.1, *Ophiocordyceps* sp.2 and *Ophiocordyceps* sp.3 could not be identified. All species were isolated as pure cultures by single spore isolation and tissue culture techniques. Each of the *Ophiocordyceps* cultures were tested for growth on various media, temperature and pH levels in the dark for 15-60 days. It was found that all of them except *O. unilateralis* grew well on potato dextrose agar at 25° C, pH 5-6 and pseudostroma were developed.

**Keywords:** Classification, *Entomopathogenic Fungi*, Field Surveying, Spatial Distribution.

**Introduction:** Thailand is one of the highest sources of various kinds of fungal especially the fungal in Cordycipitaceae, Clavicipitaceae, and Ophiocordycipidae in Division (Phylum) Ascomycota and Hypocreales. In Thailand, there are some reports revealing that the *Cordyceps* fungal in Cordycipitaceae and *Ophiocordyceps* in Ophiocordycipidae is the mostly found one [1]. There is also the report on finding three kinds of *Cordyceps* spp. destroying the cicada larvae (which must be correctly as cicada nymphs) for the first time in the North-east of Thailand. One kind was found in Kuchinarai District, Kalasin Province and two kinds were found in Muang District, Maha Sarakham Province. However, the new name was not given or separated [2] which might be *Cordyceps cicadae* found in Thailand [3]. It was the fungal called cicada flower (*Cordyceps sobolifera*) in the past or *Ophiocordyceps sobolifera* (Zhu et al., 2016, pp.619-627) at present.

Several kinds of fungal of *Ophiocordyceps* spp. could be benefited in agriculture, medical, and pharmaceutical such as being used as microbial insecticides both in formulations and the production with simple local technology for controlling the insects as same as *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces* spp, etc. [4]. Regarding medical and pharmaceutical, there is the report on the medical properties of the fungal *Ophiocordyceps* spp. especially for the fungal *Ophiocordyceps sinensis* the fungal known well as "chong cao" (Dōng-chóng-xià-cǎo or called Chóng cǎo in short) on moth caterpillar in *Thitarodes* (Lepidoptera: Hepialidae) called "ghost moth". It was reported that this kind of fungal is used as Chinese herb from the ancient Chinese era. According to Scientific data, it was reported that some kinds of fungal in *Ophiocordyceps* can produce the bioactive compounds with medical and pharmaceutical activity namely cordycepin, ophicordin, galactomannan, and polysaccharide which are the bioactive compounds detectable in fruiting body of the fungal. After having tested, it was found that these substances had the activity of antitumor, antibacterial, antifungal, etc. [5] especially *O. sinensis* which was the kind found to destroy the caterpillar in *Thitarodes* in the highland in the autonomy area of Thibet, China having the report referring to very high marketing value [6]. Moreover, there was the report of finding the essential amino acids, vitamin K, vitamin B1, vitamin

B2, and vitamin B12 in the fungal *Ophiocordyceps* [7]. Therefore, the fungal in *Ophiocordyceps* is considered a kind of microorganism beneficially for human in medical and pharmaceutical fields.

There is the report referring the numbers, kinds, and varieties of fungal *Ophiocordyceps* partly both domestically and internationally [1], [6], [8]. However, as Thailand is one of the country having varieties in the kinds of microorganism highly as well as having the data sources on the classifications of fungal *Ophiocordyceps* spp. systematically and sufficiently for further studying in order to create the knowledge for benefiting from the existing natural resources, this research project studies the culture methods to increase the quantities of *Ophiocordyceps* fungal species found in the area of Doi Inthanon National Park, Chiang Mai Province, by selecting the artificial media suitable for cultivating each kind of fungal.

**Material and Methods: Study on the culture methods to increase the quantities of *Ophiocordyceps* species found in the area of Doi Inthanon National Park, Chiang Mai Province, by selecting the artificial media suitable for cultivating each kind of fungal.**

**Pure Fungal Separation:** Bring the specimens of *Ophiocordyceps* of each species to be separted to obtain the pure culture by using the methods of single spore isolation and tissue culture.

**Single Spore Isolation:** Rinse the soil or dirt out of the specimens with clean water. Sterilize the outer surface of the specimens by using the method of triple surface sterilization as follows. Soak in alcohol 70% for 1 minute and soak in sodium hypochlorite 5.25% for 1 minute. Then, soak in alcohol 95% for 30 minutes. Wash the excessive solution out by soaking in the steriled distilled water for 1 minute and then place the specimens on the culture plate with steriled tissue paper to absorb water until getting dry. Cut the specimens on the fertile part horizontally with the razor. Drop 1-2 drops of steriled distilled water on the slide. Use the forceps to pick the specimens and turn upside down on the side with the cut on the drops of distilled water for 2-3 times. The asci will be noticed in the opaque features floating in the distilled water. Touch the asci to be separated with needle. Use Pasteur pipette to suck the spore suspensions on the slide to put into the chloramphenicol 0.01% solution in 2 ml. steriled distilled water for removing the microorganism accompanied by the spores. Incubate at the room temperature (27 ± 2° C) in the darkness for 24 hours. Examine the sprouting of spores under the microscope of combined lens. Use micro pipette to suck the spore or sprouting part-spores to put into the culture plate with potato dextrose agar (PDA) 5, 1 spore or 1 part-spore each. Place at the room temperature (27 ± 2° C) in the darkness for 15-60 days. Notice the growth of mycelia. Confirm the results of pure fungal isolation of the fungal on the culture media which have to contain similar features in all points without the contamination of bacteria or yeast. Store the pure fungal in the test tube with media of potato dextrose agar in the refrigerator (temperature around 10° C) to be used in the experiment further.

**Tissue Culture:** Rinse the soil or dirt out of the specimens with clean water. Sterilize the outer surface of the specimens by using the method of triple surface sterilization as follows. Soak in alcohol 70% for 1 minute and soak in sodium hypochlorite 5.25% for 1 minute. Then, soak in alcohol 95% for 30 minutes. Wash the excessive solution out by soaking in the steriled distilled water for 1 minute and then place the specimens on the culture plate with steriled tissue paper to absorb water until getting dry. Cut the specimens on the fertile part horizontally with the razor. Use the needles to separate the tissue inside to be placed on 5 points on the culture plate with potato dextrose agar. Incubate at the room temperature (27 ± 2° C) in the darkness for 15-60 days. Notice the growth of fiber out of the tissue. Confirm the results of pure fungal isolation of the fungal on the culture media which have to contain similar features in all points without the contamination of bacteria or yeast. Store the pure fungal in the test tube with media of potato dextrose agar in the refrigerator (temperature around 10° C) to be used in the experiment further.

**Study on The Growth of Pure Fungal on the Media:** Take the pure culture *Ophiocordyceps* of each species to be cultured in the media of potato dextrose agar. Incubate at the room temperature (27 ± 2° C) in the darkness for 15-60 days. Use the cork borer of 5 mm diameter to bore the fiber at the colony edge. Put it on the media used in studying the growth of the pure fungal namely corn meal agar(CMA), Czapek's agar, malt exract agar (MEA), oat meal agar (OA), and potato dextrose agar (PDA). Incubate at

the room temperature (27 ± 2° C) in the darkness for 15 days. For some species of pure fungal of *Ophiocordyceps* slowly grown, the period is extended to 30-60 days. Notice and measure the diameters of the colony. Record the results.

**Study on the Growth of Pure Fungal on the Media at Various Temperatures:** Take the pure culture of *Ophiocordyceps* of each species to be cultured in the media of potato dextrose agar. Incubate at the room temperature (27 ± 2° C) in the darkness for 15-60 days. Use the cork borer of 5 mm diameter to bore the mycelia at the colony edge. Put it on the media used in studying the growth of the pure culture of *Ophiocordyceps* for such species to grow best for 6 sets of experiment. Repeat each set 3 times and bring the media plate of each set to incubate in the temperatures of 20, 25, 30, 37, 45° C the room temperature (27 ± 2° C) repectively in the darkness for 15 days. For some species of pure culture of Ophiocordyceps, the period is extended to 30-60 days. Notice and measure the diameters of the colony. Record the results.

**Study on the Growth of Pure Fungal on the Media at Various pH:** Take the pure culture of *Ophiocordyceps* of each species to be cultured in the media of potato dextrose agar. Incubate at the room temperature (27 ± 2° C) in the darkness for 15-60 days. Use the cork borer of 5 mm diameter to bore the mycelia at the colony edge. Put it on the media on which such species of *Ophiocordyceps* can grow best for 6 sets of experiment. For each set, adjust pH to be 4, 5, 6, 7, and 8 by using the lactic acid or hydrogen peroxide and the set without adjusting pH, respectively. Repeat each set 3 times and bring the media plate of each set to incubate in the temperatures which the pure culture of *Ophiocordyceps* of such species can grow best in the darkness for 15 days. For some species of pure culture of *Ophiocordyceps*, the period is extended to 30-60 days. Notice and measure the diameters of the colony by using the ruler. Record the results.

**Results:** ***The results of study on the methods to increase the quantities of Ophiocordyceps found by selecting the artificial media suitable for culturing each fungal***

**Isolation of the Pure Fungal:** From bringing the specimens of *Ophiocordyceps* of each species to be sterilized on the surface by using the method of triple surface sterilization and segregate for the pure fungal using the method of single spore isolation and tissue culture in the media of potato dextrose agar, according to the initial experiment, it is found that the isolation of pure fungal by using the method of single spore isolation gives better results than the method of tissue culture as there is less contamination of bacteria or yeast. This method is selected in segregating the fungal and can segregate the pure culture of *Ophiocordyceps* of every species.

**The Growth of Pure Fungal on Various Media:** From bringing the pure culture of *Ophiocordyceps* of each species to be cultured in 5 media; corn meal agar, Czapek's agar, malt extract agar, oat-meal agar, and potato dextrose agar and leaving at the room temperature (27 ± 2° C) for 15-60 days, it is found that most pure culture of *Ophiocordyceps* grow well in the media of potato dextrose agar followed by malt extract agar, corn meal agar, oat meal agar, and Czapek's agar, respectively. When comparing the growth period of *Ophiocordyceps* of each species, it is found that *Ophiocordyceps* sp.1, *Ophiocordyceps* sp.2, and *Ophiocordyceps sp.3* grow fast in the media whereas *O. unilateralis* grow quite slowly (Table 1).

**Table 1: Diameter of colony of *Ophiocordyceps* on Various Media at the Temperature Room (27 ± 2° C) for 15-60 days**

| *Ophiocordyceps* | Period (days) | Diameter of Media Colony (mm.) | | | | |
|---|---|---|---|---|---|---|
| | | Corn meal agar | Czapek's agar | Malt extract agar | Oat meal agar | Potato dextrose agar |
| *O.irangiensis* | 30 | 15 | 7.5 | 19.5 | 17.5 | 18.5 |
| *O.sobolifera* | 30 | 7.4 | 5.5 | 10 | 7.5 | 16 |
| *O.unilateralis* | 60 | 8 | 5.5 | 15.5 | 8 | 18 |
| *O.sphecocephala* | 30 | 16 | 6.5 | 18.5 | 13 | 22.5 |
| *Ophiocordyceps* sp.1 | 15 | 47 | 24.5 | 51.5 | 46.5 | 54 |
| *Ophiocordyceps* sp.2 | 15 | 49.5 | 26.5 | 53 | 49 | 57 |
| *Ophiocordyceps* sp.3 | 15 | 48 | 25 | 52 | 47 | 54 |

**The Growth of Pure Fungal On the Media in Various Temperatures:** When bringing the pure culture of *Ophiocordyceps* of each species to be cultured in potato dextrose agar which is the media growing best and incubate at various temperatures for 15-60 days, it is found that most pure culture of *Ophiocordyceps* grow best at the tempertures of 20, 25, and room temperature (27 ± 2° C). It grows well at 25° C. However from 4 species such as *O. iranensis*, *Ophiocordyceps* sp.1, *Ophiocordyceps* sp.2, and *Ophiocordyceps* sp.3 grow well at 30° C. From the experiment, no species can grow at 37 and 45° C (Table 2).

**Table 2: Diameter of Colony of *Ophiocordyceps* on the Media of Potato Dextrose Agar Incubated At Various Temperatures for 15-60 Days**

| *Ophiocordyceps* | Period (days) | Diameter of Media Colony (mm.)* | | | | | |
|---|---|---|---|---|---|---|---|
| | | Room temperature (27 ± 2° C) | 20° C | 25° C | 30° C | 37° C | 45° C |
| *O.irangiensis* | 30 | 20.5 | 19 | 20 | 7 | - | - |
| *O.sobolifera* | 30 | 17 | 17.5 | 19 | - | - | - |
| *O.unilateralis* | 60 | 18 | 21 | 22.5 | - | - | - |
| *O.sphecocephala* | 30 | 22.5 | 23 | 25 | - | - | - |
| *Ophiocordyceps* sp.1 | 15 | 54 | 55.6 | 59 | 6 | - | - |
| *Ophiocordyceps* sp.2 | 15 | 57 | 59.5 | 63 | 37 | - | - |
| *Ophiocordyceps* sp.3 | 15 | 56 | 57 | 60 | 37 | - | - |

*- signifies no growth

**The Growth of Pure Fungal on the Media at Various pH:** When bringing the pure culture of *Ophiocordyceps* of each species to be cultured in potato dextrose agar which is the media growing best. Adjust pH to become 4, 5, 6, 7, and 8 compared to the controlling set. Incubate at 25° C which is the temperature in which the pure fungal of *Ophiocordyceps* growing best for 15-60 days, it is found that most pure culture of *Ophiocordyceps* can grow well in the media of potato dextrose agar with pH from 5-6. In the media with pH8, the pure fungal of *Ophiocordyceps* cannot grow well (Table 3)

**Table 3: Diameter of Colony of *Ophiocordyceps* on the Media of Potato Dextrose Agar at Various pH Incubated at 25° C for 15-60 Days**

| *Ophiocordyceps* | Period (days) | Diameter of media colony (mm.) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Controlling set (pH ~5.6) | pH 4 | pH 5 | pH 6 | pH 7 | pH 8 |
| *O.irangiensis* | 30 | 21 | 18 | 21 | 21.5 | 16.5 | 15 |
| *O.sobolifera* | 30 | 18 | 17 | 18.5 | 18 | 14.5 | 13.5 |
| *O.unilateralis* | 60 | 23.5 | 20 | 22 | 22.5 | 13 | 10.5 |
| *O.sphecocephala* | 30 | 25 | 23.5 | 25 | 25.5 | 22 | 21 |
| *Ophiocordyceps* sp.1 | 15 | 59 | 56 | 59 | 61 | 54 | 52.5 |
| *Ophiocordyceps* sp.2 | 15 | 63 | 59.5 | 62.5 | 63 | 56.5 | 54.5 |
| *Ophiocordyceps* sp.3 | 15 | 59 | 57 | 58 | 62 | 55 | 53 |

**Discussion:** The isolation of pure culture of *Ophiocordyceps* can be done by using the method of single spore isolation and tissue culture in the media potato dextrose agar. According to the initial study, it is found that the isolation by using the method of tissue culture takes long time in culturing from tissue to grow to fiber. In addition, the contamination from bacteria or yeast on the tissue placed on the media often occurs. Therefore, the isolation of pure culture is difficult. For segregating by using the method of single spore isolation, it is found that spore can develop to mycelia and can grow on the media more rapidly. There is also less contamination. Thus, this method may be the method suitable for isolating the pure culture of *Ophiocordyceps*.

From the experiment in culturing the culture of *Ophiocordyceps* on the media of corn meal agar, Czapek's agar, malt extract agar, oat meal agar, and potato dextrose agar at the room temperature, it is found that *Ophiocordyceps* can grow in the media of potato dextrose agar better than other media. It is because this media contains the nutrients suitable for the growth of *Ophiocordyceps* most compared to other kinds of media (corn meal agar, malt extract agar, and oat meal agar). It is possibly because the media of potato dextrose agar contains the carbon which is the single molecule sugar. The culture of *Ophiocordyceps* can be used more easily whereas the carbon source in other kinds of media must pass the decomposition process before being applied. For the artificial media (Czapek's agar), the pure fungal of Ophiocordyceps cannot grow well because this kind of media contains the difficultly-decomposed carbon sources. There is also shortage of vitamin and mineral essential for the growth of mycelia. *Ophiocordyceps* can grow very little. If the formula of Czapek's agar is adjusted to be suitable for the growth of *Ophiocordyceps*, this kind of media can be used in testing the demand of the media source of the culture of *Ophiocordyceps*. This experimental result is correspondent with the experimental result of [9] culturing the pure culture of *Ophiocordyceps* in 7 species and [10] culturing the pure fungal of *Ophiocordyceps* in 6 species. It is found to grow well in the media of potato dextrose agar.

When comparing the growth rate in media of potato dextrose agar of the pure culture of *Ophiocordyceps* and the growth of other kinds of fungi, it is found that *Ophiocordyceps* can grow quite slowly. The cause is possibly because *Ophiocordyceps* wants some nutrients from insects in the growth of mycelia. It can be seen from the experimental of [11] experimenting to culture *Ophiocordyceps militaris* in the media with rice and insect's pupa. It is found that the pure culture of *Ophiocordyceps* can grow well and creating the reproduction structure in the media.

When using the pure culture of *Ophiocordyceps* to culture on the media of potato dextrose agar with different temperature of 20, 25, 30, 37 45° C and the room temperature (27 ± 2° C), it is found that *Ophiocordyceps* of all species can grow well in the temperature range of 20, 25 and the room temperature (27 ± 2° C). It can grow at 25° C. Some species can be grown well at 30° C but they can not grow well in the temperatures of 37 and 45°C., no growth of the pure fungal is found. This experimental result is quite similar to the culture of *Ophiocordyceps* of [12] and [10] reporting that the pure culture of *Ophiocordyceps* can grow well at 22 and 20° C, respectively.

From culturing the pure culture of *Ophiocordyceps* on the media of potato dextrose agar, pH is initially adjusted to 4, 5, 6, 7, 8, and not adjust pH (pH 5.6) at 25° C. It is found that the pure culture of *Ophiocordyceps* can grow well in the media of all experimented pH. It can be said that the width of colony is similar. The media with pH 5-6 will be the media with the mycelia of the pure culture growing best. It can be seen that *Ophiocordyceps* can grow well in the media with pH both acid and base. It will grow well in the media with the soft acidic media.

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