

# Culture of *Cordyceps myrmecophila* Cesati

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## ABSTRACT

*Cordyceps myrmecophila* Cesati was isolated for culture from discharged ascospores. The ascospores germinated only in high speed shaking condition in liquid medium but not in static solid culture media. The pellets harvested from liquid medium were cultured on PDA supplemented with sugars. Trehalose was found to be the only sugar to accelerate colony growth and increased stalk number formation of *C. myrmecophila*. None of the stroma-like stalks reached maturity to produce ascus or perithecium. However, the ITS-I and ITS-II rDNA sequence of cultured pellets were identical to those of the specimens.

**Key Words:** *Cordyceps myrmecophila*, Ascospore, ITSs.

## Introduction

*Cordyceps myrmecophila* Cesati is widely distributed around the world including many areas of Europe, North America, Brazil, Ceylon, Borneo, Thailand and China (Kobayasi, 1941; Hywell-Jones, 1996). In Taiwan, *C. myrmecophila* is also a frequently collected species of *Cordyceps* in the forest with an altitude of 500–1000 m. On ant, *Palthothyreus tarsatus*, Fab Formicidae Ponerinae, *C. myrmecophila* produces tiny and slender stalk in yellow color with cinnamon-brown ellipsoid fertile part on the top of the stalk (Nnakumusana, 1987). The perithecia submerge in fertile part and its filiform ascospore separates into secondary spores after discharge (Koba-

yasi, 1982). Hence *C. myrmecophila* is an easily collected species, but due to its small size, it is difficult to obtain pure culture by mean of the method of tissue culture that usually resulted in contamination. The present report used mature specimens from central part of Taiwan and ascospores were collected for the culture of *C. myrmecophila*. The pure culture was identified by the sequences of ribosomal DNA ITS-I and ITS-II regions.

## Material and Methods

### Specimen and strain isolation

Specimens of *Cordyceps myrmecophila* were collected on the litter of the forest in vicinity of Sun-Moon Lake at an altitude of 800 m located

in Yu-Tsu Village, Nantou County, Taiwan. The fresh specimens with fertile layer were hang over underneath of the cover of plates containing water agar (1.5% w/v) to allow the discharge of ascospores onto the surface of agar. The discharged ascospores with the agar slab were moved to slants containing potatoes dextrose agar (PDA, Difco, USA) or yeast extract malt agar (YEMA, glucose 1.5%, yeast extract 1%, malt extract 1%, and agar 1.5%) and temperature was kept at 23° C. The other part of agar slabs with the ascospores were transfer to 250 mL flasks containing 100 mL of yeast extract malt broth and followed by shaking under 250 rpm ( $\phi$  1 inch) at 23° C. The swelling and germination of the ascospores was observed under dissecting microscope for 70 days.

### Stalk formation

The germinated ascospores in liquid culture gradually grow into pellets. The pellets approximately 5 mm in diameter were selected and grown on the surface of PDA slants with the supplement of 2% sugars including glucose, mannose, galactose, sucrose, maltose and trehalose, respectively. Each supplement was triplicate. The diameter of colony and number of stalk formation were recorded for 30 days. A part of pellets were lyophilized for DNA extraction.

### DNA extraction and DNA sequencing of rDNA

The methods for DNA extraction, PCR amplification and DNA sequencing were using procedure modified from Raeder *et al.* (1985), Moncalvo *et al.* (1995) and Su *et al.* (2003) In brief, the specimens and the pellets of cultured colony were lyophilized to dryness and the dried samples were washed in 70% ethanol for

5 min. and sterile deionized water for 1 min. After being dried, the samples were ground into powder in liquid nitrogen. To 1.2 mL of extraction buffer (200 mM Tris-HCl, pH 8.5; 25 mM EDTA; 250 mM NaCl; 0.5% SDS) was added to 0.1 g of the powdered sample. DNA was extracted with one vol. (1.2 mL) phenol/chloroform/isoamyl alcohol (25:24:1) twice. After centrifugation at 15,000  $\times$  g for 45 min, 1 vol. isopropanol was added to aqueous phase. The pellet was centrifuged at 12,000  $\times$  g for 20 min. washed with 70% ethanol twice, and dissolved in 200 $\mu$ L sterilized 1/10X TE. The resulting solution was used for PCR reaction. A standard double strand PCR was applied to amplify the entire ITS-I and ITS-II regions using the primers that were described by White *et al.* (1990) and the procedure for the amplification and sequencing followed the method used by Su *et al.* (2003). In brief, PCR was performed in a total 100  $\mu$ L, which consisted of 1  $\mu$ M each of the forward primer: ITS 1 and reverse primer: ITS 2 for ITS-I, or the forward primer: ITS 3 and reverse primer: ITS 4 for ITS-II, 0.8 mM of dNTP, 40 ng genomic DNA as a template, and 2.5 U *Taq* DNA polymerase (PERKIN ELMER, U.S.A.) in 1X GeneAmp PCR buffer (PERKIN ELMER, U.S.A.). Forty reaction cycles were performed at 94° C, 62° C, and 72° C for 1, 1, and 2 min, respectively. PCR products were purified using a QIAquick<sup>TM</sup> gel extraction kit (QIAGEN, Germany) and dissolved in sterile water.

## Results and Discussion

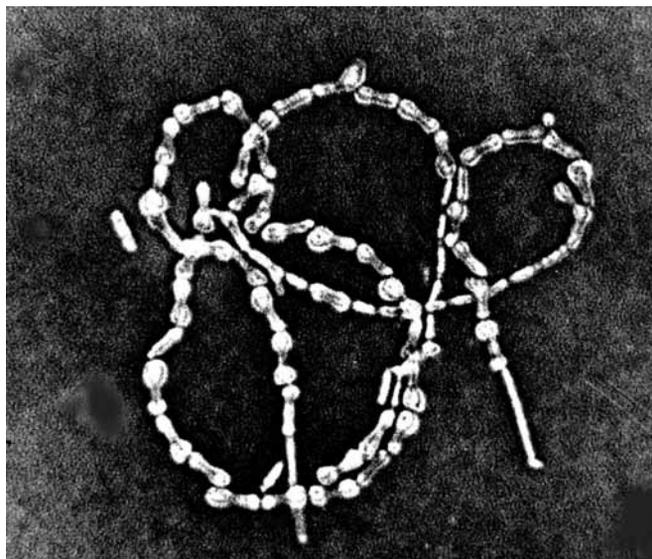
To isolate pure culture by tissue culture from collected specimens of *C. myrmecophila* had encountered difficulty with its tiny in stroma of

1 to 0.5 to 1 mm diameter. Therefore, to collect ascospores discharged from perithecia of fertile layer become one of the choices. Usually, fungal spore germination can be divided into three stages and that are activation, isotropic growth and polarized growth (d'Enfert, 1997). The fili-form ascospore collected from the agar plate is shown in Fig. 1 and the ascospore swelled and separated into secondary spores or the isotropic growth stage that were in good agreement with the description of Kobayashi (1941). However,

the ascospores on water agar or slants of PDA and YEMA showed recession in the swollen stage without further germinating into hyphae or polarized growth stage for at least 70 days after discharging (Fig 2). On the contrary, the ascospores in YEM liquid culture with shaking demonstrated vigorous growth (Fig. 3) and small pellets were produced in the liquid culture. The germination enhanced by vigorous shaking of ascospores in liquid medium might due to that some germination inhibitor existed



**Fig. 1.** Discharged ascospores of *C. myrmecophila*.



**Fig. 2.** The ascospore swelled into secondary spores with 3 to 4 days after discharge. The ascospores stayed in this stage for at least 70 days without germination on the surface of solid medium.



**Fig. 3.** Ascospores germinated in liquid shaking culture after 10 days after discharge.

on the surface of the ascospore and that was removed eventually during the shaking. The hypothesis may correlated with the proteins associate with the germination of spores (Roze *et al.*, 1999)

The pellets were transferred to slants and the growth rate and number of stalk formation are shown in Table 1. There were no differences between the growth rate as well as the number of stroma-like stalk for the groups of supplemented sugars on glucose, mannose, galactose, sucrose and maltose but only in the group of trehalose. It is presumed that insect usually stored trehalose as a main sugar type in its body and that facilitated the infection and growth of *Cordyceps*. It was also clear that the growth of mycelium and stalk formation required basically no special nutrition to achieve even this fungus was found parasitic on insect in nature.

The analyses of DNA sequence for cultured

**Table 1.** Colony diameter change and number of stalk formation of *C. myrmecophila* on PDA with 2% sugar supplements

Days of Observation	Glucose		Mannose		Galactose		Sucrose		Maltose		Trehalose	
	CD <sup>1</sup> (mm)	No. St <sup>2</sup>	CD (mm)	No. St	CD (mm)	No. St	CD (mm)	No. St	CD (mm)	No. St	CD (mm)	No. St
7	7.9 ± 0.6	1.3 ± 0.6	7.8 ± 0.9	0.3 ± 0.5	7.4 ± 1.3	1.0 ± 1.0	7.7 ± 0.7	0.7 ± 0.6	0.8 ± 0.4	1.3 ± 0.6	8.6 ± 0	3.0 ± 1.0
14	13.3 ± 1.41	3.0 ± 1.0	12.5 ± 1.1	2.3 ± 0.6	10.6 ± 1.9	3.0 ± 1.0	10.5 ± 0.8	2.0 ± 1.0	11.1 ± 1.4	4.1 ± 1	14.2 ± 1	7.7 ± 0.6
21	15.6 ± 0.7	7.3 ± 1.5	15.2 ± 0.8	7.7 ± 1.2	14.0 ± 2.6	6.3 ± 1.5	12.7 ± 0.8	6.3 ± 0.6	13.17 ± 1.1	7.7 ± 0.6	19.9 ± 2.7	14.7 ± 1.5 <sup>3</sup>
28	19.71 ± 0.9	12.0 ± 1.5	17.5 ± 1.3	10.7 ± 1.5	16.4 ± 1.0	9.0 ± 3.0	15.6 ± 1.4	10.7 ± 1.5	15.0 ± 0.2	11.6 ± 2.5	24.1 ± 0.6	19.0 ± 1.0 <sup>3</sup>

<sup>1</sup>Diameter of colony; <sup>2</sup>Number of stalk; <sup>3</sup>Significance with  $p < 0.01$

pellets derived from ascospores were identical to that of natural specimens in our previous report (Su *et al.*, 2003) and it might confirm that the isolation of the strain was in very high possibility belonging to *C. myrmecophila* without contamination. The sequences of ITS-I and ITS-II of the culture are shown in Fig. 4.

The colony produced on agar slants derived from liquid culture pellet was in a pale gray and non-spreading spherical form with a growth rate approximately 0.5–0.6 mm per day at 23° C. No obvious conidium was found in the culture; except some blastospore-like struc-

ture was detected on the surface of aging hyphal fragment (Fig. 5). The stromata initiated with bright yellow to orange color and they elongated into slender 50 to 80 mm in length that was in high similarity to the specimens collected in the nature (Fig. 6). However, no mature ascus or perithecium was observed on the stalks produced in flasks or test tubes through the experiment. To achieve maturity of the sexual organ in higher fungi usually required paired mating types in a strain as well as proper environmental stimulation (Su, 1986). The present study initiated an opportunity to

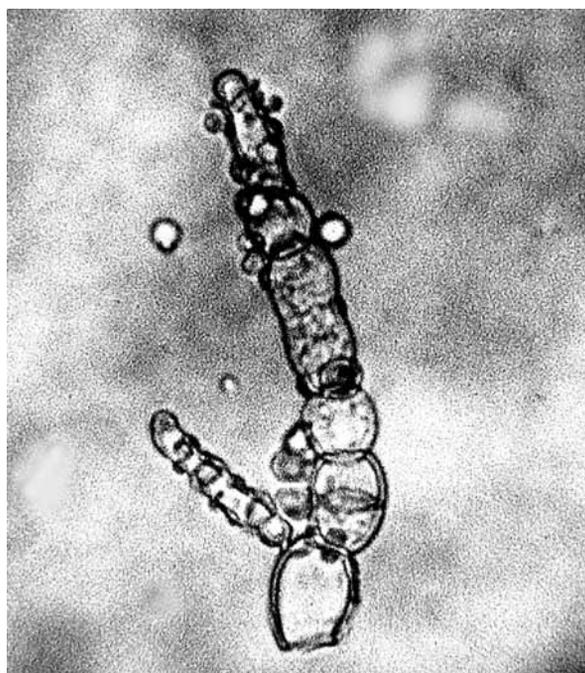
#### ITS-I

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GTAGGTGAACATGCGGAGGGATCATTAAGGAGTG-----ATAACGCGGGAAACTCTCCG-AACCTTTTGTGACCTTACC-----TATTGTTGCC
TCG-----GCAGGGAGTGTTTCTAGCGC--TT---TAAACTAGTGGCCCCGGGC---CAAAGGACCCCTA--TAACGCATT-TTTTTAGT-----GTCITCA
G.AAGGAAATAAAACA---AGTAAAAACTTTTAG.CAACGGAT-----CTCTGGCTCTAGCATCGA---TGAAGA---ACGCAGCA-TGGGAAGA--A
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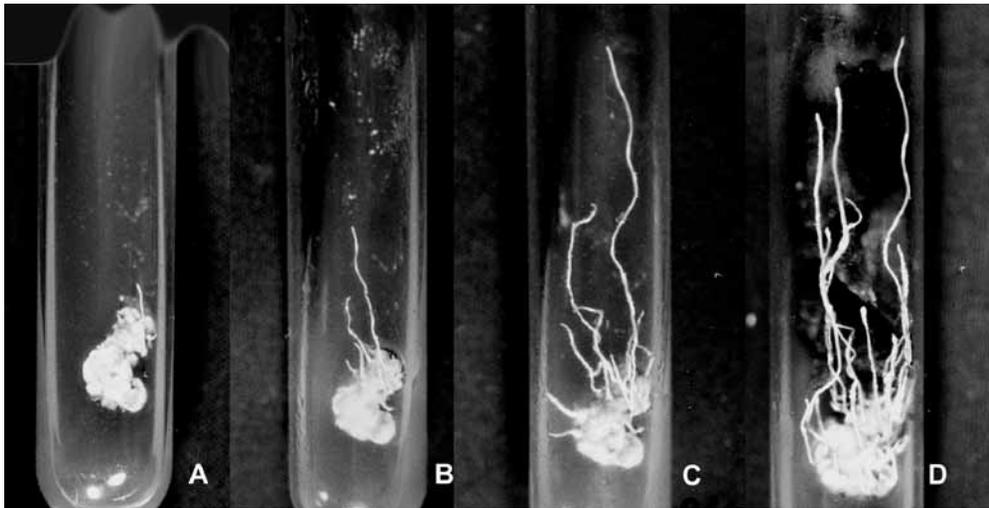
#### ITS-II

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AGCAAAATGCGAAAAGTAGTGCGAATTGCAGA--TTAGTGAGTCATCGAATCTTTGAACGCATATTGCGCCTGCTAGCAACTATAGCAGGCATGCCTG-T
CCGAGCGTTATTACATC-----ACTAGACA---GTAGTCTATGTTGGAGATTA-GTG-----GAATACTAGCT--CTG
AAATACAGTGGT-----CTAAAGCCCTTCT--ATATGCTTGTAATAGTATAA--CTTGCTTACTTGCAITGTAGAGAGTGTAGGTGCCG--AAACC
TCTTAG-----TTAACCTCGGATCAGGTAGGGATACCCGCTGAACTTAA
```

**Fig. 4.** ITS-I and ITS-II sequences of *Cordyceps myrmecophila* cultured pellets.



**Fig. 5** Blastospores-like conidia produced on the surface of aged hyphal fragment.



**Fig. 6.** Stalk formation of *C. myrmecophila* on PDA supplemented with trehalose. A. 7 days after transplanting, B. 14 days, C. 21 days, and D. 28 days.

elucidate the mating system and the behavior of parasitism of *Cordyceps* on insects.

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### 摘 要

採集自南投魚池鄉之蟻生蟲草標本以釋放之子囊孢子進行培養。搜集之子囊孢子僅在激烈震盪之液態培養基中發芽，於固態培養基之表面只形成膨大之次孢子。經液態培養之菌絲球於添加不同醣類之馬鈴薯葡萄糖培養基中發現海藻醣具有加速菌落生長及增加菌座數目之作用。在全程試驗中並無成熟之子座產生，但天然標本與培養菌球間 ITS-I 及 TS-II 之 DNA 序列比對則完全相同。

**關鍵詞：**ITS、子囊孢子、蟻生蟲草。