Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Invertebrate Pathology 85 (2004) 70–73
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Isolation of vegetable wasps and plant worms, *Cordyceps nutans*, from fruit-body tissue

Fumito Sasaki,* Toshizumi Miyamoto, Yutaka Tamai, and Takashi Yajima

Laboratory of Forest Resource Biology, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

Received 16 September 2003; accepted 20 February 2004

Abstract

We isolated *Cordyceps nutans* from the stipe and abdominal tissues of fruit bodies using a surface sterilization method. Hyphal growth was observed in inocula from both the stipe and abdominal tissue. Some strains from discharged ascospores were obtained and colony characteristics were compared to the strains isolated from the tissues. Colonies of isolates from ascospores grew quite slowly. Isolates of 43 from the 52 examined fruit bodies formed colonies similar to those from ascospores. To confirm the success of isolation, we analyzed by PCR-RFLP of the ITS regions of rDNA samples from fruit bodies, isolates from fruit bodies, and isolates from ascospores. All the isolates obtained from stipe and abdominal tissues presented identical patterns. In this study, we report the first successful isolation of *C. nutans* from fruit-body tissue using a surface sterilization method.

Keywords: *Cordyceps nutans*; *Hymenostilbe nutans*; Biological control; Entomoparasitic fungi; ITS; PCR-RFLP; Hemipteran insects

1. Introduction

Species in the genus *Cordyceps*, known as entomoparasitic fungi, kill their hosts and form fruit bodies. Some species used as herbal medicines are also expected to be highly selective biological insecticide (Ito and Hirano, 1997). Isolates of *Cordyceps* spp. are generally obtained from discharged ascospores or from ascospores in ascocarps. However, *Cordyceps* spp. may be collected as immature or sterile fruit bodies, and the isolation method from these fruit bodies is not sufficiently established. Establishment of an effective and practical isolation technique for ascospores will contribute to elucidation of the life-cycles of *Cordyceps* spp., the search for useful strains, and the classification of this genus.

Cordyceps nutans Pat. is an entomoparasitic ascomycetes belonging to the Clavicipitaceae genus *Cordceps* that parasitizes hemipteran insects and has been observed in Japan, Taiwan, China, and New Guinea among other places. The fruit bodies arise between the head and thorax or mouth of hemipteran insects. The black wiry stem (stipe) 35–100 mm long has a red to light orange-yellow clavate head that contains the ascocarp. The head is not always formed and a needle-like apex on the stipe indicates it is sterile (Shimizu, 1994) or immature. These are very similar to mature fruit body, but they do not have ascospores.

Isolation of *C. nutans* had been previously attempted. Samson and Evans (1975) collected *C. nutans* and its anamorph *Hymenostillbe nutans* in Ghana. They described morpholic feature of *H. nutans* but were unable to isolate it from the conidia. Sung et al. (1993) succeeded in isolating *C. nutans* from ascospores but not from abdominal tissue on potato dextrose agar (PDA) medium. Hywel-Jones (1995) attempted isolation from abdominal hyphal bodies and ascospores but succeeded only in isolation from conidia on PDA medium. Moreover, Hirotani and Furuya (1986) were unable to isolate *C. nutans* from abdominal hyphal bodies on media.

Thus, effective methods of isolation of *C. nutans* have not been established. Without an adequate isolation method, we cannot obtain the organisms needed to

* Corresponding author. Fax: +11-81-11-706-2536.
E-mail address: whomeet@for.agr.hokudai.ac.jp (F. Sasaki).

0022-2011/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2004.02.007

screen for practical use and to study the mechanism of genetic distribution. We present here the first reported successful isolation of *C. nutans* from stipe and abdominal tissue using a surface sterilization method.

2. Materials and methods

2.1. Sampling

Fruit bodies of *C. nutans* were collected in Tomakomai Experimental Forest, Forest Research Station, Field Science Center for Northern Biosphere, Hokkaido University from July to September, 2002. This site is located on the north island of Japan. Specimens were transported to a laboratory individually in plastic bags and stored at 10 °C before isolation within a day.

The fruit bodies were identified as *C. nutans* according to color iconography of vegetable wasps and plant worms (Shimizu, 1994).

2.2. Isolation

The stipe was cut apart from insect bodies, and then cut and divided into 5–6 segments about 10 mm long. The insect body was cut at the center of the abdomen and divided into two pieces. Half of each stipe segment and abdominal piece from each fruit body was immersed in 30% H_2O_2 for 30 s. The other halves were treated in 30% H_2O_2 for 5 min and then rinsed sufficiently in sterile distilled water and dried on the sterilized filter paper in petri dishes for a few minutes. The stipes were inoculated onto Sabouraud–glucose agar medium (glucose 2%, peptone 1%, agar 2%, streptomycin 0.002%, pH 6.5). The small pieces (about $2 \times 2 \times 2$ mm) of tissue were cut out from the center of the abdominal tissue samples with a scalpel and inoculated on Sabouraud–glucose agar medium. A total of 341 inocula from 52 fruit bodies were isolated.

We also evaluated an isolation method from discharged ascospores. Ascospores collected on a sterilized petri dish were incubated by streak culture on Sabouraud–glucose agar medium. Single colonies were transferred to new medium every 3 months and incubated at 23 °C in the dark.

Stipes were preserved by freezing at −20 °C, and part of the insect bodies were preserved at room temperature after drying at 60 °C. The fruit-body specimens and isolates examined are preserved in the Laboratory of Forest Resource Biology, Hokkaido University.

Germination of ascospores was confirmed by examination with a dissecting microscope (STZ-40TBIT, Shimadzu Rika Instruments) and an inverted microscope (IMT-2, Olympus Optical). After 30 days, the growth rate and morphology of isolates from fruit-body tissues were compared to isolates from ascospores. The color code names of the colonies were determined according to Munsell (1990).

3. DNA analysis of isolates

3.1. DNA extraction

Fungal DNA was extracted from the frozen sample of stipe (about 10 mm), a piece of isolated fungal mycelium grown on Sabouraud–glucose agar medium (about $3 \times 3 \times 3$ mm), and a piece of fungal mycelium derived from ascospores (about $3 \times 3 \times 3$ mm). We used Isoplant (Nippon Gene) following the manufacturer's guide.

3.2. PCR amplification

We used the PCR amplification method of Shih et al. (1995), with slight modifications. Extracted DNA was dissolved in 50 μl of TE buffer (Nippon Gene), and 1 μl of the DNA solution was used as template DNA. The internal transcribed spacer (ITS) region of nuclear rDNA has a highly conserved DNA sequence, so we used it as the amplification region. We employed universal primers for fungi ITS1 and ITS4 (White et al., 1990). The puRe *Taq* Ready-To-Go PCR Beads (Amersham Biosciences K.K.) and the GeneAmp PCR System 2400 (Perkin–Elmer) were used. The samples were run at an initial denaturation for 30 s at 94 °C, followed by 40 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 50 °C, and extension for 2 min at 72 °C. The amplified PCR products were electrophoresed for 55 min at 100 V (Mupid-2, Advance) on 3% NuSieve GTG agarose (FMC BioProducts) gels in 0.5× TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM $EDTA \cdot 2Na \cdot 2H_2O$, pH 8.0). A 100 bp DNA ladder (TaKaRa Bio) was used for as molecular DNA markers. After staining with 1 μg/ml ethidium bromide for 20 min, the band patterns were evaluated under UV irradiation. When only one DNA band was present in a PCR product, the product was analyzed for restriction fragment length polymorphism (RFLP).

3.3. RFLP analysis

Samples of 9 μl of the PCR products were digested separately with *Eco*RI, *Hha*I, or *Alu*I (TaKaRa Bio) following the manufacturer's guide. The restriction fragments were electrophoresed, stained, and checked by the same method as used above.

3.4. Statistical analysis

The isolation rate of *C. nutans* was calculated as the number of *C. nutans* inoculates divided by the total

number of inoculates. A χ^2 test was performed to determine whether the treatments were significantly different ($p < 0.05$). The Bonferroni method was used for multiple-comparison procedures.

4. Results

Nine of the 52 fruit bodies collected were immature or sterile. Furthermore, few of the mature stipes discharged ascospores. We obtained the strains from the ascospores of three fruit bodies.

The color of the colonies varied from pale (pale yellow (2.5Y 7/4∼8/2), white (10R 8/1), and pink (10R 8/4)) to dark (reddish brown (5YR 4/4) and dark reddish brown (5YR 3/2)). Colonies were covered with fibrous interwoven aerial hyphae. In this study, isolates from ascospores grew very slowly after germination. After 30 days of incubation, colonies attained a diameter of no more than about 5 mm and stopped growing.

In this study, some isolates were obtained from ascospores, which grew very slowly following germination. We provisionally concluded that the isolates with slow growth and morphologically similar to colonies from ascospores were *C. nutans*-like fungi.

Mycelial growth from inocula was observed in 72% of both stipe and abdominal tissue inoculated. *C. nutans*-like fungi accounted for about 38% of it.

PCR-RFLP analysis was conducted as a method for confirmation of isolates from *C. nutans.* RFLP patterns of the fruit body tissues, isolates from the ascospores, and isolates of *C. nutans*-like fungi from the fruit body were compared. DNA extracted from these samples was amplified with the primer pairs ITS1 and ITS4. All the PCR products exhibited bands of about 560 bp (Fig. 1, lane 1). These amplifications were then analyzed by RFLP, and the same band patterns appeared in all of them. *Eco*RI did not present fragments from the PCR products. Five bands of about 130, 170, 300, 340, and 380 bp resulted from *Alu*I digestion (Fig. 1, lane 2). Two bands of about 250 and 300 bp resulted from *Hha*I digestion (Fig. 1, lane 3). These results supported that the isolates were pure and not including contaminations.

The *C. nutans* isolation rates in each experiment are shown in Table 1. The different sterilization times did not significantly (52.2/56.0%) affect the isolation rates from abdominal tissue. The isolation rate from the stipes after a 5 min sterilization (27.7%) was significantly higher than that after a 30 s sterilization (7.9%).

The isolation rates from abdominal tissue were significantly higher than those from the stipe at each sterilization time. The percentages of successful *C. nutans* isolations in each treatment were 11.5% (6 per 52 fruit bodies) following a 30 s sterilization of the stipe, 47.1% (24 per 51 fruit bodies) following a 5 min sterilization of the stipe, 53.3% (24 per 45 fruit bodies) following a 30 s sterilization of the abdominal tissue, and 54.2% (26 per 48 fruit bodies) following a 5 min sterilization of the abdominal tissue.

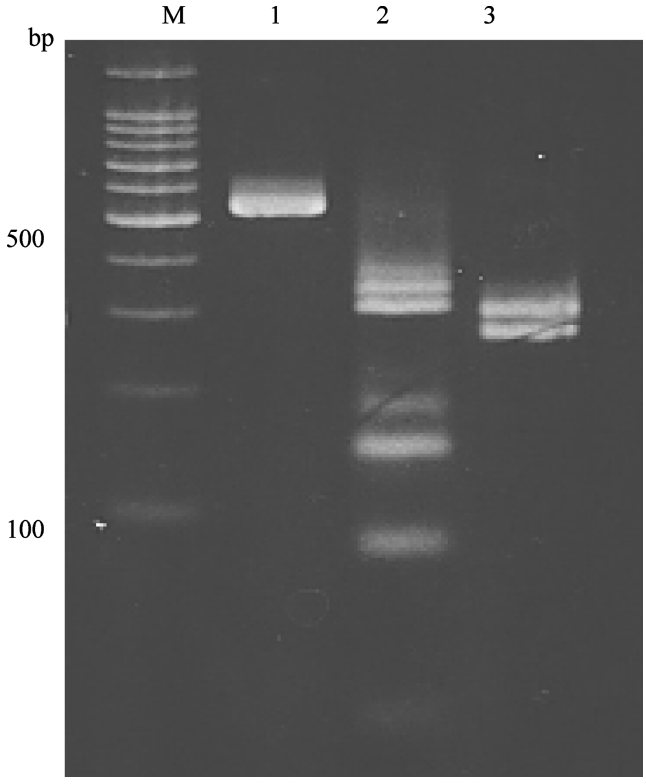

Fig. 1. Amplified DNA and RFLP patterns of ITS region of rDNA of *C. nutans*. Lane M, 100 bp ladder marker; 1, amplified DNA; 2, *Alu*I digestion; and 3, *Hha*I digestion.

Table 1
Fungal isolation of *C. nutans*

H_2O_2 treatment	Source	Isolates/inocula*
30 s	Stipe	10/126 a
	Abdominal tissue	24/46 c
5 min	Stipe	33/119 b
	Abdominal tissue	28/50 c

*Values followed by the same letter were not significantly different ($p < 0.008$) according to Bonferroni method.

The number of contaminants determined not to be *C. nutans* in each treatment were 88 and 27 following the 30 s sterilization of the stipe, 51 and 33 following the 5 min sterilization of the stipe, 6 and 15 following the 30 s sterilization of the abdominal tissue, and 7 and 15 following the 5 min sterilization of the abdominal tissue, respectively. Many more contaminants were observed in inocula from the stipe than from the abdominal tissue.

5. Discussion

In this study, isolates from ascospores grew very slowly after germination, and the colony diameters were

not more than about 5 mm after 30 days of incubation. Hywel-Jones (1995) also noted that *C. nutans* colonies grew quite slowly after germination, but following total immersion the colony diameters grew to 10 mm after 30 days. The isolated colonies were white to dark reddish brown, with entangled fibrous aerial hyphae, while Hywel-Jones described the hyphae as hyaline and sparse. We suspect that the different results related to the presence and type of hyphae were due to the different types of media used and the different characteristics of isolates.

Generally, *Cordyceps* spp. grow slowly (Sung et al., 1993). In PDA medium (Hywel-Jones, 1995) and in Sabouraud–glucose agar medium (this study), *C. nutans* also grows quite slowly. These media appear to be useful for isolation, but further evaluation of different media compositions is necessary to determine optimal media compositions for culture of *C. nutans*.

Cordyceps nutans grows naturally mainly in forests and arises from the stipe of the insect body in the humus layer. Therefore, surface sterilization is one of the important factor for its successful isolation from the fruit-body.

Sung et al. (1993) were unable to isolate *C. nutans* because of contamination. In addition, Hirotani and Furuya (1986) attempted to isolate *C. nutans* from abdominal tissue using surface sterilization for 20 s with 70% ethanol but did not observe hyphal growth.

Although it is necessary to try other surface sterilization methods (e.g., sodium hypochlorite), our results from the use of 30% H_2O_2 demonstrate that isolation is possible from tissue. The length of the surface sterilization period (30 s versus 5 min) did not affect the isolation rates from abdominal tissue. Consequently, we used a 30 s surface sterilization period with 30% H_2O_2 to isolate *C. nutans* from abdominal tissue. But the isolation rate from the stipe after a 5 min sterilization period was higher than that after a 30 s sterilization period. Thus, a longer sterilization period is required for isolation from the stipe. The isolation rate of *C. nutans* from abdominal tissue was higher than that from the stipe.

Although the isolation rates from abdominal tissues are high, small amounts of inoculum are available. In the stipe, however, many inocula from one fruit body can be used, although isolation success rates are low. Consequently, isolation should be attempted from both the stipe and abdominal tissues from a single fruit body to increase the probability of isolation.

Comparison of the contamination rates suggests that the stipe is contaminated rather heavily with filamentous fungi, while the abdomen is a more exclusive environment for other fungi.

In this study, only 9 of the 52 fruit bodies collected (about 13.5%) were immature or sterile; however, immature or sterile fruit bodies are more common in nature (Shimizu, 1994). Although we collected many mature fruit bodies, discharge of ascospores was observed in only 3 fruit bodies. We demonstrate here that isolation of *C. nutans* from the stipe and abdominal tissue is possible, and isolation from the fruit-body tissue is valid when ascospores cannot be obtained. Incidentally we tried to isolate other *Cordyceps* spp., *Cordyceps heteropoda*, *Cordyceps sphecocephala*, and *Cordyceps tricentri*, with this method, and succeeded (data not shown). So this method can be applied to the isolation of other *Cordyceps* spp.

References

Hirotani, M., Furuya, T., 1986. Animal Materials. Isolation Method of Microorganisms (in Japanese title). R&D Planning, Tokyo, pp. 211–219 (in Japanese).

Hywel-Jones, N., 1995. Notes on *Cordyceps nutans* and its anamorph, a pathogen of hemipteran bugs in Thailand. Mycological Research 99, 724–726.

Ito, Y., Hirano, T., 1997. The determination of the partial 18S ribosomal DNA sequences of *Cordyceps* species. Letters in Applied Microbiology 25, 239–242.

Munsell, A.H., 1990. Munsell Soil Color Charts, revised ed. Macbeth Division of Kollmorgen Instruments, Baltimore, Maryland.

Samson, R.A., Evans, H.C., 1975. Notes on entomogenous fungi from Ghana. 3. The genus *Hymenostilbe*. In: Proceedings, Koninklijke Noederlandse Akadamie van Wetenschappen (Series C), vol. 78, pp. 73–80.

Shih, H.L., Lin, C.P., Liou, R.F., Tzean, S.S., 1995. Complete nucleotide sequence of *Beauveria bassiana* 5.8s rRNA coding gene and flanking internal transcribed spacers. The Journal of Sequencing and Mapping 5, 381–383.

Shimizu, D., 1994. Color Iconography of Vegetable Wasps and Plant Worms. Seibundo Shinkosha, Tokyo, pp. 198–199 (in Japanese).

Sung, J.M., Kim, C.H., Yang, K.J., Lee, H.K., Kim, Y.S., 1993. Studies on distribution and utilization of *Cordyceps militaris* and *C. nutans*. The Korean Journal of Mycology 21, 94–105.

White, T.J., Brums, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols. Academic Press, San Diego, pp. 315–322.