In vitro Symbiosis between Gastrodia elata Blume (Orchidaceae) and Armillaria Kummer (Tricholomataceae) Species Isolated from the Orchid Tuber

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An Armillaria species isolated from Gastrodia elata was identified as A. gallica based on morphological characteristics of basidiomata and mating test. Symbiosis between G. elata and A. gallica was confirmed by using two-member cultures. Hyphal coils of the fungal symbiont were formed in the cortical cells of tubers and isolated from their cortical cells. Length and weight of fungus-infected tubers were significantly higher than those of non fungus-infected tubers and control (P < 0.05). These indicated that G. elata has a mycorrhizal symbiotic relation to A. gallica.

Key words: Armillaria, Gastrodia elata, mycorrhiza, symbiosis, two-member culture.

Gastrodia elata Blume is an achlorophyllous orchid that is unable to exist as an autophyte (Fig.1). Gastrodia elata therefore makes symbiotic mycorrhiza with Armillaria species and lives together (Kusano 1911). The tuber of G. elata is used as a natural medicine for headache and vertigo as “Tenma” in Kampo medicine (Hatakoshi 1947, Japanese Pharmacopoeia 2006) and as “Tian-ma” in Chinese medicine (Pharmacopoeia of the People’s Republic of China 2005). Though it has been produced by cultivation in China, there still remain many ambiguous issues about the symbiosis.

The association between G. elata and Armillaria has been reported by Kusano (1911) and Hamada (1940). Cha and Igarashi (1995) reported five Armillaria species obtained from G. elata tubers. However, these studies were conducted based on materials from a limited area in Hokkaido and no report has been done to clarify the symbiosis experimentally by using in vitro two-member culture.

Terashita (1985) reported a two-member culture between Galeola septentrionalis Rchb. f. and Armillaria. Tashima et al. (1978) recognized the symbiosis between Gastrodia verrucosa Blume and an unidentified fungus in vitro. In order to prove the symbiosis between achlorophyllous orchid and fungus, it is necessary to carry out two-member culture.

The objects of this study are: to recognize the symbiosis between G. elata and Armillaria by using two-member culture in vitro; to investigate the vegetative reproduction process of G. elata, and to identify the symbiont of G. elata in Honshu, Japan.
Materials and Methods

Collection of Gastrodia elata tuber

Tubers of *G. elata* were collected in 2000 and 2001 from *Quercus acutissima* forests in Ryugasaki City, Ibaraki Prefecture, Japan. To collect young tubers, whole plants were dug with soil and were washed carefully. There were two types of young-stage tubers. One was adhered by rhizomorphs of *Armillaria* species on the surface and the other was not. The former type collected in 2000 was used for isolation of fungal symbiont and the latter collected in 2001 was used for the association experiment (Table 1). For the subsequent experiments, all the tubers were washed twenty times with sterilized water to avoid contamination.

Isolation of the fungal symbiont

Several tubers adhered by rhizomorphs were selected for fungal isolation. After washing, the tubers were cut into serial 3 mm segments with a sterilized scalpel. The fungal symbiont was isolated by picking up hyphal coils from the cortex cells of the tuber using a sterilized platinum loop under a dissecting microscope and transferring these structures to Petri dishes containing potato-dextrose-agar medium (PDA, Difco). The Petri dishes were incubated at 25ºC in the dark.

Basidioma formation of symbiont

One hundred and twenty milliliters of the sawdust medium (beech sawdust: rice bran 3 : 1 ; 60% moisture content) was put into a 300 mL plastic cultivation bottle. An inoculation hole of 15 mm in diam. was made in the center of the medium. Then the bottles were autoclaved at 121ºC for 15 min. Ground carrot (5 g) autoclaved in the same way, was put into the bottle of the inocula-

<table>
<thead>
<tr>
<th>Rhizomorphs</th>
<th>Tubers in the field</th>
<th></th>
<th>Tubers in vitro</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>observed</td>
<td>mycorrhizal</td>
<td>observed</td>
<td>mycorrhizal</td>
</tr>
<tr>
<td>Adhesion</td>
<td>19</td>
<td>9</td>
<td>26</td>
<td>19</td>
</tr>
<tr>
<td>Non adhesion</td>
<td>104</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>123</td>
<td>9</td>
<td>26</td>
<td>19</td>
</tr>
</tbody>
</table>

Fig. 1. *Gastrodia elata* in the field. The photograph was taken at Kessoku-cho, Ushiku City, Ibaraki Pref., Japan, on 4 June 1995.
tion hole. Two grams of the fungal inoculums grown on PDA medium were put on the carrot. The cultivation bottles were kept at 25.0±0.5°C for 40 days in the dark. For fruiting induction, the bottles were subjected to 16.0±1.0°C under intermittent illumination with 350 lx alternated with the dark every 12 hours (Togashi and Takizawa 1992). Four replicates were made.

Mating test

The haploid isolates of fungal symbiont were each isolated from a single spore of basidioma. The single spore isolates of symbiont were mated with haploid tester strains of Armillaria species isolated from the basidioma collected in Japan (Table 2). Mating experiments were performed by placing inocula 1 mm apart on a 1.25% malt extract agar and incubated at 25±0.5°C for 4 weeks in the dark. The single spore isolates of an Armillaria symbiont were mated with haploid tester strains of several Armillaria species. The tester strains were first paired in all combinations in order to assay the pattern of the appearance of compatible or incompatible pairings. Results of the mating tests were judged according to Korhonen (1978). The pairings were conducted in triplicates.

Association experiment in the sawdust medium

Fifty grams of the sawdust medium was placed in a 300 mL Erlenmeyer flask and autoclaved at 121°C for 15 min. Inoculation plugs of symbiont 1 cm in diam. were inoculated on the sawdust. The culture was incubated at 25.0±0.5°C for 6 months until fungal rhizomorphs were formed actively. Eight cultures were made in this way.

Three or four tubers without rhizomorph adhesion were put into the incubating flask. The tubers were covered with the sawdust and incubated at 15±0.5°C for 3 months in the dark. As the control, eight tubers were incubated in two flasks in which no fungal symbiont was inoculated. After the incubation, the tubers were examined in length, weight, adhesion of rhizomorphs, and mycorrhizal formation. For confirmation of the mycorrhizal peloton, tubers were sectioned and observed under a dissecting microscope and scanning electron microscope (SEM). Mycorrhizal peloton was isolated from the tuber using a dissecting microscope and cultured at 25.0±0.5°C for 2 weeks on PDA.

Results

Gastrodia elata in the field

Host trees of fungal symbiont were not determined by tracing rhizomorphs because many rhizomorphs were distributed over the forest at the depth of 10–15 cm. Rhizomorphs developed well around flowering tubers. Many tubers of G. elata grew around a decayed mother tuber located just under the flowering tuber. The flowering tuber and

Table 2. Haploid testers of Armillaria species and their origins in Japan

<table>
<thead>
<tr>
<th>Biological species</th>
<th>Host tree</th>
<th>Locality</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. gallica</td>
<td>Abies veitchii</td>
<td>Mt. Simagareyama, Nagano Pref.</td>
</tr>
<tr>
<td>A. cepistipes</td>
<td>Cryptomeria japonica</td>
<td>Mt. Simagareyama, Nagano Pref.</td>
</tr>
<tr>
<td>A. ostoyae</td>
<td>Chamaecyparis obtusa</td>
<td>Mogami Town, Yamagata Pref.</td>
</tr>
<tr>
<td>A. tabescens</td>
<td>Quercus acutissima</td>
<td>Siwa Town, Iwate Pref.</td>
</tr>
<tr>
<td></td>
<td>C. japonica</td>
<td>Iwate Town, Iwate Pref.</td>
</tr>
<tr>
<td></td>
<td>Q. acutissima</td>
<td>Ami Town, Ibaraki Pref.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ami Town, Ibaraki Pref.</td>
</tr>
</tbody>
</table>
other tubers were budded from the mother tuber that was attached and penetrated by rhizomorphs (Figs. 2, 3).

Some daughter tubers were attached by rhizomorphs extended from the mother tuber (Fig. 4). In total one hundred and twenty-three tubers were collected in the field. Among them were nineteen rhizomorph-attached tubers, and nine mycorrhiza-formed ones (Table 1). Mycorrhizal formation was observed only in rhizomorph-attached tubers.

Isolation of the fungal symbiont

After 3 weeks cultivation all isolates from the hyphal coils grew well and produced dark brownish crustose colonies on PDA medium. They were confirmed to be *Armillaria* sp. based on colony morphology, mycelial growth and rhizomorph formation on PDA plates. One of these cultures was used for subsequent examinations.

Identification of *Armillaria* species

Following dark cultivation a crustose mycelium was formed on the surface of the sawdust in the 300 ml cultivation plastic bottle. Basidiomata were formed on the mycelium for a further 3 weeks incubation under the light. Scales on the pileus were dark gray. Base of the stipe was swollen. Annulus was thin, arachnoid and evanescent (Fig. 5). These characteristics corresponded to those of *Armillaria gallica* Marxmuller &
Results of the mating tests are shown in Table 3. The fluffy mycelium of fungal symbiont developed into a crustose mycelium. The symbiotic Armillaria isolated from G. elata tuber was compatible with testers of A. gallica.

Fig. 4. Daughter tubers attached and penetrated by rhizomorphs extended from the mother tuber. Bar = 4 mm.

Fig. 5. Basidiomata of Armillaria gallica. The basidioma was formed after three weeks incubation under the light.

Romagnesi.

Results of the mating tests are shown in Table 3. The fluffy mycelium of fungal symbiont developed into a crustose mycelium.
Association experiment in the sawdust medium

In Armillaria-inoculated bottles, rhizomorphs were formed on the sawdust covering the tuber by 3 months cultivation. All the tubers were adhered by the rhizomorphs to the surface (Table 1) and formed new buds (Fig. 6). Of these, 19 tubers showed mycorrhiza (Figs. 8, 9). Planted tubers did not grow themselves but produced new tubers. Therefore, the length of tubers was shown in the total length of original and budding new tubers. Mycorrhizal tubers produced new tubers actively. On the other hand, multiplication of non-mycorrhizal tubers was very limited (Figs. 10, 11). In the control, no rhizomorphs adhered to tubers nor was mycorrhiza formation observed. Though they formed some buds, the number was smaller than those of the inoculated tubers (Fig. 7).

The mean length of inoculated tubers was 7.5 times longer and mass was 4 times heavier than those of the control (P < 0.05 Table 4).

There were significant differences
Fig. 7. _Gastrodia elata_ tubers cultured without _Armillaria_ on the sawdust medium for 3.5 months. A few stem-like organ (arrows) bud from the inoculated tuber (it). Bar = 2 cm.

Table 4. Size of _Gastrodia elata_ tubers incubated with _Armillaria gallica_ for three months

<table>
<thead>
<tr>
<th>Fungal treatment</th>
<th>Number of tested tuber</th>
<th>Length (mm)</th>
<th>Fresh mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculated</td>
<td>26</td>
<td>75.8±(3.6)</td>
<td>0.8±(0.05)</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>10.0±(1.8)</td>
<td>0.2±(0.04)</td>
</tr>
</tbody>
</table>

*: Significant difference between inoculated tuber and control P < 0.05. Number in parentheses show standard error

between mycorrhizal and non-mycorrhizal tubers, and between the mycorrhizal tubes and control in length, though there was no difference between non-mycorrhizal tubers and control (P < 0.05, Fig. 10). There were also significant differences in mass between mycorrhizal and non-mycorrhizal tubers and between the mycorrhizal tubers and control (P < 0.05, Fig. 11).

The symbiotic fungi were isolated from cortical cells of the cultivated tuber again. The culture was regarded as an Armillaria species based on its colony morphology, mycelial growth and rhizomorph formation.

**Discussion**

There has been no report experimentally clarify the symbiosis between G. elata and Armillaria using in vitro two-member culture. In this study the symbiotic relationship between Gastrodia elata and Armillaria gallica was proved with the two-member culture. All of the mycorrhizal tubers were observed only in rhizomorph-attached tubers. Therefore adhesion of rhizomorphs to tubers occurred prior to mycorrhizal formation both in the field and in vitro. These tubers were attached and penetrated by rhizomorphs. Mycorrhiza were observed as hyphal coils within cortical cells of these tubers, while these coils were not observed from other parts of tubers because they have been digested in this area (Hamada 1958).

There were significant differences between mycorrhizal and non-mycorrhizal tubers, and between the mycorrhizal tubers and control in length and mass though there was no difference between non-mycorrhizal tubers and control. Mycorrhizal tubers produced a lot of new tubers though non-mycorrhizal tubers and control produced few tubers. This suggests that the tuber of G. elata obtains nutrition by digesting hyphal coils of A. gallica. On the other hand, it is not clarified whether Armillaria obtains an advantage from G. elata. The daughter tubers were not adhered by the rhizomorphs at first. Rhizomorphs from mycorrhizal

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*Fig. 9. Scanning electron micrograph of hyphal coils of an Armillaria symbiont in cortical cells of Gastrodia elata tuber observed 3 months after incubation in sawdust medium. cw: Cell wall. m: Mycelial coils. Bar = 20 μm.*
mother tuber extended to daughter tubers and adhered to them. Consequently daughter tubers were infected by symbiont. This propagation mode was the same in the field and in vitro.

_Armillaria_ is well known as an important plant pathogen (Thomas 1934). Mating tests have been commonly used for the identification of _Armillaria_ species. Several species have been delimited in Europe (Korhonen 1978, Guillaumin et al. 1993), North America (Anderson and Ullrich 1979),

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**Fig. 10.** Length of _Gastrodia_ tubers incubated with _Armillaria gallica_.

*P < 0.05 vs. non mycorrhizal tubers; +P < 0.05 vs. control.

**Fig. 11.** Weight of _Gastrodia_ tubers incubated with _Armillaria gallica_.

*P < 0.05 vs. non mycorrhizal tubers; +P < 0.05 vs. control.
Australia (Kile and Watling 1983), and Africa (Mwangi et al. 1989). Japanese species of Armillaria have been examined by Nagasawa et al. (1991), Suzuki et al. (1994), Cha et al. (1992, 1994, 1995) and Ota et al. (1998). At least 10 species are recognized in Japan. Armillaria gallica was well-known as a saprobic species and produced abundant rhizomorphs in soil (Roll-Hansen 1985). Suzuki (1994) reported that A. gallica is a weak pathogen of conifer forests and lived to decay dead hardwoods and litter, on the other hand, A. ostoyae is a strong pathogen and lived to decay healthy coniferous trees. Ota et al. (1998) showed A. gallica would be a weak pathogen and is found most commonly in the field, while A. ostoyae and A. mellea seem to be strong pathogens in Japan.

The association between Gastrodia elata and Armillaria species was reported by Kusano (1911) and Hamada (1940), however, they did not identify the Armillaria at specific level. Cha and Igarashi (1995) reported that the Armillaria species isolated from G. elata in Hokkaido were A. gallica, A. ostoyae (Romagnesi) Herink, A. jezoensis Cha & Igarashi, A. sinapina Berube & Dessureault, and A. singula Cha & Igarashi. Among them A. gallica was most common. In this study the symbiont of G. elata in Honshu, Japan was A. gallica. This result agrees with the case observed in Hokkaido (Cha and Igarashi 1995).

Mohammed et al. (1994) suggested that A. ostoyae was probably too aggressive for a symbiotic association with orchids and that A. gallica was the most common symbiont species with G. elata. They thought that this species produced abundant rhizomorphs in the soil and was a weak pathogen. These features probably have the advantage to form the symbiotic relation. It is thought that the symbiosis of G. elata and Armillaria species is formed with the balance of continuously formed mycorrhiza and mycorrhizae digestion.

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References


