Mycobiology 30(1): 22-26 (2002)

Physiological Characteristics of Symbiotic Fungi Associated with the Seed Germination of *Gastrodia elata*

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(*Received January 8, 2002*)

This study was carried out to investigate the possibility for seeds germination of *Gastrodia elata* using symbiotic fungi. Since seeds of *G. elata* are very small and lack an endosperm and other nutrients, their germination is difficult without requirement for external nutrients. Out of twenty six isolates collected from protocorms of *G. elata* and roots of native orchids inhabited in wild, two strains (H-2 and H-21) were observed to stimulate the seed germination of *G. elata*. The seed germination of *G. elata* was excellent on oak tree leaves medium. The optimal conditions for mycelial growth of symbiotic fungi were 25°C and pH 6.0, respectively. The mycelial growth of H-2 strain was excellent on YMA medium, while H-21 was poor on PDA medium. In case of carbon sources, the mycelial growth of H-2 and H-21 was good on media supplemented with glucose and dextrin, respectively. Calcium nitrate was good for mycelial growth of H-2 strain as a nitrogen sources, whereas urea was effective to H-21 strain.

KEYWORDS: ***Gastrodia elata*, Mycelial growth, Seeds germination**

Gastrodia elata Blume belongs to the Orchidaceae family. This plant is widely distributed in Korea, China and Japan. The dried roots (tuber) of *G. elata* have been used as traditional Chinese medicines for human diseases such as vertigo, blackout, headache, hemiplegia and convulsions epilepsy under the name of "Cheonma" for centries in Asian countries (Huang, 1985; Chang, 1986).

G. elata utilizes mycorrhizal fungi such as *Armillaria mellea* as an energy source (myco-heterotrophy) in a parasitic symbiosis under natural conditions because it is aphyllous and achlorophyllous orchid (Kusano, 1911; Zhang and Li, 1980). Hence, the Korean farmers have cultivated the tuber of *G. elata* using *A. mellea* as symbiotic fungi for 30 years. But the yields of *G. elata* have been recently decreased owing to the degeneration of spawn tuber arised from successive asexual reproduction. One possible way to solve this degeneration is by symbiotic seed germination, namely, sexual reproduction (Clements *et al.*, 1986). Unfortunately, since the seeds of *G. elata* are minute and possess no an endosperm, the germination rate of its seed is poor or not at all in nature (Nakamura, 1982). Also *A. mellea* inhibits the seed germination of *G. elata* (Xu and Mu, 1990). Dispite numerous researchers have attempted to germinate the seed of *G. elata*, the germination mechanism of *G. elata* remains unclear.

We isolated the symbiotic fungi associated with the seed germination of *G. elata* and investigated the mycelial growth conditions.

This is the first report referring to the seed germination of *G. elata*.

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Materials and Methods

Fungal isolation. Twenty-six fungal isolates were collected from Korea and China and were used for the seed

Table 1. The list of the fungi used in this study

Isolate	Scientific name	Origin
H-1	*Mycena osmundicolor*	IMPALD (Chinese)
H-2	*Mycena osmundicolor*	IMPALD (Chinese)
H-3	I (unidentified)	Habitat of *G. elata*
H-4	II (unidentified)	Tuber of *G. elata*
H-5	III (unidentified)	Tuber of *G. elata*
H-6	IV (unidentified)	Tuber of *G. elata*
H-7	V (unidentified)	Tuber of *G. elata*
H-8	VI (unidentified)	Tuber of *G. elata*
H-9	VII (unidentified)	Tuber of *G. elata*
H-10	VIII (unidentified)	Tuber of *G. elata*
H-11	*Mycena* sp.	Tuber of *G. elata*
H-12	IX (unidentified)	Tuber of *G. elata*
H-13	X (unidentified)	Tuber of *G. elata*
H-14	XI (unidentified)	Tuber of *G. elata*
H-15	XII (unidentified)	Tuber of *G. elata*
H-16	XIII (unidentified)	Native Orchid
H-17	XIV (unidentified)	Native Orchid
H-18	XV (unidentified)	Native Orchid
H-19	XVI (unidentified)	Native Orchid
H-20	XVII (unidentified)	Habitat of *G. elata*
H-21	XVIII (unidentified)	Habitat of *G. elata*
H-22	*Tulsanella repens*	Native Orchid
H-23	*Ceratobasidium endophytica*	Native Orchid
H-24	*Tulsanella calospora*	Native Orchid
H-25	*Tulsanella calospora*	Native Orchid
H-26	*Tulsanella repens*	Native Orchid

germination of *G. elata* (Table 1). Twenty four strains were isolated from the small tuber or habitat of *G. elata* and the roots of native orchids, and two strains (H-1 and H-2) were provided by IMPALD (Institute of Medicinal Plant Chinese Academy of Medical Sciences). These strains were cultured and maintained on yeast-malt agar (YMA).

Selection of the seeds-sprouting-promoting fungi. Raw materials such as leaves of oak tree and bamboo and waste cotton were soaked in water for overnight and pressed to expel excess water until the moisture content to be approximately 65%. The media were prepared by mixing each raw material and rice bran (8 : 2, v/v). The bottles were filled with the mixed media, sealed tightly with a plug and sterilized at 121°C (15 psi) for 2 hours in an autoclave. Each bottle was inoculated with 26 fungal sawdust spawn and incubated for 3 months at 25°C. The seeds of *G. elata* was sown on the bottle medium and cultivated at 25°C. The seed germination was observed after 4 months of sowing.

Selection of optimal media for mycelial growth. Four different culture media (Table 2) were used to investigate a favorable growth of symbiotic fungi, namely H-2 and H-21 strains. The media were autoclaved for 15 minutes at 121°C (15 psi) before pouring into a petri-dish. For inoculum, the fungi were grown for 14 days on the yeast-malt agar medium and then the mycelium of the fungi was inoculated at the center of each medium petri-dish using a sterile cork borer (5 mm dia.). Five inoculated petri-dishes were incubated at 25°C. After 14 days of incubation, the mycelial growth was measured.

Effect of temperature on mycelial growth. The effect of temperature on mycelial growth was carried out using the selected medium as the cultural media study. After autoclaving at 121°C (15 psi), the medium was septically poured into a petri-dish. The inoculation were performed as the same method described above.

For incubation, the five petri-dishes inoculated with symbiotic fungi were incubated at 15, 20, 25, and 30°C, respectively. After 14 days of incubation, the mycelial growth was observed.

Effect of pH on mycelial growth. The effect of pH on mycelial growth was studied by the mycelial dry-weight method. The liquid medium was employed the selected medium as temperature study without agar. The medium was adjusted to the range of pH 4~8 with 1 N NaOH or HCl before being dispensed into 250 m*l* Δ flasks at the rate of 50 m*l* per flask and then autoclaved for 15 minutes at 121°C (15 psi). The inoculum was obtained by growing the fungi for 14 days in petri-dish on the yeast-malt agar medium. Each flask was inoculated with a mycelial inoculum and incubated at 25°C for 14 days. The mycelia were filtered through filter paper (Whatman No. 2, dia., 9 cm), dried in an oven at 85°C for 24 hours, cooled in a desiccator and weighed.

Carbon source utilization for mycelial growth. Carbon source utilization by the seeds-sprouting-promoting fungi (SSPF) was tested by the method described by Lilly and Barnett (1951). The medium used was contained : glucose 10 g, asparagine 2 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, agar 20 g and distilled water to make 1000 m*l*. Various carbon sources were substituted for glucose. Namely, based on each molecular weight of 13 different carbon sources, each carbon source was added to the basal medium at the concentration of 0.1 M per liter.

The basal medium was adjusted to pH 6.0 and then autoclaved for 15 minutes at 121°C (15 psi). Cellobiose and xylose were sterilized by millipore filtration to avoid their breakdown to glucose and furfural, respectively. The inoculum was obtained by growing the fungi for 14 days in petri-dishes on the basal medium. Five petri-dishes of each carbon source were inoculated with 5 mm disk of the fungus and five petri-dishes of the basal medium lacking a carbon source were also inoculated for the control. The petri-dishes were incubated at 25°C for 14 days after which the colony diameter of mycelia were measured as above.

Nitrogen source utilization for mycelial growth. The ability of SSPF to utilize differernt nitrogen sources for growth was also studied. Except for the substitution of 1% gluctose as carbon source per 1000 m*l*, the basal medium was the same composition as the medium used for the carbon source requirements study. Various nitrogen sources were substituted for asparagine. Based on each molecular weight of 12 different nitrogen sources, each nitrogen source was added to the basal medium at the concentration of 0.02 M per liter. The basal medium

Table 2. Composition of media used

Ingredient	Medium (g/*l*)*			
	MCM	MEA	PDA	YMA
$MgSO_4 \cdot 7H_2O$	0.5			
KH_2PO_4	0.46			
K_2HPO_4	1.0			
Peptone	1.0	5.0		5.0
Yeast extract	2.0			3.0
Malt extract		20.0		3.0
Dextrose	20.0			10.0
PDA (Difco)			39.0	
Agar	20.0	20.0		20.0

*MCM (Mushroom Complete Medium), MEA (Malt Extract Agar), PDA (Potato Dextrose Agar), YMA (Yeast Malt Agar).

Table 3. Effect of the different culture media on seeds germination of *Gastrodia elata*

Isolate	Media				
	A	B	C	D	E
H-1	−*	−	−	−	−
H-2	+++	++	+	−	−
H-3	−	−	−	−	−
H-4	−	−	−	−	−
H-5	−	−	−	−	−
H-6	−	−	−	−	−
H-7	−	−	−	−	−
H-8	−	−	−	−	−
H-9	−	−	−	−	−
H-10	−	−	−	−	−
H-11	−	−	−	−	−
H-12	−	−	−	−	−
H-13	−	−	−	−	−
H-14	−	−	−	−	−
H-15	−	−	−	−	−
H-16	−	−	−	−	−
H-17	−	−	−	−	−
H-18	−	−	−	−	−
H-19	−	−	−	−	−
H-20	−	−	−	−	−
H-21	+++	++	+	−	−
H-22	−	−	−	−	−
H-23	−	−	−	−	−
H-24	−	−	−	−	−
H-25	−	−	−	−	−
H-26	−	−	−	−	−

Media were prepared with A: Quercus acutissima leaves, B: Quercus mongolica leaves, C: Quercus dentata leaves, D: Bamboo leaves, E: Cotton waste.

* −: non-sprouting, +: small quantity sprouting, ++: middle quantity sprouting, +++: large quantity sprouting.

was adjusted to pH 6.0 and then autoclaved for 15 minutes at 121°C (15 psi). All the other processes including the inoculation, incubation and measurement of mycelial growth were performed as the same method of carbon source.

Results and Discussions

Selection of the seeds-sprouting-promoting fungi. As the result of the seeds germination of *G. elata* was discribed in Table 3, only two strains (H-2 and H-21) out of 26 isolates was able to stimulate of seeds sprouting (Fig. 1) and the seed germination was best on Quercus acutissima leaves medium.

Selection of optimal media. Four different culture media were used to investigate a favorable growth of symbiotic fungi. It was observed that H-2 strain was good on YMA medium, while H-21 strain was poor on PDA medium (Fig. 2). The mycelial growth of the H-2 strain on 4 different media was observed in the range of 45.8~61.3 mm and mycelial growth of the H-21 isolate was observed in the range of 47.3~70.3 mm for 14 days incubation. In general, H-21 strain showed faster mycelial growth than H-2.

Effect of culture temperature. The mycelial growth of H-2 strain cultured on YMA medium for 14 days at 4 different temperature levels was favorable in the range of 20~30°C (Fig. 3). There was a slight growth at 15°C, while the best growth was at 25°C. A favorable temperature of H-21 strain for mycelial growth on MEA medium was 25 to 30°C. The result also showed that H-21 strain

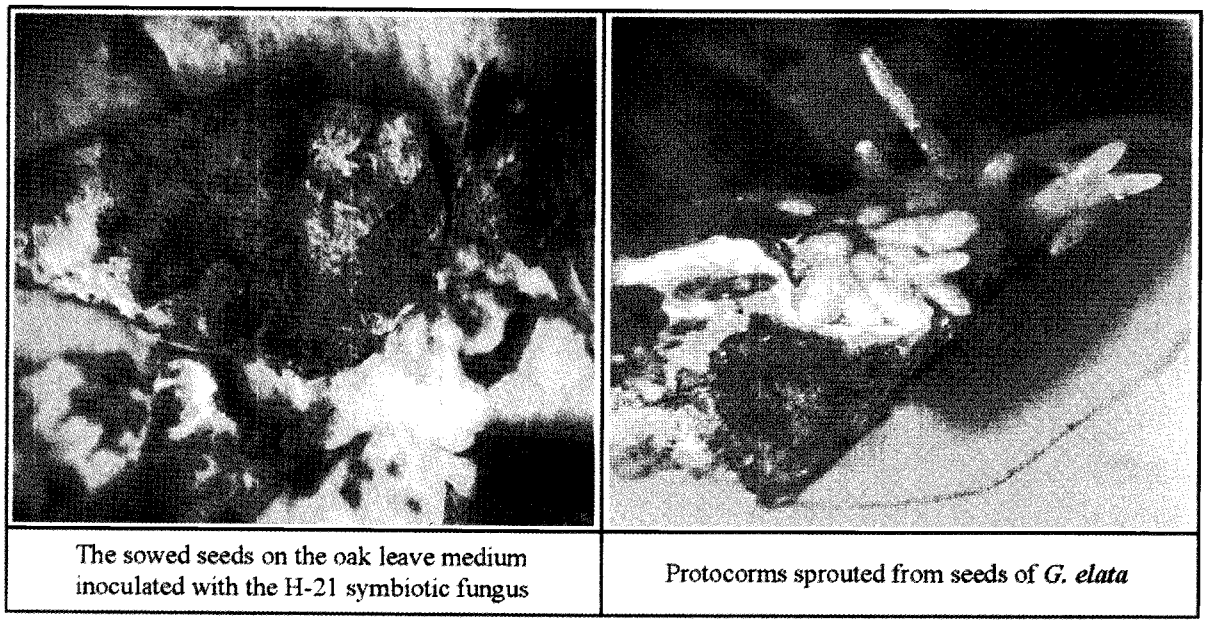

Fig. 1. Seeds germination of *Gastrodia elata* using symbiotic fungi.

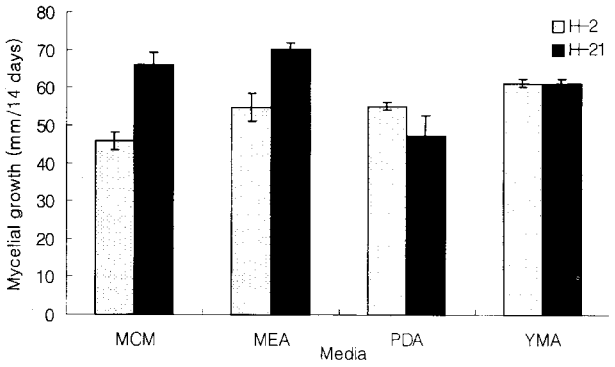

Fig. 2. Mycelial growth of symbiotic fungi associated with seed germination of *Gastrodia elata* on different culture media.

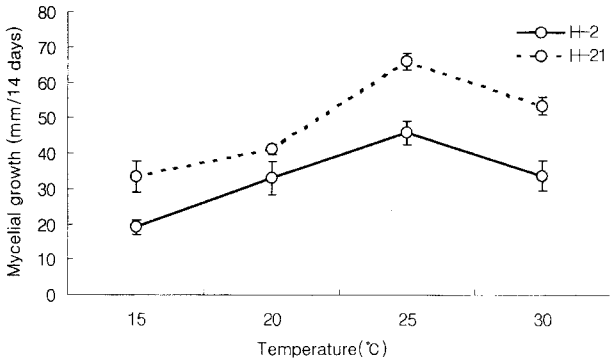

Fig. 3. Effect of temperatute on the mycelial growth of symbiotic fungi.

faster than H-2 in mycelial growth about 10 mm in 14 days culture. The most favorable temperature for mycelial growth of *A. mellea* which is another symbiotic fungi with *G. elata* was 22~25°C (Hong *et al.*, 1990).

Effect of pH. The effect of pH on mycelial growth of SSPF was studied by the mycelial dry-weight method. The pH range suitable for a favorable growth of H-2 strain was pH 5.0~6.0. The mycelial growth was most favorable at pH 6.0 and recorded dry weight of 87.0 mg, but most unfavorable at pH 8.0 (Table 4). As the fungus grows, the pH of the medium tends to decrease and to become more acidic.

The mycelial growth of H-21 strain was suitable in the range of pH 6.0~7.0 (Table 5). There was slight growth at pH 8.0, while the best growth was at pH 6.0 and recorded with 39.1 mg of dry weight. The result showed that the mycelial growth of H-2 strain faster than H-21 in the pH test. Choi *et al.* (1983) reported that pH value suitable for a favorable growth of *A. mellea* was pH 6.0.

Table 4. Effect of pH on the mycelial growth[a] of H-2 strain in YM broth medium

Item	Acidity (pH)				
Initial pH	4.0	5.0	6.0	7.0	8.0
pH after sterilization	4.09	5.08	5.95	6.66	7.18
pH after 14 days incubation	4.13	4.92	5.61	6.35	6.93
Mycelial dry weight[a]	33.6^{ab}*	81.1^{c}	87.0^{c}	45.9^{b}	21.3^{a}

[a]Mycelial dry weight was measured after 14 days of incubation.
*The different letters are significantly different at p = 0.05 according to Duncan's multiple range test.

Table 5. Effect of pH on the mycelial growth[a] of H-21 strain in ME broth medium

Item	Acidity (pH)				
Initial pH	4.0	5.0	6.0	7.0	8.0
pH after sterilization	4.10	5.09	5.92	6.61	7.26
pH after 14 days incubation	4.14	5.19	5.95	6.52	7.04
Mycelial dry weight*	29.0^{b}*	30.2^{b}	39.1^{c}	34.4^{bc}	20.2^{a}

[a]Mycelial dry weight was measured after 14 days of incubation.
*The different letters are significantly different at p = 0.05 according to Duncan's multiple range test.

Effect of carbon source. Carbon source utilization by SSPF was tested by the method described by Lilly and Barnett (1951). The best growth of H-2 strain was obtained on glucose, followed in order by fructose, mannose, galactose and xylose. Sucrose and maltose were the only oligosaccharides that supported good growth, but growth on the former was much better. The three polysaccharides, dextrin, inulin and soluble starch were good for mycelial growth, but growth on dextrin was greater (Table 6). In case of H-21 strain, there was hardly growth on galactose, mannose and xylose, while growth was very slight on arabinose and glucose in the monosaccharides. The best growth was obtained on fructose among the

Table 6. Effect of carbon source[a] on the mycelial growth

Carbon source[a]	Medium pH after sterilization	Colony diameter[b] (mm)	
		Strain	
		H-2	H-21
Arabinose	4.45	44.4^{de}*	30.5^{cd}
Fructose	4.41	52.5^{hi}	42.6^{g}
Galactose	4.52	50.1^{fgh}	27.7^{bc}
Glucose	4.61	55.3^{i}	32.4^{de}
Mannose	4.54	51.6^{ghi}	21.8^{a}
Xylose	4.48	49.6^{fgh}	27.3^{b}
Cellobiose	4.69	20.5^{a}	20.0^{a}
Lactose	4.75	36.9^{c}	20.2^{a}
Maltose	4.71	43.1^{d}	28.9^{c}
Sucrose	4.71	48.9^{efgh}	27.3^{b}
Dextrin	4.41	47.2^{defg}	48.9^{h}
Inuline	4.67	45.0^{de}	35.0^{e}
Soluble starch	5.14	46.9^{def}	39.0^{f}
Control	–	25.8^{b}	28.3^{bc}

[a]Each carbon source was added to the basal medium at the concentration of 0.1 M.
[b]The colony diameter was measured after 14 days of incubation.
*The different letters are significantly different at p = 0.05 according to Duncan's multiple range test.

Table 7. Effect of nitrogen source on the mycelial growth

Nitrogen source	Colony diameter[a] (mm)	
	Strain	
	H-2	H-21
Ammonium acetate	Non-growth	Non-growth
Ammonium chloride	44.0^{cde}*	27.0^{abc}
Ammonium citrate	38.5^{b}	39.8^{ef}
Ammonium nitrate	42.5^{cd}	32.0^{bcde}
Ammonium phosphate	18.0^{a}	29.0^{abcd}
Ammonium sulfate	45.3^{de}	33.7^{cdef}
Ammonium tartrate	41.0^{bc}	37.8^{def}
Potassium nitrate	45.5^{de}	23.8^{ab}
Calssium nitrate	49.0^{f}	19.5^{a}
Sodium nitrate	45.86^{e}	19.3^{a}
Sodium nitrite	Non-growth	Non-growth
Urea	Non-growth	42.3^{f}
Control	43.8^{cde}	28.5^{abcd}

[a]Each nitrogen source was added to the basal medium at the concentration of 0.02 M.
[b]The colony diameter was measured after 14 days of incubation.
*The different letters are significantly different at $p = 0.05$ according to Duncan's multiple range test.

monosaccharides. The oligosaccharides, cellobiose, lactose and sucrose were unable to the fungus for growth compared with the control. Among the polysaccharides tested, growth was best on dextrin, followed by soluble starch and inulin.

Effect of nitrogen source. Nitrogen source utilization by SSPF was tested by the method described by Lilly and Barnett (1951). Among the 12 nitrogen sources, the mycelial growth of H-2 strain was best on the culture media which were supplemented with calssium nitrate and recorded colony diameter of 49.0 mm, and followed by sodium nitrate, potassium nitrate and ammonium sulfate. There was no mycelial growth on ammonium acetate, sodium nitrite and urea (Table 7). In case of H-21 strain, there was no growth on ammonium acetate and sodium nitrite, while growth was hardly on ammonium chloride, ammonium phosphate, potassium nitrite, calcium nitrate and sodium nitrate compared with the control. The best growth was obtained on urea, followed by ammonium citrate and ammonium tartrate.

Acknowledgment

We are greatful to Prof. Dr. Guo, Shun-Xing, Institute of Medicinal Plant, Chinese Academy of Medical Sciences for his help and advice throughout the preparation of this article. His persistent guidance, encouragement, and assistance have been invaluable.

References

Chang, H. M. and But, P. H. 1986, Pharmacology and Application of Chinese Materia Medica, Vol. I, World Scientific, Singapore, p. 185.

Choi, M. J. and Lee, J. Y. 1983. Physiological and ecological studies on mycelial of *Armillaria mellea. Kor. J. Mycol.* **11**(2): 79-84.

Clements, M. A., Muir, H. and Cribb, P. J. 1986. A preliminary report on the symbiotic germination of European terrestrial orchids, *Kew Bull.* **41**: 437-445.

Hong, J. S., Kim, M. K., So, G. H. and Kim, Y. H. 1990. Studies on the mycelial cultivation and the rhizomorph production of *Armillaria mellea. Kor. J. Mycol.* **18**(3): 149-157.

Huang, Z. L. 1985. Pharmacologic studies and clinical applications of *Gastrodia elata* Bl. *Journal of Modern Development Traditional Media* **5**: 251-254.

Kusano, S. 1911. *Gastrodia elata* and its symbiotic association with Armillaria mellea. Imperial University of Tokyo, *Journal of the College of Agriculture* **4**: 1-65.

Nakamura, S. J. 1982. Nutritional conditions required for the non-symbiotic culture of an achlorophyllus orchid *Galeola septentrionalis. New Phytol.* **90**: 701-715.

Xu, J. T. and Mu, C. 1990. The relation between growth of Gastrodia elata protocorms and fungi. *Acta Botanica Sinica.* **32**(1): 26-31.

Zhang, W. J. and Li, B. F. 1980. The biological relationship of *Gastrodia elata* and *Armillaria mellea. Acta Botania Sinica* **22**: 57-62.