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Artificial Cultivation System for *Gastrodia spp.* and Identification of Associated Mycorrhizal Fungi

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Abstract

The Orchidaceae are the largest and most diverse family of flowering plants on earth, and include some of the most important horticultural plants. While mycoheterotrophic orchids belonging to the genus *Gastrodia* are known to be provided with carbon through mycorrhizal fungi, the relationship between the plants and fungi is poorly understood. Furthermore, it is challenging to cultivate *Gastrodia spp. in vitro*. In this study, we present an efficient method for germinating *Gastrodia pubilabiata* (*Gp)*, *Gastrodia nipponica* (*Gn*), and *Gastrodia confusa* (*Gc*) plants *in vitro*, which results in the production of a protocorm and tuber, as under natural conditions. The *Gp* and *Gc* plants produced flowers 126 and 124 days after germination, respectively, and set seed under our artificial conditions. In addition, *Gp* plants flowered up to three times a year from a single tuber. Using our artificial cultivation system, we identified some of the mycorrhizal fungi associated with these plants. *Gastrodia spp*. appear to obtain carbon from many kinds of mycorrhizal fungi. Our artificial cultivation method is a rapid and efficient means of growing *Gastrodia spp*. In addition to having applications in research and commercial nurseries, this method could be used to conserve *Gastrodia spp*. in *ex situ,* many of which are endangered.

Keywords: artificial cultivation, *Gastrodia confusa, Gastrodia nipponica, Gastrodia pubilabiata,* mycoheterotrophic plants, Orchidaceae

1. Introduction

Mycoheterotrophic plants (MHPs) have a mutualistic relationship with mycorrhizal fungi, which provide the plant with carbon (Smith & Read, 2008; Kinoshita et al., 2016). Most MHPs have a high fungal specificity (reviewed by Bidartondo, 2005), whereas others interact with a variety of species (Roy et al., 2009; Hynson & Bruns, 2009). Obligate MHPs, which include about 530 species, do not produce functional chloroplasts and are completely dependent on their associated mycorrhizal fungi as a source of carbon.

Gastrodia (Orchidaceae) is a genus of obligate mycoheterotrophic orchid that is distributed in warm areas of Madagascar, Asia, and Oceania (Paul & Molvray, 2005; Chung & Hsu, 2006). 16 species of *Gastrodia* (Orchidaceae) are indigenous to Japan, including *G. elata* (*Ge*), *G. javanica*, *G. boninensis, G. confusa* (*Gc*)*, G. nipponica* (*Gn*)*, G. pubilabiata* (*Gp*)*, G. shimizuana*, *G. gracilis*, *G. clausa, G. takeshimensis, G. uraiensis, G. fontinalis*, *G. flexistyloides*, *G. kuroshimensis*, *G. nipponicoides* and *G. okinawensis* (Tuyama, 1939; Honda & Tuyama, 1939; Tuyama, 1941, 1952, 1956, 1966, 1967; Garay & Sweet, 1974; Hatusima, 1975; Sawa, 1980; Tuyama, 1982; Kobayashi & Yukawa, 2001; Suetsugu et al., 2012, 2013, 2014; Suetsugu, 2012, 2013, 2014, 2015a, 2015b, 2016, 2017).

Ge is commercially cultivated, especially in China, as its tuber is used in herbal medicine (Xu & Guo, 2000). However, there are no reports of other *Gastrodia spp.* being successfully cultivated for more than one generation. While *Gp* and *Gc* seeds were germinated and cultured *in vitro*, using enriched agar medium supplemented with mycorrhizal fungi (Tashima et al., 1978; Umata et al., 2000), the resulting plants failed to flower and/or produce seed.

Some mycorrhizal fungi that associate with *Gastrodia spp*. have been identified, such as *Armillaria* of Physalacriaceae*,* which associates with *Ge* (Kusano, 1911); *Mycena* of Mycenaceae*,* which associates with *Gc* (Ogura-Tsujita et al., 2009); *Marasmius* and *Campanella* of Marasmiaceae*,* which associate with *G. sesamoides*

(Dearnaley & Bougoure, 2010); and *Resinicium* of Meruliaceae, which associates with *G. similis* (Martos et al., 2009).

The natural habitats of *Gp, Gn*, and *Gc* have been characterized in detail (Sugino, 1983; Sugino & Suzuki, 1985; Fukunaga et al., 2008). In the current study, we successfully cultivated *Gp, Gn*, and *Gc* in habitat-like artificial environments that were neither sterile nor enriched with nutrients. The artificial cultivation system (ACS) is simple and does not require sterile conditions or expensive equipment. We also identified some of the mycorrhizal fungal species associated with these three *Gastrodia* species, and observed that different mycorrhizal fungal species can associate with more than one of these *Gastrodia* species. Our ACS has both research and commercial applications.

2. Materials and Methods

In vitro culture was performed in laboratories at Kumamoto, Kanagawa, and Tokushima, Japan. Artificial medium was prepared from *Gp* habitats in Kanagawa, *Gn* habitats in Tokushima and Kanagawa, and *Gc* habitats in Kochi and Kanagawa. The medium included a 30-cm long segment of cedar log (*Cryptomeria japonica*; "cedar medium"), a 30-cm long segment of *Castanopsis sieboldii* log ("sudazi medium"), or sections of aboveground tissue of the bamboo *Phyllostachys edulis* ("moso medium"), respectively, and placed in a sealed plastic box with wet gauze and a vinyl sheet lining the inside of the lid (Figure 1 A-E). *Gp*, *Gn*, and *Gc* plants were grown in these different media. To promote fungal growth, the medium was surrounded with cedar cones and humus from the floor of a *Castanopsis sieboldii* forest. Seeds were sown on the surface of the medium as shown in Figure 1F. The plants were grown at 20-25°C, 80-85% relative humidity, in complete darkness. Seeds were harvested from their natural habitats in Kanagawa and Shizuoka (*Gp*), Tokushima (*Gn*), and Shizuoka (*Gc*), or obtained through self-pollination of plants in the laboratory. The seeds were directly placed in the artificial growth medium.

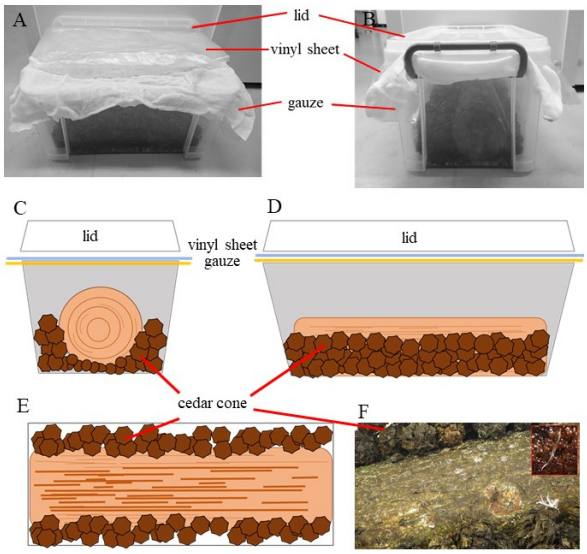

Figure 1. The artificial culture medium kit.

Medium is placed in a sealed plastic box, with wet gauze and a vinyl sheet lining the inner lid (A). To maintain moist conditions, the lid is kept closed except when watering (B). The medium is composed of a 30-cm or section of bamboo (in the case of moso medium) surrounded with cedar cones; side view (C), front view (D), and top view (E). (F) Seeds were sown directly on the medium. Inset shows magnified seed sown on the log.

DNA was extracted from protocorms that formed from the seeds using the CTAB method (Lee et al., 1988; Wu et al., 2001). The internal transcribed spacer (ITS) region of the fungal nuclear ribosomal RNA gene (rDNA) was amplified using the primers ITS1F and ITS4B (Gardes & Bruns, 1993), using TaKaRa Ex Taq Hot Start Version (Takara Bio, Japan). The PCR mixture contained 2 µL of the extracted DNA solution, 0.05 µL of Ex Taq polymerase, 10 µM of each primer, 0.25 µM of each dNTP, and 1 µL of 10 x buffer in a total volume of 10 µL. The PCR program was performed using an i cycler (Bio Rad, Japan), as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30s, 55°C for 30 s, and 72°C for 1 min, followed by a final

elongation at 72°C for 7 min. Amplified PCR products were purified using an EconoSpin column (Gene design, Inc.), and the purified PCR product was sequenced. The obtained DNA sequences were subjected to a BLAST search (Altschul et al., 1997). Multiple sequence alignment was performed using ClustalX (Larkin et al., 2007). The GenBank accession numbers of the mycorrhizal ITS sequences analyzed were as follows: *Diplomitoporus rimosus*, AB907585; *Thelepurus membranaeu*s, NR119985; *Trechispora*, JF691276; *Corticium*, KP814404; and *Mycena,* NR119985.

3. Results

3.1 Artificial cultivation of Gp

Gp seeds were germinated on cedar medium from 17-55 days after sowing (DAS; Table 1) and developed into protocorms (Figure 2). Root tuber primordia emerged from the base of bracts at the top of the spherical protocorms immediately after the latter formed. Roots also emerged from the sides of the protocorms (Figure 2). One of the roots dominated, even after flowering. Protocorms developed into tubers. The tubers spontaneously stopped developing 4 months after sowing, and the shoots bolted. Flowers opened one month after bolting. Capsules matured and opened 20-30 days after the flowers opened, a little sooner than they typically do in their natural habitat (40-50 days after flower opening). Protocorm formation, tuber formation, root emergence, floral opening, and capsule formation were observed 20-65, 25-80, 30-82, 154-299, and 194-321 DAS, respectively (Table 1).

Table 1. List of seeds, media, and growth rate in our ACS.

We could not confirm some of developments (x). We stopped some cultures, and there is no data (-).

Gastrodia	number	habitat		Experiment	Development						
spp.		media	seed	place	Sowing	Germination	Protocom	Tuber	Root	Flower open	Capsule
G.	#2	Kanagawa	Kanagawa	Kanagawa	2012.11.25	2012.12.20	2012.12.25	2012.12.28	2012.12.30	2013.05.14	2013.06.07
pubilabiata		【cedar】				25DAS	30DAS	33DAS	35DAS	170DAS	194DAS
	#3				2013.04.23	2013.05.20	2013.05.22	2013.05.30	2013.06.01	×	
	#4					27DAS	29DAS	37DAS	39DAS		
					2013.04.23	2013.05.10	2013.05.13	2013.05.18	2013.05.23	2014.02.16(1st)	2014.03.10
						17DAS	20DAS	25DAS	30DAS	299DAS	321DAS
										2014.07.22(2nd)	
										2014.10.29(3rd)	
	#13			Kanagawa	2014.12.04	2014.12.24	2014.12.28	2015.01.05	2015.01.08	-	-
						20DAS	24DAS	32DAS	35DAS		
	#14		Shizuoka	Kanagawa	2015.01.14	2015.02.07	2015.02.08	2015.02.15	2015.02.20	-	-
						24DAS	25DAS	32DAS	37DAS		
	#15		Kanagawa	Kumamoto	2015.02.18	2015.03.15	2015.03.17	2015.04.05	2015.04.09	2015.10.28	-
						25DAS	27DAS	46DAS	50DAS	252DAS	
	#16			Tokushima	2015.02.23	2015.04.19	2015.04.22	2015.04.23	2015.04.26	2015.10.25	2015.12.02
						55DAS	58DAS	59DAS	62DAS	244DAS	282DAS
	#18		self-pollinated	Kanagawa	2015.04.18	2015.05.10	2015.05.10	2015.05.15	2015.05.18	2016.10.20	-
			in vitro			22DAS	22DAS	27DAS	30DAS	185DAS	
	#20		Kanagawa	Kanagawa	2015.10.17	2015.11.27	2015.12.21	2016.01.05	2016.01.07	×	
						41DAS	65DAS	80DAS	82ads		
	#23		Kanagawa	Kanagawa	2016.11.12	2016.12.10	2016.12.12	2016.12.17	2016.12.19	2017.04.15	2017.5.30
						28DAS	30DAS	35DAS	37DAS	154DAS	199DAS
G.	#1	Kanagawa	Tokushima	Kanagawa	2015.05.16	2015.06.15	2016.06.17	201.06.20	2016.06.20	breeding	
nipponica		【cedar】				30DAS	32DAS	35DAS	35DAS		
	#2	Tokushima	Tokushima	Tokushima	2015.05.21	2015.06.03	2015.06.05	2015.07.05	2015.07.05	×	×
		【sudazi】				13DAS	15DAS	45DAS	45DAS		
	#3	Kanagawa	Tokushima	Kanagawa	2015.05.20	2015.06.15	2015.06.17	2015.06.19	2015.06.19	×	×
		【cedar】				26DAS	28DAS	30DAS	30DAS		
G. confusa	#1	Kanagawa	Shizuoka	Kanagawa	2014.11.07	2014.12.01	2014.12.10	2015.01.08	2015.01.15	-	-
		【cedar】				24DAS	33DAS	62DAS	69DAS		
	#2		Shizuoka	Kanagawa	2014.12.12	2014.12.20	2015.01.16	2015.01.25	2015.02.14	2015.07.20	2015.08.14
						8DAS	35DAS	44DAS	64DAS	220DAS	245DAS
	#4	Kochi	Shizuoka	Kanagawa	2015.11.05	2015.12.11	2015.12.18	2015.12.20	2015.12.25	2016.10.10	×
		【moso】				36DAS	43DAS	45DAS	50DAS	340DAS	
	#6	Kanagawa	Shizuoka	Kanagawa	2016.11.12	2016.12.12	2017.01.04	2017.01.30	2017.02.03	2017.04.15	×
		【cedar】				30DAS	53DAS	79DAS	83DAS	154DAS	

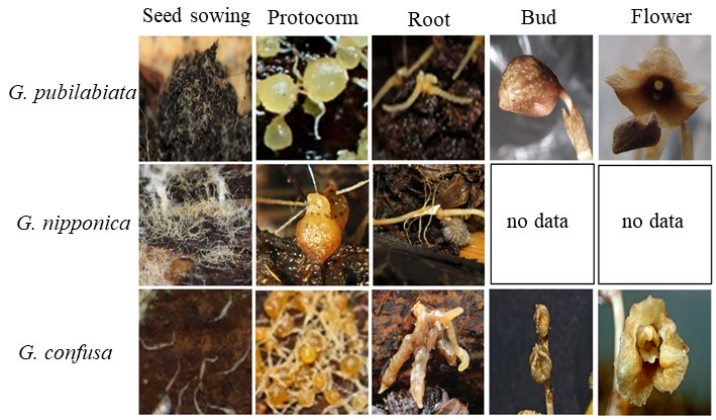

Figure 2. *Gastrodia spp.* growing steps, from seed sowing to flower opening

Protocorms are produced at 30 days after sowing (DAS), roots are produced at about 40 DAS, buds are generated at about 150 DAS, and flowers open at about 160 DAS.

The first *Gp* flower opened at 154 DAS. Artificial conditions can accelerate growth and induce early flowering. The largest of several tubers generated in our ACS produced shoots and flowers three times in a one-year period (Table 1 *Gp*#4). By contrast, *Gp* plants growing in nature only produce seed after one year, or even longer if conditions are not ideal. Thus, these plants develop more rapidly in our ACS than they do in their natural habitat, and our ACS is an efficient method for maintaining these plants *in vitro*.

3.2 Artificial Cultivation of Gn

Gn seeds were sown on cedar and sudazi medium, and germinated at 13-30 DAS. Protocorms formet at 15-32 DAS. Tubers and roots emerged at 30-45 DAS (Table 1). While *Gn* tubers did not produce flowers under our cultivation conditions, these plants. developed and grew more rapidly in the ACS than in their natural habitat.

3.3 Artificial Cultivation of Gc

Gc seeds were sown on cedar or moso medium, and germinated at 8-36 DAS. Protocorm formation, tuber formation, root emergence, floral opening, and capsule formation were observed at 33-53, 44-79, 50-83, 154-340, and 245 DAS, respectively (Table 1). *Gc* seeds sown on cedar medium were viable, giving rise to plants that flowered and set seed. Interestingly, we found that *Gc* and *Gp* were able to grow simultaneously on the same cedar log (Figure 3). This suggests that the specificity of mycorrhizal fungi is low between *Gastrodia spp.*, and that the same species of fungus can form mutualistic relationships with different species in this genus.

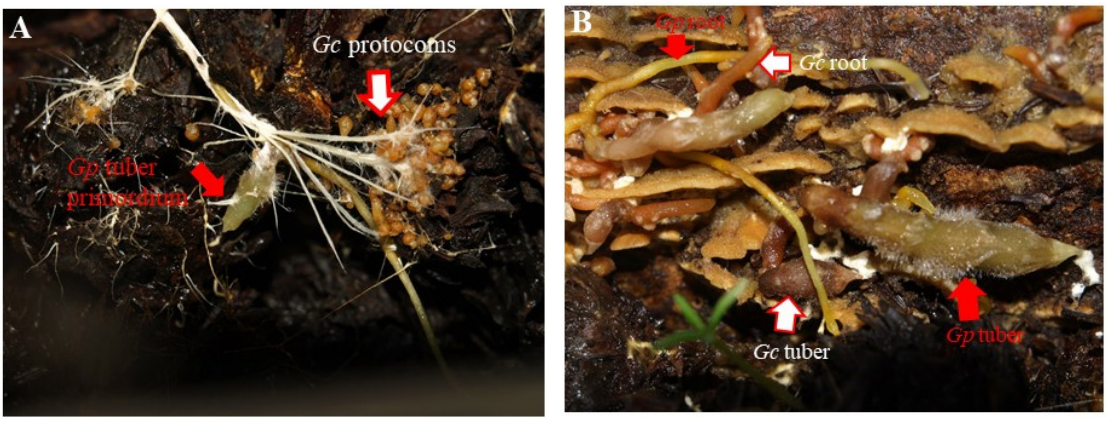

Figure 3. *Gp* and *Gc* can be cultivated together

(A) *Gc* (Shizuoka) can generate protocorms when sharing the same mycelium as *Gp* (Kanagawa). Red arrow: *Gp* tuber primordium. White arrow: *Gc* protocorms. (B) *Gp* and *Gc* grow at similar rates on the same cedar log. Red arrow: *Gp* root and tuber. White arrow: *Gc* root and tuber.

3.4 Molecular Identification of Mycorrhizal Fungi

Using our ACS, we next sought to identify the mycorrhizal fungi associated with *Gastrodia spp.* based on the internal transcribed spacer (ITS) region of the fungal nuclear ribosomal RNA gene (rDNA). We found that 99% of the sequenced region using a protocorm sample of *Gp* (cedar medium from Kanagawa) and *Gn* (sudazi medium from Tokushima) matched that of *Diplomitoporus rimosus* (558bp/559bp identity) and *Thelepurus membranaeus* (542bp/544bp identity), respectively. These fungi were also detected in the cedar medium or sudazi medium, respectively (*Diplomitoporus rimosus*, 411bp/412bp identity, or *Thelepurus membranaeus*, 548bp/553bp identity). These results strongly suggest that these fungi are responsible for the successful development of *Gastrodia spp* in our ACS. While *D. rimosus* and *T. membranaeus* are located in the same phylogenetic clade of *Polyporales*, they are not evolutionarily close to each other (Figure 4).

Furthermore, from *Gn* protocorms, we detected an ITS sequence with significant nucleotide sequence identity to the corresponding regions in *Trechispora* (509bp/582bp identity) and *Corticium* (537bp/617bp identity). We also detected an ITS sequence similar to that from *Mycena spp.* (602bp/604bp identity) as a candidate of mycorrhizal fungi associated with *Gc*. These data suggest that *Gastrodia spp.* can successfully grow in association with a number of different species of mycorrhizal fungi.

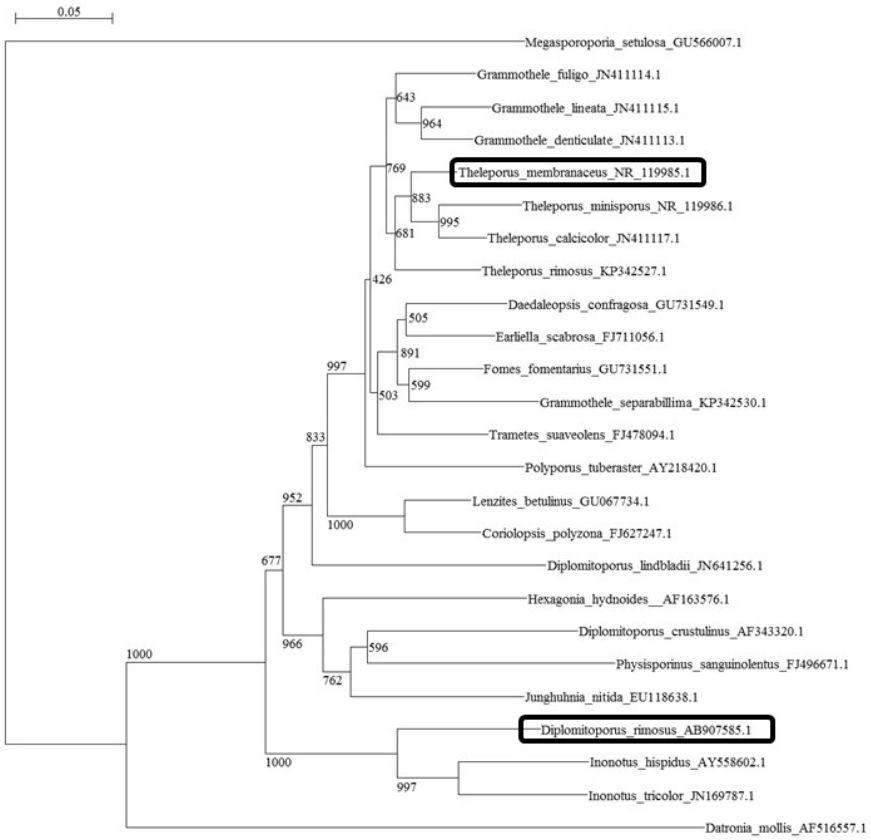

Figure 4. Phylogenetic relationship of the mycorrhizal fungi associated with *Gastrodia spp.*, based on ITS gene sequences. GenBank accession numbers follow taxa names. Support at the nodes was computed using NJ Plot with 1000 bootstrap replicates. Mycorrhizal fungi identified from *Gp* and *Gn* protocorms are *Diplomitoprus rimosus* and *Theleporus membranaceus*, respectively

4. Discussion

While it was previously suggested that most MHPs associate with specific fungal species (Leake, 1994; Bidartondo, 2005; Ogura-Tsujita et al., 2012), *Galeola altissima* was demonstrated to form a symbiotic relationship with a variety of mycorrhizal fungi (Umata et al., 2007). Furthermore, *Didymoplexis minor* and *D. pallens* share same mycorrhizal fungi (Burgeff, 1932). In addition, fungal sharing can be also detected in *Burmannia championii* and *B. cryptopetala* (Suetsugu et al. 2014). Similarly, mycorrhizal fungi associated with *Gp* or *Gn* can form a symbiotic relationship with *Gc* (Tashima et al., 1978). These findings suggest that plants of

the same genus can share mycorrhizal fungi. Mycenaceae, Masasmiaceae, Ceratobasidiaceae, and Polyporaceae were previously identified as candidate mycorrhizal fungi associated with *Gastrodia spp*. (Kinoshita et al., 2016). Based on our current findings, we propose that Polyporaceae associates with *Gp* and *Gn,* Mycenaceae associates with *Gc*, and different *Gastrodia spp.* can associate with the same species of mycorrhizal fungus.

Furthermore, we suggest that media derived from different preferred habitats for *Gastrodia spp.* can support growth of various combinations of mycorrhizal fungi and *Gastrodia spp*., indicating that these species potentially share many mycorrhizal fungi. These results suggest that mycorrhizal fungi used in ACSs do not need to be purified, and that the leaf and/or wood mold present in the habitats of saprophytic orchids can be used. Here we included cedar cones in our ACS media, as cones have a complex three-dimensional structure that maintains moisture and is therefore likely to promote fungal growth. Dryness reduces the viability and activity of rhizomes (Umata & Nishi, 2010), and maintaining a moist environment is important for the artificial cultivation of *Gastrodia spp.* While *Ge* germinates optimally at a temperature of 25°C (Hong et al., 2004), the temperature in the natural habitat of *Gastrodia spp.* ranges from <0°C to >35°C. In this study, we maintained the temperature at 20-25°C, and this ideal environment may have accelerated the growth of *Gastrodia* plants.

The mycorrhizal fungi *Mycena* and *Armillaria* were previously reported to associate with *Ge* (Xu & Guo, 2000). Our ACS of *Gastrodia spp.* may be a useful tool for analyzing mycorrhizal fungi associated with *Gastrodia spp.* and/or other orchids. This method could also be used to conserve *Gastrodia* spp. and other rare Orchidaceae plants in *ex situ,* many of which are endangered.

Furthermore, this method enabled us to obtain seeds from *Gastrodia spp. in vitro*. Using the ACS method described here, it would be possible to establish genetically homogeneous experimental lines of *Gastrodia spp.* It would be possible to regulate flowering time and to perform experimental crosses using different *Gastrodia spp*. We are currently generating inbred lines (which are now in the third generation), and plan to use these lines to perform genetic experiments *in vitro*.

Acknowledgements

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