A Preliminary Report on the Symbiotic Germination of Nine Japanese Terrestrial Orchids

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In order to develop an effective propagation method, symbiotic culture was attempted in nine species of Japanese terrestrial orchids. After 3 min of surface sterilization in sodium hypochlorite solution together with ultrasonic treatment, mature seeds were sown on slants of medium (the medium having been inoculated with orchid mycorrhizal fungi), cultured, and compared with nonsymbiotic controls. Approximately 10% to 70% of the seeds with embryos showed activity by the TTC test following the sowing. All but the *Cypripedium macranthum* var. *speciosum* were found to germinate with inoculum from a number of *Rhizoctonia* fungal strains. Three species of *Goodyera* (*G. biflora* var. *macrantha, G. foliosa* var. *laevis, G. hachijoensis* var. *matsumurana*), when inoculated with fungi from the binucleate *Rhizoctonia* fungus group among the 20 fungus strains tested, germinated with virtually the same TTC activity level as embryos. The fact that six of the species' germination rates were well below the TTC test results was thought due to the unsuitability of the test culture conditions. However, since numerous effective fungal strains proved effective with five species other than *C. macranthum* var. *speciosum,* which developed a symbiotic relationship with only one of the fungal isolates tested, it was predicted that for symbiotic germination among three species (*Aorchis cyclochila, Dactylorhiza aristata, Gymnadenia camtschatica*) the binucleate *Rhizoctonia* fungal group would be suitable, while *R. repens* would be appropriate for two other species, *Amitostigma kinoshitai* and *Ponerorchis graminifolia* var. *graminifolia.*

INTRODUCTION

Many native Japanese plants are facing extinction. The main reason orchid species in Japan are on the decrease is the indiscriminate horticultural overharvesting peculiar to this country (Japan Society of Plant Taxonomists, 1993). This is because many species collected do not have an effective propagation method to date; thus there is over-reliance on the collection of plants from natural habitats such as mountain areas. Given the threat of extinction, these orchid species must be protected and at the same time the development of effective propagation methods is essential in order to meet the demand.

As a means of both preserving and propagating temperate terrestrial orchid species which are difficult to grow, symbiotic germination using mycorrhizal fungi from the orchid root is utilized, along with the conventional nonsymbiotic germination approach (Clements et al., 1986; Rasmussen, 1995). In the symbiotic culture technique, were it possible to establish an orchid-fungus association favorable to the orchid, it would presumably provide a very practical culture method (Hadley, 1970; Rasmussen, 1995), but there are few studies on orchid-fungus associations among

Japanese orchid species (Tsutsui and Tomita, 1988; Masuhara and Katsuya, 1989; Tomita, 1995).

Thus, in the present study, we attempted a symbiotic culture with orchid mycorrhizal fungi for nine species of orchids native to Japan, and conducted a preliminary investigation for each species in terms of the suitability of the symbiotic culture method and the problems encountered.

MATERIALS AND METHODS

From July to October of 1996, seeds from the following species were collected immediately before capsule dehiscence: *Amitostigma kinoshitai* (Ohwi et Hashimoto) Hashimoto, *Aorchis cyclochila* (Franch. et Savat.) Hashimoto, *Cypripedium macranthum* Sw. var. *speciosum* (Rolfe) Koidz., *Dactylorhiza aristata* (Fisch.) Soo, *Goodyera biflora* (Lindl.) Hook. f. var. *macrantha, G. foliosa* (Lindl.) Benth. ex Clarke var. *laevis* Finet, *G. hachijoensis* Yatabe var. *matsumurana* (Schltr.) Ohwi ex Hatsushima et Amano, *Gymnadenia camtschatica* (Cham.) Miyabe et Kudo, and *Ponerorchis graminifolia* Rechib. f. var. *graminifolia*. Seeds of *Aorchis cyclochila, G. biflora* var. *macrantha*, and *G. hachijoensis* var. *matsumurana* were collected from strains cultivated in a greenhouse at Hirosaki University, while seeds of the six other taxa were cross-fertilized in their natural habitat of Aomori Prefecture, with the permission of the landowner, and the seeds collected.

The seeds were then placed in 5-ml bottles for 3 min with a sodium hypochlorite solution (1% available chlorine) to which a few drops of surface-active agent had been added. Along with surface sterilization, ultrasonic treatment of each container was performed to enhance germination (Miyoshi and Mii, 1988). Then, following several washings in sterile distilled water, the seeds were sown on the medium. The TTC test (Van Waes and Debergh,1986) was used to check activity of some of the seeds.

Oat-powdered agar (Tomita, 1995) was used for the symbiotic germination medium and T medium (Tsutsui and Tomita, 1990) for the nonsymbiotic culture medium. Seeds were sown on a slope of 20 ml of medium in a 25 mm × 150 mm test tube, with a sowing density of 200 to 300 seeds with embryos per test tube. At least five replicate tubes were prepared for each treatment.

In the symbiotic culture, a fungal block 5 mm in diameter was inoculated into the upper and lower end of the medium slope immediately after sowing. Fungi were isolated from the orchid mycorrhizae. A total of 20 strains were used (Table 1), 15 of which were identified as belonging to *Rhizoctonia;* five strains of binucleate *Rhizoctonia*, five of *R. repens*, and five strains of multinucleate *Rhizoctonia*. The other five strains were from other than *Rhizoctonia*.

The seeds were cultured for 16 weeks and the germination rate and postgermination growth were then evaluated. Seeds in which the embryos showed sufficient swelling to break the testa were considered to have germinated (Arditti, 1967). Growth was assessed on a scale of 1 to 5 as follows: (1) germination; (2) marked swelling of protocorm (equivalent to long diameter of testa); (3) shoot beginning to differentiate a protocorm; (4) bud elongation or leafing out; and (5) root differentiation. The mean values were obtained.

Culturing was done at 23±1C in the dark, but following germination moved to continuous illumination (500 lux) in the test tubes showing growth, to the 3rd and 4th stages.

Table 1. Orchid mycorrhizal fungi used in the experiment.

Isolate No.	Fungal group (anastomosis group)	Group	Origin of isolate: Host orchid	Origin of isolate: Habitat
No.614	Binucleate *Rhizoctonia*	(I)	*Dactylorhiza aristata* (Fisch.)Soo	Sapporo, Hokkaido
No.706	"	(C)	*Gymnadenia camtschatica* (Cham.) Miyabe et Kudo	Bikuni, Hokkaido
No.715	"	(unknown)	*Cypripedium macranthum* Sw. var. *speciosum* (Rolfe) Koidz.	Sapporo, Hokkaido
No.861	"	(unknown)	*Cymbidium goeringii* (Reichb. f.) Reichb. f.	Noto, Ishikawa
No.9410	"	(unknown)	*Goodyera schlechtendaliana* Reichb. f.	Ajigasawa, Aomori
No.612	*Rhizoctonia repens*	(ll)	*Cremastra appendiculata* (D. Don) Makino	Sapporo, Hokkaido
No.618	"	(l)	*Gymnadenia camtschatica*	Shakotan, Hokkaido
No.737	"	(l)	*Spiranthes sinensis* (Pers.) Ames var. *amoena* (M. v. Bieb.) Hara	Niigata, Niigata
No.864	"	(unknown)	*Spiranthes sinensis* (Pers.) Ames var. *amoena* (M. v. Bieb.) Hara	Bibai, Hokkaido
No.9407	"	(unknown)	*Cymbidium goeringii*	Hirosaki, Aomori
No.101	Multinucleate *Rhizoctonia*	(unknown)	*Gymnadenia camtschatica*	Zenibako, Hokkaido
No.621	"	(l)	*Dactylorhiza aristata*	Sapporo, Hokkaido
No.634	"	(l)	*Gymnadenia camtschatica*	Oohira, Hokkaido
No.714	"	(unknown)	*Oreorchis patens* (Lindl.) Lindl.	Chitose, Hokkaido
No.9412	"	(unknown)	*Gymnadenia camtschatica*	Hiraka, Aomori
No.606	Non *Rhizoctonia* Isolates	(l)	*Calanthe discolor* Lindl.	Shinoyama, Hyogo
No.620	"	(l)	*Gymnadenia camtschatica*	Sapporo, Hokkaido
No.622	"	(l)	*Calanthe discolor*	Sapporo, Hokkaido
No.640	"	(l)	*Calanthe discolor*	Kitakata, Gifu
No.726	"	(unknown)	*Calanthe tricarinata* Lindl.	Chitose, Hokkaido

RESULTS

Table 2 presents the TTC test results for mature seed immediately after sterilization along with the symbiotic and nonsymbiotic germination results. The results shown for symbiotic culture include at least one orchid species seed from the strains listed in Table 1 which had been established with a fungal strain. From the color reaction results in the TTC bath water solution, the percentage of active seeds from among the tested seeds with embryos ranged widely from 10% to 70%.

Germination was recognized in symbiotic culture with *Rhizoctonia* isolates in all nine orchid species tested. In the non-*Rhizoctonia* fungi-inoculated types, no germination whatsoever was observed although embryo swelling was found. In the multinucleate *Rhizoctonia* fungi-inoculated cultures, aside from *G. biflora* var. *macrantha*, eight species showed no germination. Moreover, in the eight species excluding *C. macranthum* var. *speciosum,* germination was achieved from inoculation with a number of *Rhizoctonia* fungal isolates.

Amitostigma kinoshitai. There was no germination in the nonsymbiotic controls. Germination was evident in both the binucleate *Rhizoctonia* and *R. repens* fungi-inoculated groups. Germination rate and postgermination growth were better in the *R. repens* group than in the binucleate *Rhizoctonia* fungi-inoculated group. The percentage of embryo staining in all seeds with embryos was 35.5% by TTC test, and No. 9407 isolate showed the highest germination rate of 8.6%.

Aorchis cyclochila. Symbiotic germination was only found with binucleate *Rhizoctonia* inoculation. No difference in the germination rate was noted between the symbiotic and nonsymbiotic culture techniques, but seedling growth was better with symbiotic germination. From the TTC testing, the embryo staining in all seeds with embryos reached 70.5%, against the maximum germination rate of 20% among nonsymbiotic culture seeds.

Cypripedium macranthum* var. *speciosum. Symbiotic germination in this orchid species was only achieved with isolate No. 706. The nonsymbiotic culture achieved a higher germination rate and subsequent seedling growth. Even in the nonsymbiotic approach, however, germination was only 10% of the TTC test embryo staining ratio.

Dactylorhiza aristata. Symbiotic germination resulted only with the binucleate *Rhizoctonia* fungal isolates. There was more actual growth than by nonsymbiotic germination, especially with isolate No. 614.

Goodyera biflora* var. *macrantha. The embryo staining rate was 59.8% by TTC test. There was over 50% germination with either of the binucleate *Rhizoctonia* fungal isolates, and seedling growth after germination was better than by nonsymbiotic germination. Of the nine orchid species tested, germination was achieved only by the multinucleate *Rhizoctonia* isolate No. 9412.

***Goodyera foliosa* var. *laevis*.** In this species the culture reaction tended to be the same as for *G. biflora* var. *macrantha,* except that no germination resulted from inoculation with the multinucleate *Rhizoctonia* fungal group. The germination rate and actual growth with *R. repens* were virtually the same as in the nonsymbiotic culture, but the results were better with the binucleate *Rhizoctonia* fungi.

Table 2. Effects of fungal endophytes on seed germination and seedling development of nine Japanese terrestrial orchids.

Mycorrhizal fungi isolate	Orchids: Germination percentage (A) and developmental index (B)							
	Amitostigma kinoshitai		*Aorchis cyclochila*		*Cypripedium macranthum*		*Dactylorhiza aristata*	
No.	A	B	A	B	A	B	A	B
614	5.4b[Z]	2.4b	15.9a	2.5a	0b	-	26.3a	3.6a
706	2.3b	2.1b	16.5a	2.3a	0.4b	1.0b	13.0b	2.8a
9410	3.6b	2.2b	17.0a	2.3a	0b	-	22.6a	3.2a
618	8.5a	2.8b	0b	-	0b	-	0c	-
864	8.2a	3.0ab	0b	-	0b	-	0c	-
9407	8.6a	3.6a	0b	-	0b	-	0c	-
9412	0c	-	0b	-	0b	-	0c	-
Asymbiotic control	0c	-	20.0a	1.7a	1.1a	2.0a	27.4a	1.5b
Colored embryo with TTC test (%)[Y]	35.5		70.5		10.6		59.5	

Symbiotic culture results are shown for at least one of orchid species seeds observed to have germinated from among the fungi incubated in Table 1. Symbiotic culture was performed on oat powdered medium (Tomita, 1995). Tsutsui and Tomita medium (1990) was used for asymbiotic control.

[Z] Mean separation within column by Duncan's multiple range test at 5% level.

[Y] The seeds after sterilization were soaked in 1% 2,3,5-triphenyl tetrazolium chloride(T.T.C.) for 24 h at 30C in the dark (Van Waes and Debergh, 1986). At least 400 seeds tested.

***Goodyera hachijoensis* var. *matsumurana*.** The germination rate and growth of this species when symbiotically cultured with the binucleate *Rhizoctonia* isolates was better than in the nonsymbiotic culture. Germination did not result in the *R. repens* or multinucleate *Rhizoctonia* fungal inoculates.

***Gymnadenia camtschatica*.** Symbiotic germination resulted only when the symbiont was a binucleate *Rhizoctonia* isolate. Post-germination growth was better by symbiotic culture, but there was a higher germination rate with nonsymbiotic culture.

***Ponerorchis graminifolia* var. *graminifolia*.** The germination rate and actual seedling growth were almost the same in the control or seeds cultivated symbiotically with binucleate *Rhizoctonia* fungi isolates. Both the germination rate and seedling growth were better with *R. repens* than in either the control or binucleate *Rhizoctonia* fungal inoculation approach.

Orchids									
	Germination percentage (A) and developmental index (B)								
Goodyera biflora var. *macrantha*		*G. foliosa* var. *laevis*		*G. hachijoensis* var. *matsumurana*		*Gymnadenia* camtschatica		*Ponerorchis graminifola*	
A	B	A	B	A	B	A	B	A	B
57.1a	4.6a	52.4a	3.8a	34.0ab	3.5a	10.2ab	1.3a	17.6ab	1.5b
54.5a	4.6a	45.6a	3.7a	45.8a	4.0a	7.6b	1.8a	12.5b	1.3b
50.6a	4.6a	46.8a	3.9a	30.3ab	3.9a	0c	-	10.8b	1.6b
34.4b	2.0b	40.3a	1.6b	0c	-	0c	-	18.2ab	1.9b
46.2ab	1.9b	36.1ab	1.3b	0c	-	0c	-	23.1a	2.8a
45.0ab	2.0b	45.8ab	1.5b	0c	-	0c	-	20.5a	2.5a
13.6c	1.4b	0c	-	0c	-	0c	-	0c	-
46.1a	1.9b	51.3a	2.1b	30.1b	2.1	13.5a	1.0b	16.8ab	1.3b
59.8		61.5		52.2		27.0		43.5	

DISCUSSION

From the results of the TTC test, which have a close relation to germination activity (Van Waes and Debergh, 1986), the tested seeds showed 10% to 70% staining immediately after being sown, and were thus considered to have high germination activity. However, given the germination test results (Table 2), only the three *Goodyera* species showed virtually the same germination rate as the active seed percentage indicated by the TTC test. The other species, either in symbiotic or nonsymbiotic conditions, showed less than half or a much lower germination rate than that indicated by the TTC test, so the test conditions were considered inappropriate for germination. *Dactylorhiza majalis* and several other terrestrial orchids exhibited the effects of culture temperature on the symbiotic seedling growth (Rasmussen, 1995). *Ponerorchis graminifolia* was reported to undergo a germination promotion effect after sowing by the addition of low-temperature treatment (Ichihashi, 1989). Besides the screening tests for orchid seeds and mycorrhizal fungi, it was clear that due consideration should be given to the seed culture, environmental, and other conditions as well.

Many reports credit the binucleate *Rhizoctonia* group (a species related to *Ceratobacidium cornigerum*) with a strong growth-enhancing effect on the symbiotic seedlings of *Goodyera* species (Alexander,1982; Rasmussen, 1995; Tomita, 1995), a finding corroborated in the present investigation. Even within the *Goodyera* genus, the range of symbiotic fungal groups in the three tested species was different, and in *G. biflora* var. *macrantha*, a symbiotic relationship developed with the same multinucleate *Rhizoctonia* fungal strain as with *G. oblongifolia* (Harvais, 1974).

The difference in the range of fungal strains resulting in symbiosis even within the same genus is of great interest in investigating the orchid-fungus association for developing a propagation method using symbiotic germination.

In symbiotic culture, another issue is the nourishment dependence on the fungus of the orchid for propagation (Tsutsui and Tomita, 1988). As with *Bletilla striata* (Masuhara and Katsuya, 1989) or *Pectelis radiata* (syn. *Habenaria radiata* Spreng)(Tsutsui and Tomita, 1988), species that readily germinate even asymbiotically and form a symbiosis with many fungal strains and groups, the dependence on the fungus is thought to be low. In such orchid species, there is not much to be gained from using symbiotic culture for seedling propagation.

Among the nine orchid species tested in the present study, the three belonging to the *Goodyera* genus were easily germinated by nonsymbiotic culture, and *G. biflora* var. *macrantha* in particular showed somewhat less fungal dependence than the other orchid species. However, since in all three of the species belonging to the *Goodyera* genus, seedling growth was promoted by inoculation with the *Rhizoctonia* fungal group isolate when compared to the nonsymbiotic culture method, we consider the symbiotic method to be an effective means to propagate seedlings.

Based on the foregoing, with the exception of the three *Goodyera* species, the test culture conditions may well have not been ideal for the germination of the other six orchid species. However, one may assume a suitable fungal group exists from the fact that numerous fungal isolates proved effective. The present findings suggested that the binucleate *Rhizoctonia* fungal group is suitable for the three species, *A. cyclochila*, *D. aristata*, and *G. camtschatica*, while the *R. repens* fungal group is suitable for *A. kinoshitai* and *P. graminifolia* var. *graminifolia*. In forthcoming screening of the mycorrhizal fungi applicable for symbiotic culture of various orchid species, we believe that effective fungi can be efficiently selected by testing the fungal groups which appear suitable for symbiotic culture. From careful study of the culture conditions for germination, seedling production from symbiotic germination is also possible.

In only one line of *C. macranthum* var. *speciosum* was there any symbiotic germination, and it would be risky to assume a suitable fungal group based on only the present experimental results. The use of the symbiotic germination method as a means to propagate this orchid species would necessitate both study of the seed culture conditions and the screening of more fungal strains. In parallel with this, the asymbiotic method should be investigated because there is a need to find a more effective culture method.

On the basis of the above findings, it was clear that the use of the symbiotic germination method was actually of use as a means to propagate the three species belonging to the *Goodyera* genus among the nine orchid species tested. Detailed studies are needed of all culture conditions, aside from the fungus inoculated, in order to prove the use of the symbiotic germination method as a means to propagate the other six orchid species.

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LITERATURE CITED

Alexander, C. 1982. The biology of mycorrhiza in the orchid *Goodyera repens* BR. Ph. D. Thesis. University of Aberdeen, Aberdeen, U.K.

Arditti, J. 1967. Factors affecting the germination of orchid seeds. Botanical Rev. 33:1-97.

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

Harvais, G. 1974. Notes on the biology of some native orchids of Thunder Bay, their endophytes and symbionts. Can. J. Bot. 52:451-460.

Hadley, G. 1970. Non-specificity of symbiotic infection in orchid mycorrhiza. New Phytol. 69:1015-1023.

Ichihashi, S. 1989. Seed germination of *Ponerorchis graminifolia*. Lindleyana 4:161-163.

Japan Society of Plant Taxonomists. 1993. Red Data Book. pp141. Nouson Bunka-Sha, Tokyo.

Masuhara, G. and **K. Katsuya.** 1989. Effects of mycorrhizal fungi on seed

germination and early growth of three Japanese terrestrial orchids. Scientia Hort. 37:331-337.

Miyoshi, K. and **M. Mii.** 1988. Ultrasonic treatment for enhancing seed germination of terrestrial orchid, *Calanthe discolor*, in asymbiotic culture. Scientia Hort. 35:127-130.

Rasmussen, H. 1995. Terrestrial Orchids — From seed to mycotrophic plant. Cambridge University Press, Cambridge.

Tomita, M. 1995. Symbiotic seed germination and development of *Goodyera schlechtendaliana* in vitro. Plant Tissue Culture Letters 12:151-155.

Tsutsui, K. and **M. Tomita.** 1988. Differences in the symbiotic capacity among isolates of mycorrhizal fungi on some terrestrial orchids. J. Fac. Agr. Hokkaido Univ. 63:345-353.

Tsutsui, K. and **M. Tomita.** 1990. Suitability of several carbohydrates as the carbon sources for symbiotic seedling growth of two orchid species. Lindleyana 5:134-139.

Van Waes, J. and **P.C. Debergh.** 1986. Adaptation of the tetrazolium method for testing the seed viability and scanning electron microscopy of some Western European orchids. Physiol. Plant. 66:435-442.