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Symbiotic Seed Germination and Development of *Goodyera schlechtendaliana In Vitro*

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Symbiotic and non-symbiotic germination of *Goodyera schlechtendaliana* seeds were tested on a Knudson C medium and on an oat-powdered agar medium inoculated with or without one of four *Rhizoctonia* strains isolated from orchid mycorrhizae. A binucleate *Rhizoctonia* isolate No. 706 obtained from *Dactylorhiza aristata* was the most effective symbiont for *G. schlechtendaliana*. Symbiotic culture with the isolate No. 706 was effective in promoting seed germination and protocorm development compared with non-symbiotic culture. All acclimated plantlets derived from the seeds which were inoculated with No. 706 were successfully established.

Introduction

Many tropical orchid species and hybrids are now propagated using a range of non-symbiotic seed germination techniques or tissue culture procedures[1]. However, many temperate terrestrial species have proven difficult to propagate by non-symbiotic methods[2]. Some species can germinate and grow well under non-symbiotic conditions, but they have proven difficult to transfer to a soil-based medium in pots[3]. Many terrestrial orchids with beautiful and often unique shaped flowers are becoming rare as their native habitats are reduced through human interference. For both conservation and commercial production, it is desirable to determine the most effective method of propagating these species.

In nature, a symbiotic relationship with a fungus, mainly *Rhizoctonia* species, is essential for at least the later stages of germination and plantlet development of orchids[2,4]. Studies showed that fungal isolates from mature orchids could promote germination in a wide variety of European[5], Australian[6] and North American species[3,7]. It has been hoped that symbiotic germination utilizing orchid mycorrhizal fungi might be a way to overcome the difficulty of germination and to enhance the growth of the seedlings. But there are only a few comparable studies of the symbiotic germination of terrestrial Japanese orchids[8,9].

The object of this study was to compare symbiotic and non-symbiotic germination and the subsequent development of seeds of *Goodyera schlechtendaliana*, a common Japanese wild orchid.

Materials and Methods

1. *Orchid seed*

Seeds of *Goodyera schlechtendaliana*, which were cultured in a green house, were collected from fully ripened capsules and stored at 4°C in a small airtight vial after complete air-drying.

2. *Fungal isolate*

Fungal isolates used for symbiotic germination are listed in **Table 1**. These fungi were isolated

Table 1. Orchid endophyte data.

Isolate number	Fungal group (Anastomosis group)		Host orchid*	Collection site
706	Binucleate *Rhizoctonia*	(AG-C)	*Dactylorhiza aristata*	Bikuni, Hokkaido
614	〃	(AG-I)	*Gymnadenia camtschatica*	Sapporo, Hokkaido
871	*Rhizoctonia repens*	(unknown)	*Spiranthes sinensis*	Himi, Toyama
618	〃	(AG-I)	*Gymnadenia camtschatica*	Shakotan, Hokkaido

* Species of orchid from which mycorrnizal fungus was isolated.

from the roots of orchid species. Cultures were maintained on potato dextrose agar[8].

3. *Seed culture*

Dry seeds were surface sterilized in a sodium hypochlorite solution (0.5% available chlorine) for 2 minutes, then washed in 5 changes of water, and sown on slants of 30 m*l* medium in 25×150 mm test tubes. The Knudson C medium[10] was used for the non-symbiotic germination test, and oat-powdered agar (OPA: Oat powder, 3 g; distilled water, 1,000 m*l*; agar 10 g) was used for both symbiotic and non-symbiotic germination tests. For the symbiotic germination tests, a small fungal inoculum (5 mm in diameter) was added to the upper side of the slope. Five replications (tubes) of each treatment were seeded. Cultures were maintained in a 16-hour light and 8-hour dark regimen at 20°C.

Seeds in which the protocorm showed sufficient swelling to break the testa were defined as germinated. Germination and protocorm development was assessed on a scale 0-6 as follows: 0) no germination; 1) embryos swollen, testa cracking; 2) embryo 2-3 times enlarged, rhizoid apparent; 3) protocorm stage, protocorm as lomg as or longer than the testa; 4) protocorm considerably longer than the testa; 5) shoot beginning to differentiate; and 6) leaf becoming green[5,7]. At the end of the investigation, seedlings at stage 6 were thinned out and transplanted to 30 m*l* fresh OPA

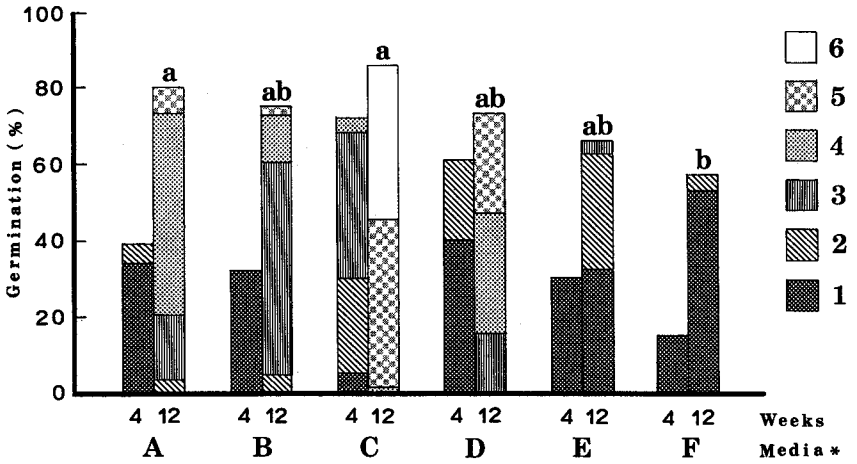

Fig. 1 Germination and development of *Goodyera schlechtendaliana* seeds as affected by orchid endophytes under non-symbiotic (A-B) and symbiotic (C-F) conditions.
Germination and protocorm development was assessed as follows: 1) Embryos swollen, testa cracking; 2) Embryo 2-3 times enlarged, rhizoid apparent; 3) Protocorm stage, protocorm as long as or longer than the testa; 4) Protocorm considerably longer than the testa; 5) Shoot beginning to differenciate; 6) Leaf becoming green.
Germination percentages marked by the same letter on the top of bars are not significantly different when tested by Duncan's multiple range test at 5% level.
A: Knudson C, B: OPA, C: OPA with isolate No. 706, D: OPA with isolate No. 614, E: OPA with isolate No. 871, F: OPA with isolate No. 618.

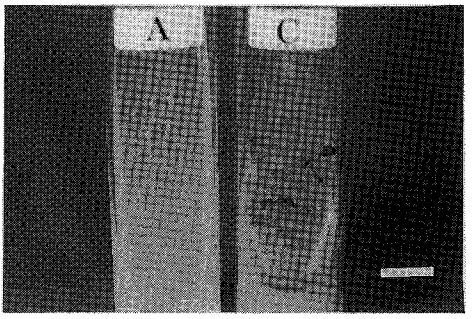

Fig. 2 Germination and development of *Goodyera schlechtendaliana* seeds on both non-symbiotic and symbiotic medium 12 weeks after sowing.
A: Knudson C (non-symbiotic)
C: OPA with isolate No. 706
bar = 1 cm

media in 100 m*l* Erlenmeyer flasks. Further, rooting plantlets were transferred to sand-based media.

Results

The results are summarized in **Fig. 1**. Germination took place in both non-symbiotic and symbiotic media. At the end of the experiment (12 weeks after sowing), the germination percentages of the seeds of *G. schlechtendaliana* under symbiotic conditions were not significantly different from those of the non-symbiotic controls with one exception (isolate No. 618, **Fig. 1-F**). The effect of fungal isolates on seedling growth varied according to their origin. Compared with non-symbiotic conditions (**Fig. 1-A, B**), inoculation of Iso. No. 706 or No. 614 stimulated seedling growth (**Fig. 1-C, D**) while No. 871 and No. 618 inhibited seedling growth (**Fig. 1-E, F**). The first sign of the stimulative effect of No. 706 and No. 614 on the orchid growth was the rapid germination of a large proportion of the seed sown. The results obtained from Iso. No. 706 were very impressive and protocorms (stage 3-4) developed 4 weeks after sowing, when many seeds on other media had only attained stage 1-2 (**Fig. 1**). After 12 weeks of sowing, the growth of the seeds inoculated with No. 706 was more remarkable as shown in **Fig. 2**.

After 12 weeks of sowing, seedlings (at stage 6) derived from seeds inoculated with No. 706 were subcultured. After 16-18 weeks of sowing, they produced roots, while the non-symbiotic seedlings had only reached stage 4-5 as shown in **Fig. 3-a**. After 18 weeks of sowing, the rooting plantlets were rinsed with tap water and transplanted into a potting mixture composed of river sand. All acclimated plantlets survived 26 weeks after sowing as shown in **Fig. 3-b**.

Discussion

There is some question concerning the degree (presence or absence) of specificity in the relation between orchid and mycorrhizal fungus[2,4]. Currently, the view that there is no species-to-species specificity between an orchid and its mycorrhizal fungus has been expressed by many researchers[5-9]. Iso. No. 706, isolated from a root of *Dactylorhiza aristata*, is known to be a good symbiont of *Spiranthes sinensis*[8]. In this study, No. 706 also proved to be a good symbiont of *Goodyera schlechtendaliana* as well. Zettler and McInnis reported that a fungus isolated from *Platanthera ciliaris* is a good symbiont of both *S. cernua* and *G. pubescens*[7]. This non-specificity has also been reported for other genera[5,8,9].

Among the fungal isolates in a fungal group, the effect of each isolate on orchids varied remark-

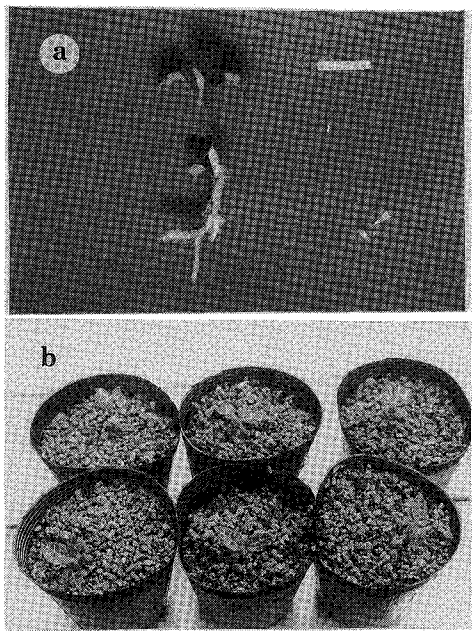

Fig. 3 Development of symbiotic seedlings of *Goodyera schlechtendaliana*.
a. Comparision with non-symbiotic condition 16 weeks after sowing.
left: Seedling cultured on OPA medium with isolate No. 706.
right (arrow): Protocorm cultured on Knudson C medium. bar=1 cm
b. Acclimated plantlets 26 weeks after sowing.

ably from non-effective to highly effective (**Fig. 1**). Warcup found that different isolates of *Tullasnella calospora* (*Rhizoctonia repens*) differed markedly in the efficiency with which they stimulated germination of the species of *Diuris* and *Thelymitra*[6]. These facts suggest that it is both possible and necessary to determine fungal isolates with a higher symbiotic capacity.

Symbiotic seedlings inoculated with No. 706 were easily transferred to sand-based media and all of them survived. Anderson found that only 2 of 40 non-symbiotic seedlings of *S. magnicamporum* survived soil transfer under septic conditions, whereas 40 of 40 symbiotic seedlings survived and flowered under the same conditions[3]. This indicates that plantlets produced using symbiotic method can acclimatize without difficulty.

The present results demonstrate that the symbiotic method is a feasible means of cultivating *G. schlechtendaliana* when seeds are inoculated with an endophyte Iso. No. 706. Further investigation is needed to improve methods for propagating the vast majority of terrestrial orchids using symbiotic fungi.

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《和文要約》

ミヤマウズラの *in vitro* 共生発芽と生長

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ミヤマウズラ(*Goodyera schlechtendaliana*)完熟種子をエンバク粉末(OPA)培地上に播種し，ラン科植物菌根より分離した *Rhizoctonia* 属菌を接種して共生培養をおこない，Knudson C 培地および OPA 培地に無菌播種した対照区と比較した．ハクサンチドリより分離した 2 核 *Rhizoctonia* 分離番号 706 はミヤマウズラに対して生育促進効果が高く，接種区では無菌対照区を著しく上回る生育を示した．706 接種区由来の実生は順化個体全てが容易に活着した．