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DIFFERENCES IN THE SYMBIOTIC CAPACITY AMONG ISOLATES OF MYCORRHIZAL FUNGI ON SOME TERRESTRIAL ORCHIDS

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Introduction

The symbiotic culture of orchid seeds *in vitro* using mycorrhizal fungi has been given much attention as a promising way of raising seedlings of terrestrial orchids which are difficult to culture by conventioinal asymbiotic methods. Recently successful results by this method were reported using some European, Australian and Japanese orchids by several authors (1, 2, 3, 6, 10). It has been stressed that finding the most effective symbiont for each orchid species is vitally important for this method to be practical (2, 5). In order to perform the screening tests efficiently, the general relationships between orchids and fungal species or isolates must be clarified. On the other hand, 'to be practical' in this method requires that not only a stable orchid-fungus association is established at a high rate inducing seed germination but also the growth of seedlings should be adequately stimulated.

In this study the relationships between symbiotic capacity of mycorrhizal fungal isolates and orchid species were examined from the above view point regarding the successful orchid-fungus associations found in the course of screening practices.

Materials and Methods

Seed and Culture

Mature seeds of *Sprianthes sinensis* Ames, *Liparis nervosa* Lindl., *Epidendrum ibaguense* HBK, *Amitostigma kinoshitae* Schltr. and *Habenaria*

Propagation of orchids by symbiotic culture. III.

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radiata Spreng. were used. The seeds were collected in July-September, 1986, and stored at 4°C in a desiccator containing silica gel after complete air drying.

Dry seeds were surface-sterilized in a sodium hypochlorite solution of 0.5% available chlorine for 2 minutes then washed in 3 changes of sterile water. Sterilized seeds of 50–150 according to orchid species were sown on the slope of 30 ml midum in 30 mm × 150 mm test tube. The test tubes were stoppered with double sheets of aluminium foil. At least 3 replicate tubes were prepared for each orchid-fungus combination. One week after seeding, a small fungal inoculum was added to the upper end of slope. All cultures were maintained at about 25°C in a 16-hour light and 8-hour dark regime.

An oat medium was used throughout. Hull-less oat grains of 25 g were boiled in 1 litre of distilled water for 1 hour and filtrated through 4 sheets of gauze. The filtrate was solidified with 1% agar. The pH of the medium before autoclaving was about 6.0 which was not adjusted further. As the asymbiotic control, the medium containing inorganic salts of T-formula described in a previous paper (10) and 2% sucrose (T1) and one to which was added 0.02% yeast extract to T1 (T2) were prepared.

Symbiotic capacity of all fungi was evaluated by the germination percentage and the developmental index of seedlings 6–31 weeks after inoculation according to orchid species as shown in Table 2 with the exception of *Spiranthes sinensis* for which the fresh weight of seedlings was measured 12 weeks after inoculation.

Seed in which the protocorm showed a sufficient swelling to break testa was defined as germinated. Developmental index of seedlings was obtained as a weighed average of the grades given to the following 5 developmental stages: 1. protocorm apparently swollen but still in the testa, 2. protocorm stage with some epidermal hairs, 3. protocorm with many haris and the growing end usually somewhat green, 4. a green shoot appearing but still without root, 5. seedling stage with leaves and root(s).

Mycorrhizal fungi

The methods for isolation and culture of mucorrhizal fungi were identical to those used in a previous paper (10). Mycorrhizal fungi used in the present study were 32 isolates obtained from the mycorrhizae of 10 orchid species: *Coeloglossum viride* Hartm. var. *bracteatum* Richter, *Cremastra appendiculata* Makino, *Cymbidium goeringii* Reichb. fil., *Cymbidium* cv., *Dactylorhiza aristata* (Fisch.) Soo, *Gymnadenia camtschatica* Miyabe et Kudo, *Liparis nervosa* Lindl., *Oreorchis patens* Lindl., *Paphiopedilum* cv. and

Spiranthes sinensis Ames. These are shown in Table 1 together with their origin. The fungal isolates could be devided into 2 groups: the first one indicated as B group was binucleate *Rhizoctonia*, and the other one indicated as R group was *R. repens* Bernard.

TABLE 1. Orchid mycorrhizal fungi used in the experiment

Fungal group and Isolate No.	Origin of isolate	
	Host orchid	Habitat*
Binucleate *Rhizoctonia* (B group)		
613	*Dactylorhiza aristata*	Sapporo, Hokkaido; W
614 (AG-I)	*Gymnadenia camtschatica*	Sapporo, Hokkaido; W
616	*Oreorchis patens*	Sapporo, Hokkaido; W
617	*Coeloglossum viride*	Sapporo, Hokkaido; W
619	*Coeloglossum viride*	Jozankei, Hokkaido; W
6312	*Oreorchis patens*	Kuromatsunai, Hokkaido; W
706 (AG-C)	*Gymnadenia camtschatica*	Bikuni, Hokkaido; W
Rhizoctonia repens (R group)		
503	*Liparis nervosa*	Taketoyo, Aichi; W
504	*Spiranthes sinensis*	Taketoyo, Aichi; W
603	*Cymbidium* cv.	Tokai, Aichi; C
605	*Cymbidium goeringii*	Mita, Hyogo; W
607	*Paphiopedilum* cv.	Chita, Aichi; C
608	*Spiranthes sinensis*	Sasayama, Hyogo; W
612	*Cremastra appendiculata*	Sapporo, Hokkaido; W
618	*Gymnadenia camtschatica*	Shakotan, Hokkaido; W
623	*Cymbidium goeringii*	Fuchu, Toyama; W
624	*Spiranthes sinensis*	Fuchu, Toyama; W
627	*Dactylorhiza aristata*	Jozankei, Hokkaido; W
628	*Dactylorhiza aristata*	Kuromatsunai, Hokkaido; W
637	*Dactylorhiza aristata*	Kuromatsunai, Hokkaido; W
639	*Spiranthes sinensis*	Tonami, Toyama; W
813	*Spiranthes sinensis*	Taketoyo, Aichi; W
821	*Spiranthes sinensis*	Taketoyo, Aichi; W

* C = cultivated plant; W = wild plant

Results

Actually, about 50 isolates of mycorrhizal fungi consisted of 12 isolates of binucleate *Rhizoctonia*, 17 isolates of *R. repens*, 9 isolates of multinucleate *Rhizoctonia* including *R. solani* and 9 isolates of fungi other than Rhizoctonia had been obtained from orchid mycorrhizae, and their symbiotic capacity

TABLE 2. Effects of fungal isolates on the germination and seedling development of some terrestrial orchids

Mycorrhizal fungi		Orchids, time after inoculation, germination % (A) & fresh weight (B, mg) or developmental index (C)									
		Spiranthes sinensis		*Liparis nervosa*		*Epidendrum ibaguense*		*Amitostigma kinoshitae*		*Habenaria radiata*	
Group	Iso. No.	12 weeks		6 weeks		19 weeks		31 weeks		22 weeks	
		A	B	A	C	A	C	A	C	A	C
B	613	30.1	38.4	0	—	0	—	0	—	20.5	4.4
»	614	37.5	43.5	0	—	8.3	5.0	0	—	12.5	2.6
»	616	34.2	36.6	0	—	15.0	4.3	0	—	37.3	3.8
»	617	14.8	—	0	—	7.9	2.4	0	—	33.3	2.4
»	619	26.0	34.9	0	—	0	—	2.4	2.0	8.6	3.4
»	6312	17.6	25.1	0	—	14.9	3.3	0	—	0	—
»	706	35.6	40.6	0	—	14.8	5.0	0	—	48.1	3.8
R	503	1.9	—	0	—	0	—	0	—	32.9	2.9
»	504	0	—	0	—	0	—	0	—	31.9	2.8
»	603	10.5	—	0	—	7.9	3.0	0	—	62.5	3.5
»	605	0	—	0	—	5.1	3.0	0	—	69.0	2.2
»	607	21.5	8.4	0	—	20.6	2.6	0	—	37.8	2.1
»	608	4.1	—	34.8	2.7	5.6	2.3	0	—	33.3	2.9
»	612	18.4	44.9	0	—	20.6	4.1	1.4	3.5	45.1	3.2
»	618	18.4	32.3	0	—	15.6	3.6	0	—	49.1	2.6
»	623	21.8	21.8	0	—	6.9	5.0	1.9	3.0	63.5	3.4
»	624	17.9	34.2	72.1	3.6	20.6	5.0	4.6	5.0	60.7	4.8
»	627	9.9	31.6	0	—	26.4	3.9	0	—	54.4	3.1
»	628	12.9	44.2	0	—	17.1	4.7	0	—	64.2	2.9
»	637	14.1	—	0	—	4.7	4.0	2.1	3.8	67.1	2.9
»	639	22.4	18.1	0	—	8.1	2.6	0	—	44.3	2.4
»	813	18.8	18.7	0	—			0	—	45.3	3.2
»	821	12.2	—	0	—			0	—	34.8	2.9
Asymbiotic control											
T1		0	—	0	—			0	—	71.7	4.0
T2		0	—	0	—	18.6	3.4	0	—	66.7	4.0

was examined with the seeds of 30 orchid species including some cultivated ones. The fungal isolates listed in Table 1 are those that caused germination of at least one orchid species. The rest, which is omitted in this paper, all failed to cause germination among the investigated orchids excepting *Habenaria radiata* which germinated by the inoculation of 2 isolates of multinucleate *Rhizoctonia* and an isolate of non-*Rhizoctonia* besides those shown in Table 1.

The results of symbiotic cultures with these fungal isolates shown in Table 1 are summarized in Table 2.

Spiranthes sinensis The highest germination percentages of about 35% were obtained by the inoculation of isolates No. 614 and No. 706 of B group. These isolates were also most effective in stimulating seedling growth. In contrast to these, the germination percentages of those inoculated with the isolates of R group were lower than these (about 20%) even in the highest cases. The R group contained such isolates as No. 503,504 and 605 which induced utterly or nearly no germination. This group was obviously inferior to B group as a whole in the germination percentage. However, it contained some isolates such as No. 612 and No. 628 which were equal to the best ones of B group in the stimulating effect on seedling growth. No germination occurred in the asymbiotic culture at the time of observation. The fate of seeds in this case was already described in a previous paper (10).

Liparis nervosa The germination of this orchid was only observed by the inoculation of isolates No. 624 and No. 608 of R group; especially the former was far superior in the stimulating effect both in the germination and seedling growth. Although the observation of this orchid was made earlier than the others (6 weeks after inoculation), no additional germination was observed from that time on except in the asymbiotic culture. In the asymbiotic culture, germination began several months later, but the growth of seedlings was far slower than those of symbiotic cultures although the germination percentage was as high as that of No. 624.

Epidendrum ibaguense The germination of this foreign orchid was best when inoculated with 4 isolates of R group, and isolate No. 624 was again the best among these isolates also in the enhancing effect on seedling growth. Although the stimulating effect on seedling growth was equally great by 2 isolates in B group and an isolate in R group, the germination was not so good. This orchid was germinated under asymbiotic conditions as well as the best inoculated ones, but the growth rate of seedlings was much lower than symbiotic cases.

Amitostigma kinoshitae This orchid failed to germinate in asymbiotic

culture, and even by the inoculation of the most effective symbiont, isolate No. 624 of R group, the maximum germination was less than 5%. The germination occurred by the inoculation of 3 isolates in R group and an isolate in B group beside this, but the germination percentages and growth indices of seedlings were much lower than those by No. 624.

Habenaria radiata All the tested fungal isolates with exception of No. 6312 more or less caused germination of this orchid, and as already mentioned the germination also occurred by some fungal isolates belonging to the fungal groups other than B or R. However, the germination and seedling growth were excellent under asymbiotic conditions. The germination by the inoculation of B group fungi was usually much inferior to that of asymbiotic culture but nearly half the number of fungal isolated of R group showed high germination percentages equal to that of asymbiotic culture. Nearly equal or slightly superior seedling growth to asybiotic culture was seen with fungal isolates No. 613 in B group and No. 624 in R group. But the latter was the only one that brought about both good seedling growth and a high germination percentage comparable to the asymbiotic culture.

Discussion

The presence or absence of specificity in the relation between orchid and mycorrhizal fungus has long been a matter of concern in this field. Recent reports all agree in the point that there is not such a strict specificity as species-to-species, nor is the orchid-fungus relation completely random (2, 4, 7, 8, 9, 10, 11, 12). The results of the present paper also agreed with those in that point. The germination percentage and developmental index of seedlings used as the parameters for the symbiotic capacity of fungal isolates did not necessarily accompany each other but there were various combinations of them: no germination occurred, germination occurred but both were low, either of the two was high or both were high. Therefore, in the screening practice for effective fungal isolates, it will be necessary to examine not only the effect on germination but also that on seedling growth.

Among the fungal isolates in a fungal group, their effects on an orchid varied remarkably from non-effective to highly effective. WARCUP (12) found that different isolates of *Tulasnella calospora* (*Rhizoctonia repens*) differed markedly in the efficiency with which they stimulated germination of the species of *Diuris* and *Thelymitra*. This fact suggests that it will be necessary and possible to find out those having a higher capacity.

Whether the differences in effectiveness among isolates are consistent or vary with the associating orchids arouses one's interest. Certainly in a

strict sense the answer was negative. For example isolate No. 6132 which was effective on *Spiranthes* to some extent was utterly ineffective on *Habenaria* which was sensitive to the other isolates than this, and isolate No. 608 which was one of the 2 isolates effective on *Liparis* was almost ineffective on *Spiranthes* and *Amitostigma*.

Notwithstanding these inconsistencies in detail it may be possible to generalize that the differences in effectiveness among isolates in a fungal group as a whole are fairly consistent and the disturbances by associating orchids are relatively small. This leads to the idea that the symbiotic capacity of an isolate in a fungal group primarily depends upon the characteristics of the isolate and is modified secondarily by the orchid with which it associates.

On the other hand, the most effective isolates in terms of both germination and seedling growth were isolates No. 614 and No. 706 of B group for *Spiranthes* and isolate No. 624 of R group for the other 4 orchid species in common. Although *Liparis* associated only with the isolates of R group, other orchids associated with those of both R and B groups. But if it is taken as a criterion to contain the most effective isolates and many isolates which induce high germination percentages, the most effective fungal group may be identified for each orchid species. Thus *Spiranthes sinensis* can be classified as B group and the other 4 orchids as R group.

The fact that the most effective isolate was in common with the 4 orchids supports the idea of relative consistency in the degree of effectiveness among isolates in a fungal group. If this be true, it would be useful in increasing the efficiency of screening practices. To cite one example of R group, it will be sufficient to use only one of the most effective isolates such as No. 624 as a representative of this group when screening of fungi is made against an unexamined orchid, and in the same manner it will be sufficient to use only one orchid specific to this group such as *Liparis nervosa* when screening is made to search for a more effective isolate in this group.

As a matter of course such a rule of inductive nature as this should be drawn from sufficient accumulation of data. Therefore, further investigations with more fungal isolates and orchids may be necessary, and further progress of taxonomical studies of *Rhizoctonia* should be anticipated as well before we can visualize the whole picture. But this idea may be useful as a working hypothesis at least for R group for the time being.

Summary

The symbiotic capacity of 7 isolates of binucleate *Rhizoctonia* (B group) and 16 isolates of *R. repens* (R group) obtained from various orchid mycorrhizae were evaluated by the 2 parameters of seed germination and seedling growth with *Spiranthes sinensis* and 4 other terrestrial orchids *in vitro* on a oat medium. Since the germination percentage and the seedling growth did not necessarily accompany each other, it was considered necessary to examine both parameters in the screening practice. Among the isolates in a fungal group the effects on an orchid varied considerably from non-effective to highly effective. These differences in effectiveness among isolates in a fungal group were considered to be fairly consistent as a whole, although they could be modified to some extent by the difference of orchids to associate in detail. Thus the most effective fungal group for each orchid species could be identified by the criterion that it contained the most effective fungal isolates and many isolates which induced higher germination percentages than the other groups. *Spiranthes sinensis* could be classified as B group and *Liparis nervosa*, *Epidendrum ibaguense*, *Amitostigma kinoshitae* and *Habenaria radiata* as R group.

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