SYMBIOTIC GERMINATION OF *SPIRANTHES SINENSIS* AMES ASSOCIATED WITH SOME ORCHID ENDOPHYTES

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Introduction

At that time when KNUDSON succeeded in raising *Cattleya* from seeds non-symbiotically, it was considered that the problem of seed germination for orchids would soon be solved by this method. Since then the study on the germination of orchids has been centered around this method and a large number of reports on non-symbiotic germination of various cultivated and wild orchids have appeared as seen in the reviews by ARDITTI (1, 2). In spite of the fact that the non-symbiotic method has become a practical way of propagation for many important cultivated orchids of tropical origin, there are still many orchids which fail to germinate or grow only very slowly if not fail under non-symbiotic conditions. Especially, this is true of wild terrestrial orchids in the temperate zone. Many species among these orchids have been threatened with extinction by human impact so that it has become an urgent question to develop a new method of seed germination as the means of practical propagation for these orchids.

It has been hoped that the symbiotic germination utilizing orchid mycorrhizal fungi can be a way to overcome the difficulty in germination and to enhance the growth of seedlings. On the mycorrhiza of orchids a considerable amount of knowledges have been accumulated through research work carried out from the scientific interest in this phenomenon (2, 12). Reflecting the above mentioned situation the work attempting to utilize orchid mycorrhizal fungus as a tool for parctical propagation, however, has almost been terminated by the classic work of BURGEFF (4). It may be necessary to resume the study such as this on the basis of such fundamental knowledges

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1) An outline of this paper was read at the annual meeting of the Japanese Society for Horticultural Science, Spring 1985.

as HADLEY (8) has stressed this in his article on this matter.

The information directly related to this problem may be summarized as follows: (a) no strict species-to-species specificity has been found between an orchid and a mycorrhizal fungus, (b) an orchid species has one or more compatible symbionts favourable to its germination and growth in different degrees, (c) the establishment of the symbiotic association can be affected considerably by the nutritional condition of the culture medium. In order to establish such a practical method of germination, it may be necessary firstly to find out the most suitable symbionts through screening of various kinds of Rhizoctonia and secondly to investigate the effect of culture media on the symbiosis.

This paper reports the results of the tests carried out in this context with *Spiranthes sinensis* Ames, one of the most common wild orchids in Japan, and also touches upon the comparison between the symbiotic and non-symbiotic germination of this species.

Materials and Methods

Isolation and culture of mycorrhizal fungi

Isolation of endophytes was attempted with the following orchid species from June to August, 1984: *Gymnadenia camtschatica* MIYABE et KUDO, *Cremastra appendiculata* MAKINO, *Oreorchis patens* LINDL., *Coeloglossum viride* HARTM., *Cypripedium japonicum* THUNB., and *Spiranthes sinensis* collected from their natural habitats, and *Calanthe reflexa* MAXIM., *C. discolor* LINDL., *Cymbidium goeringii* REICHB. f. and *Phajus minor* BL. grown in pots.

Roots of orchid were thoroughly washed in running water and those with healthy appearance were selected. Some hand sections from each root were microscopically examined for the presence of endophytes. If hyphae were found, portions 3–10 mm long depending on the thickness of roots were cut and surface sterilized in sodium hypochlorite solution (0.5% available chlorine) for 2 minutes and washed in three changes of sterile water. A portion of the root was placed in 2–3 drops of sterile water in an isolating dish and teased apart using two pairs of sharp pointed tweezers, and plated with cooled, molten WARCUP's isolating medium described by CLEMENTS *et al.* (5) and incubated at 25°C in the dark. After subculturing on the same medium, the pure culture was transferred onto potato dextrose agar slopes.

Seed culture

Seeds of *Spiranthes sinensis* from plants in the collection of The Botanic

Garden, Faculty of Agriculture, Hokkaido University were collected and stored ar 4°C in small airtight vials after complete air-drying. Dry seeds were surface sterilized in sodium hypochlorite solution (0.5% available chlorine) for 5 minutes and washed in three changes of water. For symbiotic culture a strip of 20 mm × 50 mm sterilized filter paper was placed on the slope of 30 ml medium in a 30 mm × 150 mm test tube. Sterilized seeds were sown on the filter paper, a small inoculum (4 mm in diameter) was added to the upper edge of filter paper, and the tube was stoppered with double sheets of aluminium foil. Seed cultures were kept at 25°C in the dark for 2 weeks before being transferred to a 16-hour light and 8-hour dark regimen at 25°C unless otherwise specified. All culture media were solidified with 1% agar and adjusted to pH 5.5 before autoclaving. Media for non-symbiotic culture contained 2% sucrose regardless of the original formulae except in Experiment 3.

Experiment 1. Symbiotic capability of fungal isolates

Seeds were sown on October 17, 1984, on the oat medium of CLEMENTS *et al.* (5) for symbiotic cultures and on KNUDSON C (KC) or MURASHIGE and SKOOG's medium of half strength in major mineral nutrients (1/2 MS) for non-symbiotic cultures. One-half of non-symbiotic cultures using KC and 1/2 MS were incubated in the light, *i.e.* 16-hour light/8-hour dark, and the other half in complete darkness.

Experiment 2. Effects of media in symbiotic culture

Defined and undefined media containing 545 mg KH_2PO_4, 470 mg $Ca(NO_3)_2 \cdot 4H_2O$, 245 mg $MgSO_4 \cdot 7H_2O$, 200 mg KNO_3, 120 mg NH_4NO_3/1, NITSCH's micro-element excepting Fe, and Fe of MS's formula as the basic ingredients (T-formula) were compared with oat medium. Various media were prepared by adding 1% starch or 1% cellulose powder and different amounts of sucrose and yeast extract to this basic formula as is shown in Table 2. Light and dark non-symbiotic cultures were also made using the following 5 kinds of media: T-formula (T1), T-formula +0.02% yeast extract (T2). T-formula+organic supplement of MS (T3), KC and hyponex medium (3 g/l). Seeding and inoculation were carried out on December 1.

Experiment 3. Effects of the addition of mycelial powder and fungus culture filtrate to non-infected culture

Two fungal isolates, No. 706 and No. 813, which were found to be symbiotic with *Spiranthes sinensis* in the preceding tests were cultured in 500 ml liquid medium of T-formula containing 2% sucrose at 25°C in the dark for 20 days, then filtered through a suction filter. The mycelia on the

filter paper were dried in a ventilated oven at 90°C and ground into fine powder with a mortar and pestle. The filtrates were adjusted to pH 5.5. The following agar media were prepared using these mycelial powder and culture filtrates:

A. 50 mg mycelial powder and 2% sucrose are added to 50 ml T-formula before autoclaving,
B. 50 ml filtrate and 1.5% sucrose are added to 50 ml T-formula before autoclaving,
C. 1.0% sucrose is added to 100 ml filtrate before autoclaving,
D. 50 ml filter-sterilized filtrate is added to 50 ml T-formula containing 1.5% sucrose after autoclaving, and
T. T-formula control containing 2.0% sucrose.

In this experiment a rubber stopper with a 5 mm hole which was filled tightly with cotton was used instead of aluminium foil. Seeding and inoculation were made on December 17, 1984.

Results

Symbiotic capability of fungal isolates

Of the 10 orchid species examined 6 did not yield any *Rhizoctonia* strains. The *Rhizoctonia* isolates obtained are listed in Table 1.

Gymnadenia camtschatica yielded multinucleate *Rhizoctonia* isolate No. 101, and the same species from different habitats yielded binuleate *Rhizoctonia* AG-C isolate No. 706. From the plants of *Spiranthes sinensis* from different

TABLE 1. Isolated mycorrhizal fungi and their host orchids

Isolate	*Rhizoctonia*	Host orchid	Habitat & note
No. 101	Multinucleate	*Gymnadenia camtschatica* MIYABE et KUDO	Zenibako, Hokkaido
No. 706	Binucleate AG-C	*Gymnadenia camtschatica* MIYABE et KUDO	Bikuni, Hokkaido
No. 813	Binucleate (*R. repens*?)	*Spiranthes sinensis* AMES	Taketoyo, Aichi
No. 821	Binucleate (*R. repens*?)	*Spiranthes sinensis* AMES	Taketoyo, Aichi, habitat different from that of No. 813
No. 861	Binucleate AG-I?	*Cymbidium goeringii* REICHB. f.	Collected from Noto, Ishikawa and cultivated
No. 881	Binucleate	*Phajus minor* BL.	Cultivated; origin not known
No. 886	Binucleate	*Phajus minor* BL.	From the same plant as that No. 881

habitats two binucleate *Rhizoctonia* isolates No. 813 and No. 821 were obtained, which were identical species, possibly *Rhizoctonia repens*. Cultivated *Cymbidium goeringii* yielded binucleate *Rhizoctonia* (possibly AG-I) isolate No. 861. A cultivated plant of *Phajus minor* yielded two diperent kinds of binucleate *Rhizoctonia* isolates No. 881 and No. 886 in which the anastomosis groups were not clear.

The experiment of symbiotic germination of *Spiranthes sinensis* was carried out with these 7 *Rhizoxtonia* isolates. The result of observation on the germination and seedling development 80 days after seeding is shown in Table 2.

Since ungerminated seeds on filter paper were difficult to distinguish even under a binocular microscope, the total number of seeds sown directly on agar medium in each tube was counted for all the non-symbiotic cultures and the average number of all counts (in this case it was about 150) was used as the basis for the calculation of estimated germination percentages. The figures in Table 2 are mean values of the three replicate tubes rounded to 5% intervals.

To indicate the degree of seedling development it was classified into 5

TABLE 2. Germination and development of *Spiranthes sinensis* seeds as affected by orchid endophytes (Average of 3 replicate tubes in which sown about 150 seeds each; 80 day after seeding)

Medium	Inoculum	Estimated germination percentage	No. of germinated seeds at each developmental stage			
			<B	B~	C~	D-E
Oat	No. 101	?	?	?	0	0
»	No. 706	25	0	0	2.0	37.5
»	No. 813	10	2.0	0	0.6	8.0
»	No. 821	25	35.7	1.0	2.3	0.6
»	No. 861	20	15.7	14.3	3.6	0
»	No. 881	?	?	?	0	0
»	No. 886	5	10.5	0.5	0	0
»	Non-ino.	10	10.6	2.0	0	0
KC (light)	»	10	8.6	4.0	0	0
» (dark)	»	5	4.3	1.3	0	0
1/2MS (light)	»	10	14.5	1.5	0	0
» (dark)	»	10	7.4	3.6	0	0

?=Observation being difficult because of vigorous growth of aerial hyphae.

stages as shown by outline drawings in Fig. 1, *i.e.* A: ungerminated, B: protocorm stage with some epidermal hairs, C: protocorm with many hairs and the growing end somewhat green, D: green shoot appearing but still without root, and E: seedling stage with root(s). In Table 2 the actual number of seeds classified into each developmental stage is given by the mean value of 3 replicate tubes.

As is evident from the data, the estimated germination percentage on oat medium inoculated by isolate No. 706, No. 821 and No. 861 was 20%-25% and was higher than that on non-inocultated oat medium which was about 10%. Among these three isolates No. 706 showed an outstanding enhancing effect on seedling development: most germinated seeds attained to D-E stages. On non-inoculated oat medium, the germination percentage was low and most of the germinated seeds had not yet reached B stage. In contrast to this, germinated seeds inoculated by isolates No. 821 and No. 861 more or less contained of those at B or more developed stages.

On the other hand, cultures inoculated by isolate No. 813 were as low as those of non-inoculated oat medium in germination percentage but germinated seeds contained many of those at D-E stages; the enhancing effect on seedling development of this isolate was greater than that of isolate No. 821 or No. 861. There was no difference between the inoculation by No. 886 and non-inoculated oat medium. Although in the case of No. 101 and No. 881 it was difficult to obtain accurate counts of germinated seeds at less developed stages because of the vigorous growth of the fungi forming dense aerial hyphae, it was thought that they were at the same level as that of non-inoculated oat medium in germination and seedling development.

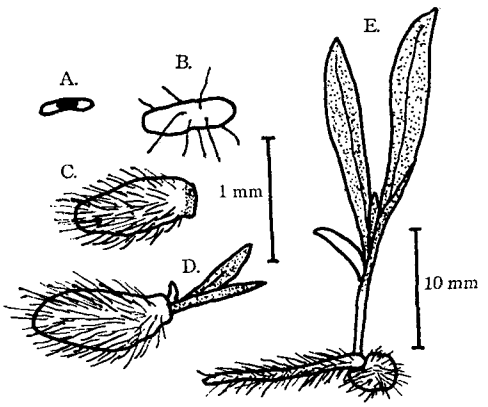

Fig. 1. Outline drawings of developmental stages of germinating *Spiranthes sinensis* seed.

The germination and seedling development in non-symbiotic cultures on KC or 1/2 MS were also at the same level as that of non-inoculated cultures on oat medium.

Effects of media in symbiotic cultures

The effect of media on the germination and seedling development was investigated in symbiotic cultures inoculated by the 7 *Rhizoctonia* isolates. The observation on germination and seedling development was made 40 days after seeding in the same way as described above. The average number of seeds sown in a tube was about 50 in this case.

The result is shown in Table 3 only about the cultures inoculated by isolates No. 706 and No. 813. The cultures inoculated by the other isolates scarcely germinated irrespective of media at the time of observation.

Among the 6 kinds of media inoculated by isolate No. 706 the one containing mineral salts of T-formula, 1% cellulose, 0.1% sucrose and 0.05% yeast extract was the best for the seedling development; almost all of the germinated seeds were at D–E stage. The omission of sucrose from this medium lowered the seedling development, and further omission of sucrose and yeast extract lowered it more to a level of less than that of oat medium.

TABLE 3. Effects of culture media on the symbiotic germination of *Spiranthes sinensis*
(Average of 3 replicate tubes in which sown about 50 seeds; 40 days after seeding)

Symbiont	Medium	Estimated germination (%)	No. of seeds at each developmental stage			
			<B	B~	C~	D–E
No. 706	T+1% Ce	20	7.6	1.3	0.6	0
»	T+1% Ce+0.02% YE	20	1.3	1.3	1.3	6.0
»	T+1% Ce+0.1% Su+0.05% YE	30	0	1.6	0.6	12.6
»	T+1% Ce+0.2% Su+0.1% YE	5	3.0	0	0	0
»	T+1% Starch	30	14.3	0	0	0
»	Oat medium	35	17.3	13.3	4.0	0
No. 813	T+1% Ce	0	0	0	0	0
»	T+1% Ce+0.02% YE	10	3.3	0	3.3	0
»	T+1% Ce+0.1% Su+0.05% YE	10	4.0	0	0.6	0
»	T+1% Ce+0.2% Su+0.1% YE	0	0	0	0	0
»	T+1% Starch	5	2.6	0	0	0
»	Oat medium	10	4.3	3.3	0	1.0

T=T-formula given in the text; Ce=cellulose; Su=sucrose; YE=yeast extract,

The increase of both sucrose to 0.2% and yeast extract to 0.1%, however, retarded the seedling development markedly, which was probably due to the detrimental effect of the high level of yeast extract on fungal growth. On the medium containing only mineral salts and 1% starch seedling development was inferior to that on oat medium. The cultures inoculated by isolate No. 813 were low in germination percentages but the general tendency was similar to those inoculated by isolate No. 706.

There was an obvious tendency that the enhancing effect of fungus on the seedling development followed an increase of apparent growth of fungus on the culture medium except for oat medium. The seedling development on oat medium was better than that on the medium containing only mineral salts and cellulose or starch notwithstanding the fact that the fungal growth on oat medium was less vigorous.

As in non-symbiotic controls the germination was much delayed and the development of protocorms was very slow, a direct comparison with that of symbiotic cultures on the same date was impossible. Hence, the germination percentage of seeds at each developmental stage one year after the seeding is shown in Table 4. The average germination percentage of all tubes at that time was about 40%, which was somewhat higher than that of the symbiotic cultures 80 days after seeding. However, no statistically

TABLE 4. Effects of culture media on the germination of *Spiranthes sinensis* in non-symbiotic culture (One year after seeding; average of 3 replicate tubes)

Medium and Light condition		Germination percentage at each developmental stage				
		<B	B~	C~	D-E	Total
T 1	lignt	15.7	14.8	3.7	1.9	36.1
	dark	18.8	23.5	9.4	0.0	51.8
T 2	light	9.6	16.8	7.2	4.5	37.6
	dark	5.8	19.8	6.6	5.0	37.2
T 3	light	16.4	23.0	2.5	3.2	45.1
	dark	15.5	17.8	0.6	0.6	34.5
KC	light	8.5	13.1	2.6	0.6	25.2
	dark	32.4	31.3	0.0	0.0	63.7
Hyp.	light	15.0	15.9	1.9	0.0	32.7
	dark	18.2	11.6	1.0	1.0	31.8

T 1=T-formula, T 2=T-formula+0.02% yeast extract, T 3=T-formula+organic supplement of MS-formula, Hyp.=Hyponex medium (3 g/l)

significant differences in germination were found among the media or between dark and light conditions.

Effects of the addition of mycelial powder and filtrates of fungal culture on non-symbiotic cultures

The germination percentages at each developmental stage one year after seeding are given in Table 5. No statistically significant differences among treatments were found. Neither, the addition of mycelial powder nor that of culture filtrates of isolate No. 706 or No. 813 to the medium containing mineral salts of T-formula and sucrose exerted any appreciable effects upon the germination and protocorm development. The development of protocorms was very slow as in the non-symbiotic cultures of the preceding experiments. In this experiment in which rubber stoppers were used the average germination percentage was about 55% and apparently higher than the preceding one in which aluminium foil was used.

TABLE 5. Effects of supplement of mycelial powder and culture filtrate of *Rhizoctonia* isolate No. 706 to culture media on the germination of *Spiranthes sinensis* in non-symbiotic culture
(One year after seeding; average of 5 replicate tubes)

Treatment	Germination percentage at each developmental stage				
	<B	B~	C~	D-E	Total
A.	17.1	20.7	7.8	6.7	52.3
B.	20.2	23.9	4.9	4.5	53.5
C.	42.8	16.4	5.8	1.3	63.4
D.	13.9	26.4	4.2	5.6	50.0
T.	21.5	21.0	12.1	1.5	56.1

Treatments are described in the text.

Discussion

In terms of the enhancing effect on seedling development the most effective symbiont to *Spiranthes sinensis* was binucleate *Rhizoctonia* AG-C isolate No. 706 obtained from *Gymnadenia camtschatica*. Although the 2 binucleate *Rhizoctonia* isolates obtained from *Spiranthes sinensis* itself were symbiotic to this orchid, they were far less effective and there was a considerable difference between the two in the effectiveness. These facts support the view that there is no species-to-species specificity between an orchid and its mycorrhizal fungus as has been expressed by many workers (6, 7, 9), and

suggest that the screening of the most capable symbiont should be as extensive as possible taking account of that there can be a considerable variation in symbiotic capability among isolates even in a *Rhizoctonia* species.

In the symbiotic culture associated with a capable symbiont, the protocorms which had not attained to the state of producing a green shoot by a two month culture after seeding, further growth ceased because of the parasitic effect of the fungus. From the practical view point, this is important because the symbiotic culture will be meaningless as a practical way of propagation if seedlings could not attain a sufficient growth for transplanting even if the fungus is symbiotic. The symbiotic isolates other than isolate No. 706 were cases in point.

TOKUNAGA and NAKAGAWA (13) obtained 18 fungal isolates from the mycorrhizae of various Japanese wild orchids and NISHIKAWA and UI (11) 56 isolates from wild orchids in Hokkaido and carried out inoculation tests to orchid protocorms. They stated that a large number of those isolates caused symbiotic infection judging from the penetration of hyphae through epidermal hairs and the formation of pelotons in the protocorm tissues. However, it is not known how many of those isolates were sufficiently symbiotic in a sense being practically useful. HARVAIS and HADLEY (9) testing symbiosis using various *Rhizoctonia* isolates obtained from *Dactylorchis purpurella* and others native to England indicated that isolates from roots generally infected the protocorms but did not always stimulate the growth of the species from which they originated. HADLEY (7) tested the interactions between protocorms of 10 orchids and 32 *Rhizoctonia* isolates; and demonstrated that compatible infection of epidermal hair cells was not necessarily followed by vigorous symbiosis, and that there were various degrees of symbiosis. These may provide circumstantial evidence for the necessity of the study such as the present undertaking.

The importance of the nutritional status of culture medium in symbiotic cultures has often been stressed. HARVAIS and HADLEY (10) demonstrated that the concentration and supply of carbohydrates to cultures of *Dactylorchis purpurella* protocorms infected by *Rhizoctonia solani* has a marked effect upon both growth of the protocorm and parasitism by the fungus. More practically, CLEMENTS and ELLYARD (5) reported that in the symbiotic cultures of Australian terrestrial orchids oat medium was better than WARCUP's medium or ZAK's medium. In the present study the medium containing mineral salts, 1% cellulose, 0.1% sucrose and 0.02–0.05% yeast extract was better than the oat medium. The fact that the omission of sucrose and/or yeast extract from this medium deteriorated the symbiotic capability, indicates

that the advantage of the medium is mainly dependent upon the these supplements. The following facts obtained also suggest that these supplements must act upon the orchid seeds indirectly through their effect on the growth of gungus: in non-symbiotic cultures addition of yeast extract or organic supplement of MS-formula did not show any improving effects, and in symbiotic cultures there was a tendency that the stimulating effect of fungus on the protocorm growth followed an increase of the fungal growth on the medium.

In symbiotic cultures the nutritional status of the medium exerted a considerable effect on the results, but the symbiosis may depend on the symbiotic capability of the fungus so that it may remain within the limits to modify the degree of symbiosis to some extent. Accordingly, as the procedure to establish a practical method of seed propagation for an orchid, it will be reasonable to make the screening first for the most effective *Rhizoctonia* isolate for the orchid using an appropriate culture medium easy to prepare such as oat medium, and then to select the most suitable medium for the isolate.

BURGEFF (3) showed that seeds of *Vanda* etc. which were very poor germinators on medium containing mineral salts and sucrose in non-symbiotic culture germinated and grew well by sowing them on heat sterilized culture of compatible fungus or by adding of the extract of the fungus, and that the effect could be attributed to vitamins produced by the fungus. *Spiranthes sinensis* may not be the case since the addition of mycelial powder or culture filtrate of the compatible fungus did not bring about any improvements of protocorm growth in non-symbiotic culture. In this case the effect of fungus may act on protocorm growth only through the process of infection of fungus into the protocorm tissue and digestion or lysis of the hyphae.

Spiranthes sinensis seeds were found to germinate in non-symbiotic cultures irrespective of light conditions even on a simple medium containing only mineral salts and sugar but the protocorm development was extremely slow, which could not be improved even by the addition of any supplements. It required more than 16 months from seeding to grow large enough to transplant. However, in symbiotic cultures with the appropriate symbiont the seedlings grew to the same stage within 3 months after seeding although the increment of germination percentage might not be anticipated because it was dependent mainly upon the viability of the seeds.

In many cases of wild temperate terrestrial orchids in which non-symbiotic cultures are unsuccessful the seeds germinate and grow to the protocorm stage but further growth beyond this does not seem to occur. Thus

symbiotic culture of orchids, if only a capable symbiont could be found, may generally be an excellent way of the seed propagation for such orchids that are difficult to grow non-symbiotically.

Summary

With 7 *Rhizoctonia* isolates obtained from 4 orchid species out of 10, symbiotic capability to *Spiranthes sinensis* seeds was tested on oat medium. A binucleate *Rhizoctonia* AG-C isolate obtained from *Gymnadenia camtschatica* was the most effective symbiont to this orchid and the germinated seeds grew large enough to transplant within 3 months after seeding. Two binucleate *Rhizoctonia* isolates, possibly *Rhizoctonia repens*, obtained from *Spiranthes sinensis per se* were symbiotic to this orchid but far less effective. And there was a considerable difference between the two in their effectiveness.

The effect of culture media on the symbiosis was considerably large but it could not change the essential nature of the symbiosis. For this orchid the medium containing mineral salts, 1% cellulose, 0.1% sucrose and 0.02–0.05% yeast extract was better than the oat medium for symbiotic culture.

The addition of mycelial powder or culture filtrate of the compatible fungus to non-symbiotic culture did not stimulate the germination or protocorm growth. Although *Spiranthes sinensis* was found to germinate non-symbiotically on a wide range of media irrespective of light conditions, the protocorm growth was very slow and it required more than 16 months from seeding to attain sufficent growth for transplanting.

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