Effect of Plant Density on the Growth of Seedlings of *Spiranthes sinensis* Ames and *Liparis nervosa* Lindl. in Symbiotic Culture

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**Summary**

The effect of plant density on the growth of seedlings of *Spiranthes sinensis* Ames and *Liparis nervosa* Lindl. in symbiotic cultures with *Rhizoctonia* isolates, effective for these orchids, was investigated using an oat medium containing the decoction of 25 g/l oat grains and 1% agar. In high plant densities, the weight of individual seedlings increased in proportion to the quantity of medium per plant, but the rate of fresh weight increase was lowered with a decrease in plant density. The point where maximum fresh weight per individual seedling was obtained, i.e. the threshold where the density effect disappeared, was at a very low plant density: 60 ml/plant for *S. sinensis* and 30 ml/plant for *L. nervosa*. The dry weight of mycelia per culturing flask also increased with a decrease of plant density. It is considered that the effect of plant density on the growth of seedlings is, with respect to the oat medium, mainly dependent on the amounts of nutrients in the medium. Competition among aerial parts did not take part in the effect.

**Introduction**

In a previous paper, the authors reported that the seedling growth of *Spiranthes sinensis* Ames was remarkably enhanced in symbiotic culture with a compatible *Rhizoctonia* isolate, and pointed out that this method could be practical for overcoming the difficulties in growing seedlings of hard-to-germinate orchid species. During the experiments, we noticed that the growth of seedlings could be retarded at early stages of development when the plant density was high. Since no information is available, the effect of plant density on the growth of seedlings in symbiotic culture must be clarified. This paper describes the results of a series of experiments using *S. sinensis* and *Liparis nervosa* Lindl. with their symbionts, to solve this problem.

**Materials and Methods**

Seeds of *S. sinensis* and *L. nervosa* were collected at Taketoyo, Aichi prefecture in August, 1986 and stored at 4°C in a desiccator containing silica gel after complete air drying.

As the symbiont for *S. sinensis*, binucleate *Rhizoctonia* isolate No. 706 (8) was used. For *L. nervosa*, binucleate *Rhizoctonia* isolate No. 624 (possibly *R. repens*) was used, which was most effective one for this orchid among those so far tested (unpublished).

Dry seeds were surface-sterilized in sodium hypochlorite solution of 0.5% available chlorine for 2 minutes then washed in 3 changes of sterile water. About 150 sterilized seeds on average were sown on the slope of 30 ml medium in 30 mm × 150 mm test tubes. One week after seeding, a small inoculum of fungus was added to the upper end of the slope. Three weeks (*S. sinensis*) or 6 weeks (*L. nervosa*) after inoculation, protocorms showing signs of sprouting were transplanted onto the medium in the culturing vessels.

An oat medium was used throughout.
less oat grains of 25 g were boiled in 1 litre of distilled water for 1 hour and filtered through 4 sheets of gauze. The filtrate was solidified with 1% agar. The pH of the medium before autoclaving was about 6.0. All the culturing vessels were stoppered with double sheets of aluminium foil.

In each experiment or each part of an experiment, at least 10 replicate cultures were made. All cultures were kept at about 25°C in a 16-hour light and 8-hour dark regime. The growth of seedlings was generally examined 10 weeks (S. sinensis) or 16 weeks (L. nervosa) after inoculation.

The following 3 experiments were carried out. Each consisted of one or more parts performed separately. In the first part of the first experiment, both orchids were used, but in the other cases only S. sinensis was used. Plant density is expressed as the volume of oat medium per plant (ml/p.) for the convenience of direct comparison among different parts of experiments.

**Experiment 1: Relation between plant density and seedling growth.**
1. Ten, 5, 3 (S. sinensis only), 2 and 1 protocorm (s) were transplanted on 60 ml oat medium in 100 ml Erlenmeyer flasks.
2. Two protocorms were transplanted on 120 ml medium, 1 protocorm on 90 ml medium and 1 protocorm on 120 ml medium using 300 ml flasks.
3. Ten, 5 and 2 protocorms were transplanted on 30 ml oat medium in 100 ml flasks, and sampled at 2-week intervals after transplanting.

**Experiment 2: Dominant factor in density effect.**
The relation between plant density and seedling growth was compared among the following 3 parts of the experiment in a higher density range than that of Experiment 1.
1. Thirty, 20, 15 and 5 protocorms each were transplanted on 30 ml oat medium in 100 ml flasks.
2. Five, 7.5, 15 and 30 ml oat medium were added to plain agar that had been poured into 100 ml beakers and cooled beforehand to reach an ultimate volume of 30 ml, and 5 protocorms were transplanted to each beaker.
3. One, 1.5, 3, 5 and 6 ml oat medium were poured into 16 mm x 40 mm tubes, and a set of 5 tubes was placed in a 100 ml beaker. These tubes were fixed by pouring plain agar into the beaker about 10 mm deep. One protocorm was transplanted to each tube.

**Experiment 3: Effect of the extent of hyphal distribution in the medium.**
To change the height of medium, 15 ml oat medium was poured into test tubes of different inside diameters, i.e. 20 mm, 24 mm and 34 mm; and 1 protocorm was transplanted to each tube.

**Results**
The results of Experiment 1-1 with S. sinensis are shown in Table 1. The growth of seedlings showed a general trend in which every parameter of growth increased with the decrease in plant density. Above all, this was most remarkable in the fresh weight of seedlings: the differences between any two treatments were statistically significant. The fresh weight of seedlings increased in proportion to the decrease in plant density, but the rate of increase was reduced with the decrease in plant density. The weight of roots was about 1/2 that of the aerial part including a protocorm,

<table>
<thead>
<tr>
<th>No. of plants per 60 ml medium</th>
<th>Medium quantity/plant (ml)</th>
<th>Leaf</th>
<th>Root</th>
<th>Fresh weight of seedling</th>
<th>Dry weight of mycelia/flask (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>Length (mm)</td>
<td>No.</td>
<td>Length (mm)</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>3.8b</td>
<td>18.1b</td>
<td>1.4</td>
<td>11.8a</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>3.9b</td>
<td>21.3b</td>
<td>1.8c</td>
<td>13.3a</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>4.3b</td>
<td>19.5b</td>
<td>2.2bc</td>
<td>12.8a</td>
</tr>
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<td>30</td>
<td>4.9a</td>
<td>23.9a</td>
<td>2.4ab</td>
<td>14.3a</td>
</tr>
<tr>
<td>1</td>
<td>60</td>
<td>5.0a</td>
<td>25.6a</td>
<td>2.8a</td>
<td>13.9a</td>
</tr>
</tbody>
</table>

* Mean separation by Duncan’s multiple range test at 5% level.
and the proportion of roots became slightly higher with the lowering of plant density. However, these two parts did not basically differ in their response to plant density.

The dry weight of mycelia in each flask shown in the last column of Table 1 also clearly increased with the lowering of the plant density.

To determine the point where the density effect disappears in *S. sinensis*, the effect of plant density in a lower range (60–120 ml/p.) than the above was examined in Experiment 1–2. But there was no significant difference among the treatments (data omitted). Thus the maximum growth of an individual seedling was obtained around 60 ml/p.

From Experiment 1–3, the changes of fresh weight of seedlings at 3 different plant densities are shown in Fig. 1. Five weeks after inoculation the rate of fresh weight increase became greater with the lowering of plant density. The higher the plant density, the earlier the weight of a seedling reached its maximum: it was attained 9 weeks after inoculation at the density of 3 ml/p., 13 weeks at 6 ml/p. and 15 weeks at 15 ml/p.

The results of Experiment 1–1 with *L. nervosa* are shown in Table 2. Generally, the seedling weight of *L. nervosa* was about 1/2 that of *S. sinensis*. However, the effect of plant density on the growth of seedling was similar to that of *S. sinensis* except for the point where the effect of density disappeared. The maximum weight of an individual seedling was obtained at a much higher density than that of *S. sinensis*, i.e. at 30 ml/p.

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**Table 2. Effect of plant density on the growth of seedlings of *Liparis nervosa* in symbiotic culture, 16 weeks after inoculation.**

<table>
<thead>
<tr>
<th>No. of plants per medium 60 ml medium</th>
<th>Medium quantity/plant (ml)</th>
<th>Protocorm diameter (mm)</th>
<th>Leaf</th>
<th>Root</th>
<th>Fresh weight of a seedling (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Length (mm)</td>
<td>Width (mm)</td>
<td>No.</td>
<td>Length (mm)</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>3.25 c</td>
<td>5.31 c</td>
<td>1.47 b</td>
<td>6.37 b</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>4.09 b</td>
<td>6.71 b</td>
<td>2.00 a</td>
<td>8.17 b</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>5.07 a</td>
<td>8.81 a</td>
<td>2.06 a</td>
<td>11.07 a</td>
</tr>
<tr>
<td>1</td>
<td>60</td>
<td>4.93 a</td>
<td>8.69 a</td>
<td>2.13 a</td>
<td>11.53 a</td>
</tr>
</tbody>
</table>

* Mean separation by Duncan's multiple range test at 5% level.
EFFECT OF PLANT DENSITY ON THE GROWTH OF SEEDLINGS OF SPIRANTHES SINENSIS

From Experiment 2, the relation between the quantity of medium per plant and fresh weight of individual seedlings is shown in Fig. 2. Although there was a slight inconsistency since the 3 parts of this experiment were performed separately, a strict linear relation was observed between the fresh weight of individual seedlings and the quantity of medium per plant, except for 6 ml/p. in Experiment 2-3.

The depth of growing front of fungus in the small tubes of each treatment of 5 ml/p, or 6 ml/p., which did not show any difference in the fresh weight of individual seedlings, was 25.5 mm on an average, against the height of medium which was 30 mm and 36 mm, respectively. As this fact suggested that the effectiveness of the medium was restricted to the volume of medium in which mycelia were distributed, this was further examined in Experiment 3.

The results of Experiment 3 are shown in Table 3. The distribution ratio of fungus was calculated as the ratio of the weight of medium containing mycelia to total medium weight. In 34 mm tubes the ratio was 100%, but that of 24.5 mm tubes was 83% and that of 20 mm tubes was only 62%. On the other hand, the differences in the growth of seedlings among tubes with different diameters were not so outstanding. A significant difference in the fresh weight of seedlings was only detected between 34 mm and 20 mm tubes.

**Discussion**

In asymbiotic culture of orchid seeds, as a matter of course, plant density affects the growth of seedlings. Ichihashi (4) has reported that in asymbiotic culture of Bletilla striata the fresh weight and root number of seedlings decreased but the height of seedlings increased at higher plant densities. However, the effect of plant density in the present study was far greater than that in asymbiotic culture. Moreover, a phenomenon as increase in plant height at high density was not observed.

The effect of plant density on the growth of individual seedlings was mainly dependent on the volume of medium, i.e. the quantity of nutrients in medium per plant, as far as the oat medium was concerned. In fact, even at a plant density so high that it might induce severe competition for light among seedlings, the growth of seedlings ceased before they reached the size at which competition for light could occur.

It seems that the growth of seedlings in symbiotic culture has an essentially different physiological character from the asymbiotic condition, in that they are supplied with all necessary nutrients from medium through the activities of fungus. This may be the reason why such seedlings can grow so rapidly.

Beyrle et al. (2) successfully germinated some species of Dactylorhiza in symbiotic cultures inoculated with Rhizoctonia stahlii in vitro. In this case, the seeds were sown in a Petri dish divided into 2 sections which contained nutrient agar in one section and water agar in the other. They found that the germination and further growth of protocorms were excellent both on nutrient agar and on water agar when the nutrient agar was suitable for the orchid-fungus associations, while the germination was extremely poor on either medium when the nutrient agar was unsuitable. These facts support the above view.

The relationships among the quantity of medium per plant, the fresh weight of individual seedlings and total weight of seedlings per 30 ml medium are illustrated in Fig. 3.

**Table 3. Differences in growth among Spiranthes sinensis seedlings in symbiotic culture, on equal quantities of medium (15 ml) in test tubes of different diameters.**

<table>
<thead>
<tr>
<th>Inside diameter of test tube (mm)</th>
<th>Volume ratio of fungus distribution (%)</th>
<th>Protocorm diameter (mm)</th>
<th>Leaf No.</th>
<th>Leaf Length (mm)</th>
<th>Root length of a seedling (mm)</th>
<th>Fresh weight of a seedling (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>62.4</td>
<td>3.26 a</td>
<td>4.30 a</td>
<td>15.7 a</td>
<td>13.6 b</td>
<td>96.4 b</td>
</tr>
<tr>
<td>24.5</td>
<td>83.2</td>
<td>3.28 a</td>
<td>4.30 a</td>
<td>15.3 a</td>
<td>15.3 a b</td>
<td>128.6 a b</td>
</tr>
<tr>
<td>34</td>
<td>100</td>
<td>3.47 a</td>
<td>4.10 a</td>
<td>15.4 a</td>
<td>15.6 a</td>
<td>129.9 a</td>
</tr>
</tbody>
</table>

* Mean separation by Duncan's multiple range test at 5% level.
sities, but it gradually decreased with the lowering of plant density. As is evident from tracing the growth process, the time needed for seedlings to reach maximum growth was delayed as the plant density decreased. It required 15 weeks after inoculation even at 15 ml/p. Since the data in Fig. 3 are those of 10 weeks after inoculation, an active increase of seedling weight must have been proceeding at low plant densities. If the seedling weight had been measured when it reached its maximum, the line for individual weight in Fig. 3 would approach line A, and the line for total weight would approach the 0.4 g horizontal line B as shown by the arrows in Fig. 3.

Roughly speaking, this means that there is a limit to the total yield of seedlings which a fixed quantity of medium can produce, and that the effect of plant density is to partition it among individuals. For practical propagation, it is desirable to obtain seedlings as large as possible for a given period. The quantity of oat medium necessary for maximum growth of individual seedlings was 60 ml/p. for *S. sinensis* and 30 ml/p. for *L. nervosa*. This difference between two species also coincided well with the fact that the fresh weight of seedlings of *L. nervosa* was about 1/2 that of *S. sinensis*.

On the other hand, a clear-cut tendency in which the dry weight of mycelia in a cultur-flask increased with lowering of the plant density, implies that the partition of nutrients in medium between fungus and seedlings inclines to be favorable to the former with the lowering of plant density. It is possible that this may be related to some extent to the decrease in total weight at low plant densities.

There is limit to the depth to which the mycelia of fungus growing on a solid medium can reach. A tendency in which the effect of medium quantity on the growth of seedlings was lowered when the height of medium exceeded the limit, was observed. However, this effect was not so strict as to reflect directly the ratio of mycelia distribution in the medium. This may be because the giving and receiving of substances between fungus and medium deeper than the mycelial front may take place through diffusion. Practically, it is desirable that the height of medium does not exceed this limit.

If the growth of seedlings is primarily dependent on the quantity of nutrients in medium per plant, maximum growth of seedlings must be obtained at a plant density much higher than the threshold value of the present study, by increasing the nutrient concentration of medium. There is a experimentally supported consent that the type and amount of nutrients available to the fungus are important for a balanced symbiosis and either excess or depletion of nutrients for fungal growth will enhance parasitism of the fungus (1, 3, 5, 6, 7). It will be necessary to investigate the effect of nutrient concentrations on the growth of seedlings in symbiotic culture taking this aspect into consideration.

**Literature Cited**

4. Ichihashi, S. 1978. Studies on the media for orchid seed germination II. The effects of...


ネジバナおよびコクラン共生実生の生育に及ぼす個体密度の影響

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摘 要

エンパク 25g/l の蒸汁に寒天 1％ を加えた培地を用いて、ネジバナおよびコクラン種子それぞれに有効な共生リソクトニア菌を接種し、シュート発生開始時のプロトコームを移植した in vitro での共生培養条件下で、実生の生育に及ぼす個体密度の影響を検討した。

高密度範囲では、実生個体新鮮重は個体当たりの培地量に比例して増加したが、密度が低くなるにつれて重量増加率は徐々に低下した。最大の実生個体重量が得られる点。すなわち密度効果がみられなくなる点は、ネジパナでは個体当たり培地量 60ml, コクランでは 30ml という低密度にあった。また、容器当たりの菌体乾重も、個体密度の低下に伴って増加した。

実生の生育に及ぼす個体密度の影響は、このエンパク培地を用いる限り、むしろ個体当たりの培地養分量に支配され、地上部の競合はこれに関与しないと考えられた。