Effects of Cold Pretreatment and Potting Materials on Growth of Acclimated Plantlets of *Cypripedium macranthum* var. *speciosum*

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To achieve effective seedling production of the endangered orchid, *Cypripedium macranthum* var. *speciosum*, the effects of cold pretreatment duration and potting substrate type on seedling growth and survival were investigated. With over 12 weeks of cold treatment at 5°C, approximately 90% of the in vitro plantlets formed shoots and rooted. However, following 24 weeks or more of cold treatment, the acclimated plantlets showed a lower survival rate after 26 weeks of cultivation and less new bud formation. Potting substrate influenced survival rate and new bud formation. A sandy loam soil mixed with a soil rich in clay and/or rock wool was more effective than the single use of each potting material.

INTRODUCTION

Cypripedium spp. (Orchidaceae) have become endangered around the world due to indiscriminate overharvesting and destruction of their habitat. Cypripediums native to Japan are gravely affected (Japan Society of Plant Taxonomists, 1993). In recent years, there have been a number of reports dealing with the in vitro production of the Japanese cypripediums from the standpoint of gene resource conservation and in response to nursery demand (Takahashi and Tsutsui, 1992; Hoshi et al., 1994; Tomita, 1997; Tomita and Tomita, 1997b). A cold treatment before acclimation has been reported to be effective for in vitro plantlet acclimation (Frosch, 1985; Malmgrem, 1996; Ramsay and Stewart, 1998; Stewart and Mitchell, 1991). Few studies on plantlet acclimation have been reported on the Japanese cypripediums (Takahashi and Tsutsui, 1992; Tomita, 1997; Tomita and Tomita, 1997a, 1997b). Also, no reports have been made on the influence of cold treatment on the growth of the plantlets post-acclimation or the cultivation conditions required have not been reported. One method of protecting wild terrestrial orchids is by growing them in cultivation in order to supplement the natural population (Ramsay and Stewart, 1998). In order to form a basis for development of an effective method to propagate orchids of the *Cypripedium* genus, in vitro plantlets of *C. macranthum* Sw. var. *speciosum* (Rolfe) Koidz. were used to investigate the effects of both the duration of cold treatment before acclimation. The effect of the composition of potting materials on post-acclimation plantlet growth was also investigated.

MATERIALS AND METHODS

Immature seeds of *C. macranthum* var. *speciosum* were sown on ½ Norstog medium (Tomita and Tomita, 1997b) in July of 1994 and 1995. The resulting plantlets were transferred every 12 weeks onto the same medium. All cultures were incubated in

the dark at 20°C. The effect of cold treatment was investigated in 1996 on plantlets derived from seeds sown in 1994, and the effect of potting materials was investigated in 1997 on plantlets from seeds sown in 1995.

Experiment 1. Effects of Cold Stratification on Growth of Acclimatized Plantlets. Plantlets derived from seed sown in 1994 were utilized. Plantlets of uniform size (dormant bud size, 5 to 10 mm; fresh weight, 200 to 300 mg) were selected at weekly intervals from July of 1995 through January of 1996, and removed from flasks. The plantlets were placed in sterilized vermiculite in vinyl bags. The plantlets were subjected to cold treatment at 5°C. Control (untreated) plantlets were selected in March 1996. After cold stratification for 0 (untreated control), 8, 12, 16, 20, 24, 28, and 32 weeks the plantlets were placed in 12-cm-diameter pots with "towadasuna" (3 plantlets were placed in each pots) in March. Towadasuna is a kind of sandy loam, the particle size used was 5 mm or less, and it was sterilized by autoclaving after lumps were removed (Tomita, 1997). Plantlets were moved to a shaded (below 10,000 lux), nonheated greenhouse, watered regularly to prevent drying out, and dilute liquid fertilizer (Hyponex; 5N : 10P : 5K) was applied every 2 weeks.

Experiment 2. Effect of Potting Materials on Acclimated Plantlet Growth. Plantlets derived from seeds sown in 1995 were used for this experiment. As for the first experiment, plantlets of uniform size were selected between November through December of 1996. Cold treatment at 5°C was conducted for 12 weeks. The date of initiation of the cold treatment varied between different replicates within each treatment, between late February of 1997 and mid-March of the same year. Following cold treatment three plantlets were placed per each 12-cm-diameter pot. Each replicate was made up of 4 pots (a total of 12 plants). For each treatment four replicates were used. Six different potting materials were used for cultivation:

A) Towadasuna (same as in Experiment 1).
B) Kanumatsuchi (clay-rich soil, 5- to 10-mm granules after dust removal).
C) Rockwool fiber.
D) Towadasuna + kanumatsuchi (1:1, v/v).
E) Towadasuna + rock wool (1:1, v/v),
F) Towadasuna + kanumatsuchi + rock wool (1 : 1 : 1, by volume).

All materials were sterilized by autoclaving. Cultivation conditions were the same as those in Experiment 1. Shoot formation was considered to have occurred when buds pushed their way to the surface, turned green, and the first leaves appeared (Takahashi and Tsutsui, 1992). Plantlets were observed after shoot formation, and dug up after 26 weeks of cultivation. The survival rate and fresh weight of survivors, along with the new bud formation rate, were recorded.

RESULTS

Experiment 1. Effects of Duration of Cold Treatment on Plantlet Growth. The results are shown in Table 1. No shoot formation was observed on untreated control. The shoot formation rate where the cold treatment was applied for more than 12 weeks was significantly higher than in the 8-week cold treatment. No significant differences were noted in leaf number or height of the shoot (data not shown) between the treatments. After 18 weeks of cultivation, there were noticeable

differences between the treatments. Plants which were exposed to 20 or more weeks of cold treatment showed some discoloration of the leaves. Moreover, when the investigation ended in the 26th week of cultivation, the above-ground portion of the plantlets had withered away. The survival rate, fresh weight of survivors, and new bud formation rate of plantlets after acclimation and 26 weeks of cultivation were high in the cold-treatment plots of 20 weeks or less duration. With longer treatment, these levels decreased as the cold treatment time was prolonged. In the 32-week treatment, all plantlets had withered and died when dug up, and there was no bud formation.

Experiment 2. Effect of Potting Materials on Acclimated Plantlet Growth. The results are shown in Table 2. The rate of shoot production was around 90% with no significant difference between the treatments. This was also true of the height and number of leaves after shoot formation (data not shown). Discoloration of the above-ground portion was recognized earliest in treatment B after 16 weeks of cultivation. With treatment F, yellowing was not observed until 23 weeks of cultivation. When dug up after 26 weeks of cultivation, over half the plantlets still had green foliage (data not shown). At 26 weeks, the survival rate, plantlet fresh weight, and new bud formation rate tended to be higher using treatments D-F, where composites were used, than in treatments A-C, in which only a given type of soil was used in each. In treatment B, there was poor formation of new roots, and many of the roots formed were discolored. Moreover, at 26 weeks, these plantlets had a significantly reduced survival rate, fresh weight, and new bud formation rate when compared with the other five treatments.

DISCUSSION

In vitro plantlets of *Cypripedium* genus have a type of epicotyl dormancy (Takahashi and Tsutsui, 1992) and cold treatment is necessary for shoot formation (Frosch, 1985; Malmgrem, 1996; Ramsay and Stewart, 1998; Stewart and Mitchell, 1991; Takahashi and Tsutsui, 1992; Tomita, 1997). In the present experiments using *C. macranthos* var. *speciosum*, as for other cypripediums, dormancy breaking was achieved in the in vitro plantlets given 12 or more weeks of cold treatment at 5°C.

Given the post-acclimation-growth efficiency, the acclimation period should preferably be winter for the in vitro plantlets given cold treatment (Malmgrem, 1989). However, in cypripediums the rate of germination and development is highly variable and the uneven growth of the post-germination plantlet (Ballard, 1987; Butcher and Marlow, 1989; Tomita, unpublished data) may be considered to hinder effective seedling production. Thus, in Experiment 1, we attempted to adjust the plantlet acclimation phase to spring, for the convenience of following cultivation, by prolonging the cold treatment time, and then investigated the effects on plantlet growth after acclimation. In the cold treatments given for 12 weeks or more, no difference was noticed in the shoot formation rate or ostensible growth following formation of the shoot. But when the treatment was prolonged to 24 weeks or more, there was an adverse effect on the survival rate and new bud formation in the plantlets following acclimation. This was thought to be caused by the weakening of the plantlets by the long cold treatment. There is the need to achieve greater seedling production efficiency by considering the various conditions in cultivating plants. For example, one can adjust the acclimation time in the in vitro condition by maintaining its growth through various adjustments, or post-acclimation methods such as use of fertilizer are worth considering.

Table 1. Effects of time exposure to cold treatment time on the growth of acclimated plantlets of *Cypripedium macranthum* var. *speciosum*.

Cold treatment period (weeks)	Shoot formation rate (%)[1]	After 26 weeks of cultivation		
		Survival rate (%)	Fresh weight (mg)	New bud formation (%)
0	0 c[2]	0 c[2]	-	-
8	66.7 b	83.0 a	198 a[2]	63.1 a[2]
12	89.6 a	80.9 a	216 a	58.1 a
16	91.7 a	70.1 a	212 a	49.8 a
20	89.6 a	70.1 a	200 a	46.6 a
24	87.5 a	38.2 b	174 ab	34.2 ab
28	89.6 a	30.0 b	140 b	6.3 b
32	87.5 a	0 c	-	-

[1] Percentage of plants with buds appearing on surface, turning green and producing leafs (Takahashi and Tsutsui, 1992).
[2] Mean separation within column by Duncan's multiple range test at 5% level.

Table 2. Effects of potting materials on growth of acclimated plantlets of *Cypripedium macranthum* var. *speciosum*.

Cold treatment period (weeks)	Shoot formation rate (%)[2]	After 26 weeks of cultivation		
		Survival rate (%)	Fresh weight (mg)	New bud formation (%)
A	93.8 NS[3]	77.3 a[3]	261 b[3]	55.7 b[3]
B	89.6	32.8 b	125 c	14.6 c
C	91.7	45.5 b	301 b	45.0 b
D	91.7	86.4 a	333 ab	81.4 a
E	91.7	90.9 a	397 a	80.4 a
F	93.8	91.1 a	415 a	83.2 a

[1] A: "towadasuna"; B: "kanumatuchi"; C: rock wool; D: "towadasuna" + "kanumatuchi" (1:1); E: "towadasuna" + rock wool (1:1); F: "towadasuna" + "kanumatuchi" + rock wool (1:1:1).
[2] Percentage of plants with buds appearing on surface, turning green and producing leafs (Takahashi and Tsutsui, 1992).
[3] Mean separation within column by Duncan's multiple range test at 5% level; NS: not significant.

There are few examples in the literature of the detailed cultivation conditions required for the post-acclimation plantlets of cypripediums (Malmgrem, 1996; Ramsay and Stewart, 1998). InExperiment 2, the type of potting materials used had a marked effect on plantlet growth. As a potting medium for cultivation of the seedlings of *C. macranthos* var. *speciosum*, it was found that the sandy loam mixed with clay-rich soil, and/or rock wool was most effective, rather than the sandy-loam-based media reported as being successful for other *C. macranthos* variants (Takahashi and Tsutsui, 1992; Tomita, 1997; Tomita and Tomita, 1997a). The physico-chemical characteristics of these potting materials were considered to have influenced the plantlet growth like other terrestrial orchids (Mckendrick, 1996). Further studies will be needed.

To establish an effective method to reproduce the endangered *Cypripedium* genus, the present investigators intend to continue their studies, based on the above results, of the culture conditions from 2 years after acclimation until flowering.

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