# Effects of Media and Time of Seed Collection on Seed Germination of *Cypripedium macranthum* var. *rebunense*

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**To obtain basic information for maximizing the *in vitro* seed germination of *Cypripedium macranthum* var. *rebunense* the suitability of media and the optimum time of seed collection were investigated. Seed capsules were collected at weekly intervals ranging from 5 to 8 weeks after pollination and inoculated on four tested media (Harvais, 1/2 MS, 1/2 Norstog, and T). Seeds collected at 6 weeks after pollination had the highest germination regardless of the medium components. Germinations and subsequent growth on both °Norstog and T medium were better than those on Harvais and ° MS medium. Eighty weeks after inoculation, seedlings were transplanted to soil-based media, and a preliminary investigation was made of the relation of cold treatment to sprouting (shoot elongation).**

## INTRODUCTION

Most terrestrial orchids have become increasingly rare owing to the destruction of their native habitat by human encroachment. In fact, *Cypripedium macranthum* var. *rebunense* has become the symbol for the conservation of endangered plant species in Japan (Japan Society of Plant Taxonomists, 1993). The propagation method that has received the most attention for *Cypripedium* species is *in vitro* seed germination (De Pauw and Remphrey, 1993), and many attempts have been made to germinate seeds of North American *Cypripedium* species (Arditti, 1982; Harvais, 1982; De Pauw and Remphrey, 1993). Immature seeds were often used for the propagation of 'hard-to-germinate' orchids (Arditti, 1982). However, little is known about the germination of Asiatic taxa (Hoshi et al., 1994; Takahashi and Tsutsui, 1992; Tomita and Kanbara, 1995). Nagashima (1995) only reported the germination ability of mature seeds of *C. macranthum* var. *rebunense*, but no other practical report, especially about the culture of immature seeds of this species, is known. For both conservation and commercial production, it is desirable to find practical, efficient methods of propagation.

To obtain basic information for maximizing the *in vitro* seed germination of *C. macranthum* var. *rebunense*, the suitability of media and the optimum time of seed collection were investigated. In addition, the relationship between cold treatment and sprouting (shoot elongation) of juvenile plantlets was preliminarily investigated.

## MATERIALS AND METHODS

After pollination, seed capsules were collected at intervals ranging from 5 to 8 weeks during July and August, 1994. Capsules were rinsed with tap water, burned in flame after spraying with 70% ethanol, soaked in sodium hypochlorite solution (1% available chlorine) containing 2 to 3 drops of Tween 20 for 20 min, then rinsed in

sterile distilled water (Tomita and Kanbara, 1995). Capsules were cut open in a sterile Petri dish and seeds were transferred to culture media with an inoculation loop. Sowing density was approximately 100 to 180 seeds per test tube. Four to five replications of each treatment were seeded. The media used were Harvais (1982), 1/2 MS [the major inorganic elements of MS (Murashige and Skoog, 1962) were reduced by one-half], 1/2 Norstog [the major inorganic elements of Norstog medium (Norstog, 1973) were reduced by one-half], or T (Tsutsui and Tomita, 1990). All media were supplemented with 10 g litre$^{-1}$ sucrose, adjusted to pH 5.5 and solidfied with 8 g litre$^{-1}$ agar. Twenty milliliters of medium was distributed to each 25 × 150-mm test tube, and cooled in a slanted position after autoclaving. All test tubes were incubated in the dark at 20C. At 20 weeks after sowing, germination was assessed. After the assessment of germination, protocorms were subcultured onto fresh medium (30 ml of medium in 100-ml flasks, solidfied with 3 g litre$^{-1}$ Gelan Gum) every 20 weeks for 80 weeks. After culturing, seedlings derived from seeds 6 weeks after pollination and cultured on 1/2 Norstog medium were thinned out. They were divided by bud size (5 mm <, 5 mm-10 mm,10 mm >), and transplanted to pots containing soil-based media [sand : volcanic ash soil : vermiculite (1 : 1 : 1, by volume)]. They were then treated for 12 weeks at 5C (cold treatment) for vernalization. Following cold treatment, potted plants were transferred to a greenhouse with 70% shading. After 16 weeks of culture, sprouting was investigated.

## RESULTS AND DISCUSSION

Germination had occurred on both 1/2 Norstog and T media within 2 weeks after inoculation. On the other hand, germination on both Harvais and 1/2 MS medium

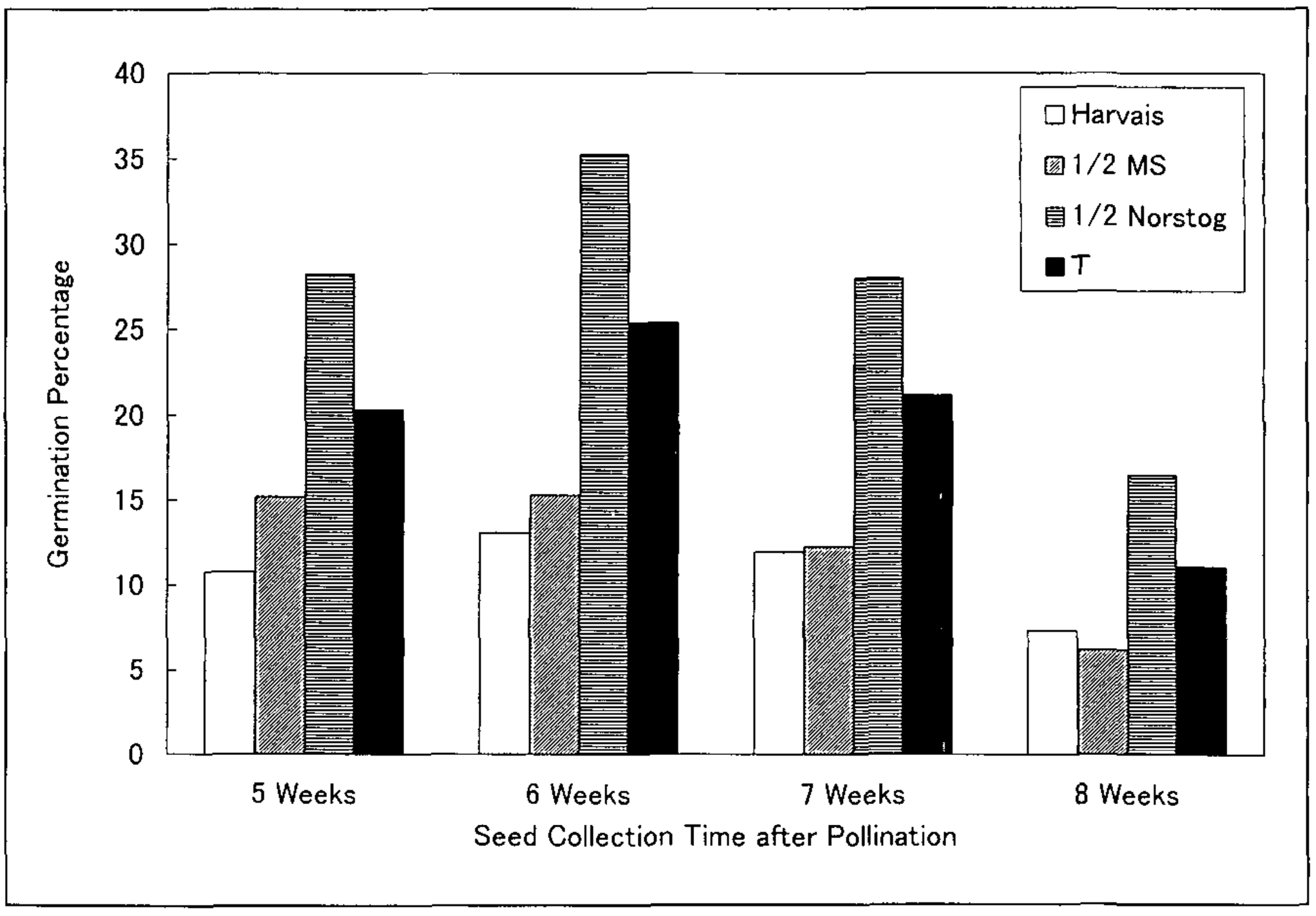


**Figure 1.** Effect of media and seed collection time on the germination of *Cypripedium macranthum* var. *rebunense*.

was very slow, with the first germination observed at 3 to 5 weeks after inoculation. The results of the initial germination of *C. macranthum* var. *rebunense* after 20 weeks of culture are summarized in Fig.1. The time of seed collection affected the initial germination of *C. macranthum* var. *rebunense*. Seeds collected at 6 weeks after pollination had the highest germination regardless of the medium components. Seeds collected after 7 weeks showed a decrease in germination for all media. Nagashima (1995) reported that the germination of mature seeds of *C. macranthum* var. *rebunense* was very poor (under 1%). The present study shows that the decrease in the germination ability of seeds, according to the seed maturation, starts 7 weeks after pollination. Many factors have been suggested as causing the poor germination ability of mature seeds (De Pauw and Remphrey, 1993). Further study of the physiological changes in the seed maturing process is needed. The germination on both 1/2 Norstog and T media was better than that of the other two media. Among four tested media, the germination was most successful on 1/2 Norstog medium. All germinated protocorms, 8 to 16 weeks after inoculation, were a bright maize colour. At the period of assessment (20 weeks after inoculation), protocorms which germinated on both Harvais and 1/2 MS media changed to a creamy brown, and some of them died. After the assessment of initial germination, all live protocorms were transplanted to fresh medium at 20 week intervals. All protocorms cultured on both Harvais and 1/2 MS medium were dead by 40 weeks after inoculation. On the other hand, most protocorms on both 1/2 Norstog and T media survived to reach seedling stage by 80 weeks after inoculation (Fig.2).

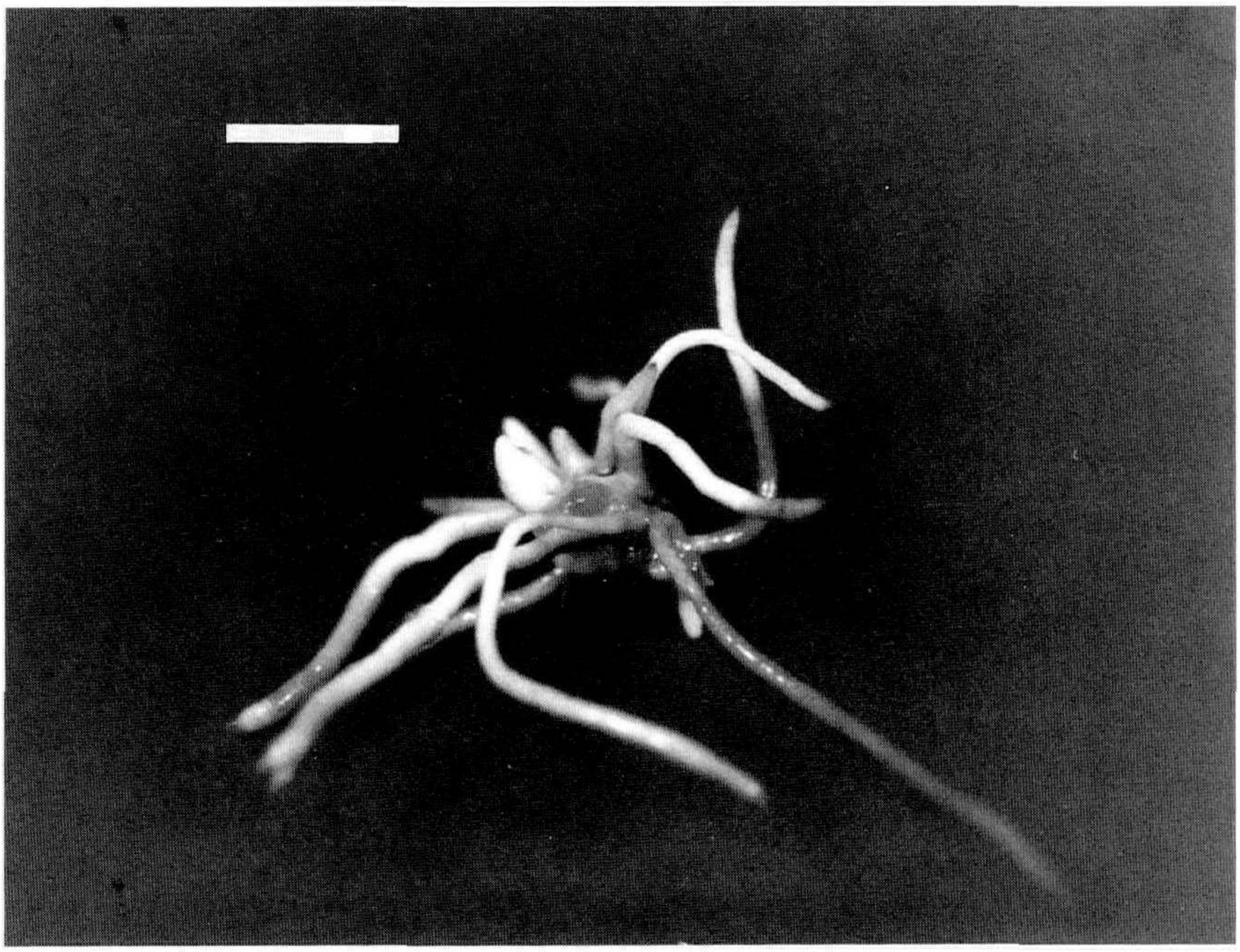

**Figure 2.** Plantlet of *Cypripedium macranthum* var. *rebunense* after 80 weeks of culture on 1/2 Norstog medium. Scale bar = 10 mm.

The latter two media were used for germination of some Asiatic *Cypripedium* species, and showed good results on *C. macranthum* var. *speciosum* (Takahashi and Tsutsui, 1992; Tomita and Kanbara, 1995) and *C. macranthum* var. *taiwanianum* (Tomita and Kanbara, 1995). It seems that these two media were also useful for initial germination and subsequent protocorm growth of *C. macranthum* var. *rebunense*. The 1/2 Norstog medium would be more suitable for research to determine the organic components essential for asymbiotic germination and seedling growth of *C. macranthum* var. *rebunense* because it is a completely defined medium.

After 60 weeks of culture, some plantlets were transferred to light conditions (16 h day regime under 1500 lux). However, no plantlets sprouted buds, and were dead by 80 weeks after inoculation (data not shown). It was suggested that the juvenile plantlets of *C. macranthum* var. *rebunense* had a kind of dormancy. In nature, dormancy ensures that seedlings do not sprout until climatic and temperature conditions are optimal for seedling survival. Takahashi and Tsutsui (1992) showed that asymbiotic seedlings of *C. macranthum* var. *hotei-atsumorianum* had epicotyl dormancy which requires low temperature for sprouting. They investigated the relationship between the bud size of juvenile plantlets of *C. macranthum* var. *hotei-atsumorianum* and their reaction to low temperature, and reported that shoot development following cold treatment affected bud size. Small buds (<5 mm), in turn, delayed both sprouting time and subsequent seedling growth. Then, for the purpose of overcoming dormancy, the effect of cold treatment on the sprouting time of *C. macranthum* var. *rebunense* as it relates to their bud size was preliminarily investigated. The results are summarized in Table 1 and Fig. 3. The present study agreed with the results of *C. macranthum* var. *hotei-atumorianum* (Takahashi and Tsutsui, 1992) that the plantlets of *C. macranthum* var. *rebunense* required cold

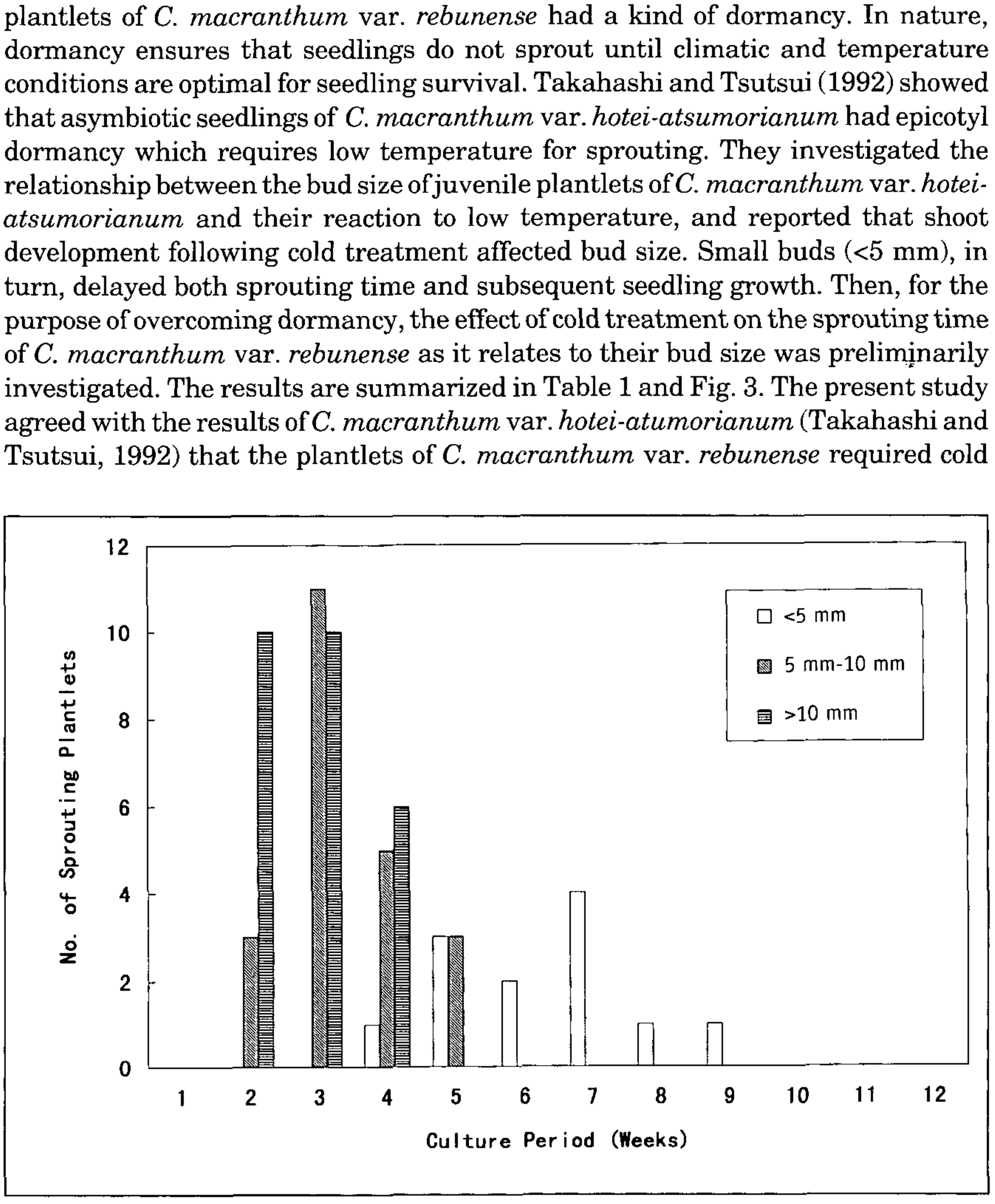


**Figure 3.** Effect of bud size on sprouting time after 12 weeks of cold treatment.

treatment to overcome dormancy (sprouting), and that the sprouting time was related to their bud size when exposed to cold treatment.

**Table 1.** Effect of bud size on sprouting time of *Cypripedium macranthum* var. *rebunense* after 16 weeks of culture in greenhouse following 12 weeks of cold treatment.

| Bud size (mm) | No. of sprouting plantlets /no. of tested plantlets | Sprouting percentage | Mean sprouting time (weeks) |
|---|---|---|---|
| <5 mm | 12/30 | 40 | 6.3 |
| 5 mm to 10 mm | 22/30 | 73.3 | 3.4 |
| >10 mm | 26/30 | 86.7 | 2.8 |

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