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Propagation of *Cypripedium* Species from Seed *in vitro* for Production, Breeding and Conservation

by

Kazumitsu MIYOSHI*

三吉一光*：生産，育種および保全を目的とした *in vitro* におけるアツモリソウ属植物の種子繁殖

Orchidaceous plants are among the favorite plants of gardeners and scientists. Among terrestrial species of orchids from temperate regions, members of the genus *Cypripedium* have become popular as both garden and potted plants. Unfortunately, information about germination of seeds of *Cypripedium* species *in vitro* is fragmented. Thus, the commercial production of plants, efficient breeding for horticultural purposes, and conservation *ex situ* by propagation from seeds have been hampered. In this review, we describe procedures for the enhancement of seed germination of *Cypripedium* species for production, breeding and conservation. The mechanisms that control germination of this genus are also discussed.

1. The importance of propagation of *Cypripedium* species from seed for production, breeding and conservation.

1-1 Production of plants

Cypripedium species have fascinated generations of gardeners in Europe, North America and East Asia. As early as 1597, John Gerard, a herbalist, was growing the Lady's Slipper Orchid in his garden in Europe (Cribb and Bailes 1989). In Japan, *Cypripedium guttatum* var. *yatabeanum* was already cultivated in 1695 (Iwasa 1975). *Cypripedium* plants are presently grown in gardens and in pots and have great commercial potential as ornamental plant for gardens as well as potted plants. The elucidation of appropriate culture conditions, namely, growth media, nutrition and fertilization, temperature and light, for *Cypripedium* species is indispensable for successful production of plants from seeds.

Little information about requirements for culture of *Cypripedium* is available, as compared to the information that is available for culture of epiphytic orchids of tropical and subtropical origin. Recently, Kim *et al.* (1996a) reported the effects of chilling treatment on the growth of *Cypripedium macranthos*. The timing and duration of chilling treatment at 0-4℃ were important for the enhancement of growth and flowering. Kim *et al.* reported that plants subjected to chilling treatment prior to 26 Sept. grew poorly and failed to flower. Maximum growth and flowering was achieved with 50 days of chilling treatment at 4℃. They found too that fertilization also critically influences growth and

*The Japanese Society for Orchid Sciences, c/o Research Institute of Evolutionary Biology, Kamiyoga, Setagaya-ku, Tokyo 156. Address for correspondence : Green Haitsu 401, Wada 32-2, Kakegawa, Shizuoka, 436. ラン懇話会. 〒156 東京都世田谷区上用賀2-4-28　進化生物学研究所内. 著者連絡先：〒436 静岡県掛川市和田32-2　グリーンハイツ401号.

flowering in this species. They analysed the physico-chemical properties of soils from three natural habitats of *Cypripedium macranthos* and found that such soils had a pH of 5.0–5.2, with 10.3–14.1% organic matter and 12–27 ppm of phosphorus. They examined the effects on the growth and flowering of this species of a solution of 1,000 ppm of Hyponex® (N:P:K＝6.5:4.5:19.0; The Hyponex Company, Inc., Copley, OH, USA) and of "ball fertilizer", which is a solid organic fertilizer that consists of 60% rape seed meal, 20% flax seed and 20% rice meal (overall N:P:K＝4:2:1) alone or in combination. Growth of new shoots and flowering percentages were maximal with a combination of ball fertilizer and the application of Hyponex. Tip burn of leaves, a physiological disorder, was reduced with fertilization (Kim *et al.* 1996b). Further studies to determine the requirements for culture of each species are needed for the efficient establishment of seedlings and further growth, if we are to maximize propagation from seed in this genus.

1–2 Breeding

Some hybrids that occur in nature, such as *C.* ×*andrewsii* Fuller, have been reported (Homoya 1993). However, the first artificial interspecific hybrids were registered in 1987 by Whitlow (Cash 1991). Since then more than 50 hybrids have been registered. Some hybrid in second generations, such as Hedi (Ingrid × *macranthos*) have also been registered (Royal Horticultural Society 1997). For the efficient breeding of inter- as well as intra-specific hybrids, efficient procedures for propagation from seed are prerequisite. Some species in this genus may have a great impact on breeding programs. Even though it has not emphasized, improvement of heat tolerance by inter-specific hybridization will be important for horticultural exploitation of this genus. *Cypripedium* species are now cultivated only in temperate regions with cool summers. However, *C. japonicum* which is native to Japan and surrounding countries, grows vigorously in temperate regions with hot summers where temperatures exceed 25℃ at night and occasionally reach 35℃ in the daytime. *C. irapeanum, C. dickinsonianum* and *C. male* from Mexico and Central America may have potential as breeding materials for introduction of heat tolerance. Further observations are needed to evaluate this possibility. Incorporation of the trait for heat tolerance from these species into heat-sensitive species will enable us to expand the region in which *Cypripedium* plants can be grown without any special equipment in summer. Some other traits, such as dwarfism of *C. debile*, multi-flowering on stems of *C. californicum*, and the large flowers of *C. macranthos,* are also attractive features for the breeding of this genus.

1–3 Conservation

Almost all *Cypripedium* species are now extinct and/or endangered in Europe, in Central and North America, and in East Asia. Although, from an ecological perspective, conservation *in situ* is most important for conservation of orchids, conservation *ex situ*, for example, by propagation from seed, should also be considered for effective conservation. One of the most important goals of conservation *ex situ* is to make rare plants available to propagators so that they can produce large numbers of artificially propagated plants as rapidly as possible. Such production will reduce the threat of collection of plants from natural habitats to meet the horticultural demand. Another important role for conservation *ex situ* is the recovery of destroyed natural habitats and reintroduction of seedlings derived from seeds.

C. calceolus is found in Britain, where it has been reduced to a single specimen in the wild as a result of over-collecting. Stewart and colleagues, working on a conservation project at the Royal

Botanic Gardens at Kew, successfully germinated seeds of British *C. calceolus* for reintroduction into the wild (Cribb 1993).

In the United States, continuing efforts aimed at protection of habitat, research into propagation, education about the importance of conservation, population studies, and monitoring of *Cypripedium* species and other terrestrial orchids have been reported (Jesup 1996). As a consequence, seedlings of some of North American species, such as *C. reginae,* are available on a commercial scale and should prevent the impending destruction of habitats due to over-collection of plants (Steele 1995).

In Japan, several groups are working on the conservation of *Cypripedium* species both *in situ* and *ex situ*. Seeds were produced by hand-pollination of *C. macranthos* var. *rebunense* plants in the natural habitat and were dispersed there. Some seedlings that putatively originated from those seeds were observed several years after the dispersal of the seeds (The Asahi 1990). In Japan, researchers in some administrative districts in Iwate Prefecture, such as Kawai-mura and Sumita-cho, were successful in the artificial propagation of *C. macranthos* from seeds, which was aimed both at reintroduction into the natural habitat and the horticultural market (personal communication from; Prof. Mii, M Chiba University, Matsudo, Japan).

2. Asymbiotic germination of seeds of *Cypripedium in vitro.*

2-1 Germination of immature and mature seeds

In *Cypripedium* species, successful germination has sometimes been achieved by culturing immature seeds and germination percentages of 60-95% have been reported (de Pauw and Remphery 1993, St-Arnaud *et al.* 1992, de Pauw *et al.* 1995). De Pauw and Remphery (1993) reported an optimum time of about 8 weeks after pollination for germination in *C. candidum, C. reginae* and *C. caluceolus* var. *parviflorum*, with a sharp decrease in germination *in vitro*. It has been suggested that an increase in the hydrophobic nature of the seed coat during development might inhibit germination of mature seeds of terrestrial species of orchids (van Waes and Debergh 1986a).

The period during which immature seeds are available asymbiotic germination is short and occurs only once a year. In addition, it is difficult to determine the correct timing for the harvest of fruits that have reached the optimum stage of development. By contrast, mature seeds can be collected from fruits of orchidaceous plants in large quantities and such seeds can be stored and are available year-round (Miyoshi 1988, Lauzer *et al.* 1994). Moreover, transmission of plant viruses, which was reported in the culture of immature seeds of orchids (Lawson and Hsu 1995, Brunt *et al.* 1996), can be avoided by the culture of mature seeds exclusively. Successful germination of seeds of *C. calceolus* var. *pubescens* (Chu and Mudge 1994), *C. reginae* (Steel 1995), and *C. macranthos* (Miyoshi and Mii 1998) was recently demonstrated. Thus, mature seeds appear to have great potential as a source material for propagation.

2-2 Chilling treatment

Previous reports on the requirements for chilling of seeds of *Cypripedium* prior to germination are contradictory. Pre-chilling of seeds for 2 to 5 months stimulated the germination of seeds of *C. reginae* (Ballard 1987) and *C. calceolus* (Chu and Mudge 1994), whereas no such effect was observed in the case of seeds of *C. acaule* (Lauzer *et al.* 1994) and *C. calceolus* (van Waes and Debergh 1986b). Miyoshi and Mii (1998) demonstrated that chilling at 4℃ for 8-12 weeks was very effective in

stimulating the germination of seeds of *C. macranthos*. The differences in the effects of chilling on seed germination observed among the various species might be due to genetic differences, such as inter- and intra-specific variation, and/or to differences in the physiological conditions of seeds, as affected by the time of harvest, the extent of dehydration and the duration of storage at low temperature. Therefore, detailed analysis of the requirements of the seeds of each species in this genus for chilling are indispensable, if we are to improve techniques for propagation from seeds.

In symbiotic cultures of seeds of *Epipactis palustris*, incubation at 20℃ prior to chilling treatment increased the germination frequency (Rasmussen 1992). It was suggested that the initial incubation at 20℃ enabled the seeds to imbibe water, to establish contact with the fungus and, possibly, to after-ripen before the start of cold treatment. Rasmussen (1995) also suggested that the previously reported failure of seeds to respond to chilling might have been associated with the exposure to low temperature immediately after sowing. However, Miyoshi and Mii (1998) showed that the reaction of seeds to chilling decreased with prior culture at 20℃ and they concluded that, at least, seeds of *C. macranthos* are fully mature at fruit maturity and have no requirement for an elevated temperature for after-ripening. We can also speculate that a requirement for initial culture at 20℃ prior to chilling treatment might be restricted to seeds in symbiotic culture.

2-3 The effects of solutions of sodium and calcium hypochlorite on germination

Solutions of hypochlorite are commonly used as disinfectants for orchid seeds and the stimulatory effects on seed germination of NaOCl (Miyoshi and Mii 1981, 1995, Frosch 1982) and of $Ca(OCl)_2$ (van Waes and Debergh 1986b) have been reported. Van Waes and Debergh (1986b) demonstrated that germination of *C. caluceolus* was stimulated by prolonged soaking of seeds in a solution of calcium hypochlorite. After treatment with $Ca(OCl)_2$ for 4h, 38% of seeds germinated, but only 0.8% germinated after such treatment for only 15 min. Rasmussen (1995) noted that frequencies of germination of orchid seeds are usually higher after sterilization with $Ca(OCl)_2$ than with NaOCl, but gave no reason for this difference. Miyoshi and Mii (1998) reported, however, that the maximum germination frequencies of seeds of *Cypripedium macranthos* that they obtained with solutions of these hypochlorites were almost the same (*ca.* 70%), irrespective of differences in the concentration of available chlorine and in the time required for maximum germination. Miyoshi and Mii suggested that, although it is unclear why the two solutions ultimately had similar effects on seed germination, it might be better to use NaOCl rather than $Ca(OCl)_2$ because of the ease of preparation of solutions and the shorter time required for treatment with NaOCl.

2-4 The effects of plant hormones on the germination *in vitro*

In general, the responses of orchid seeds to cytokinins vary from species to species (Arditti and Ernst 1984). In *Cypripedium*, a requirement for kinetin during germination of *C. reginae* has been demonstrated (Harvais 1982) and benzyladenine (BA) is required for germination of seeds of *C. calceolus* (van Waes and Debergh 1986b). A preference for a particular cytokinin for germination *in vitro* of immature seeds of *C. candidum* has also been demonstrated: the frequency of germination was increased by BA and 6-(α, α-dimethylallylamino)-purine (2iP) but not by kinetin (de Pauw *et al.* 1995). Miyoshi and Mii (1998) evaluated the effects of six cytokinins on the germination of seeds of *C. macranthos* and they found that kinetin had the highest promotive effect on the germination of such seeds. The differences among the responses to kinetin in the various studies suggest that the maturity

of seeds and/or species-specific differences in germinability might be responsible for the varying degrees of sensitivity of seeds to kinetin. Cytokinins have been reported to act as substitutes for chilling treatment in some plants (Brown and van Staden 1973, Brown and van Staden 1975, Reda 1976). However, Miyoshi and Mii (1998) showed that germination of seeds of *C.macranthos* was enhanced by culturing chilled seeds in the continuous presence of a cytokinin. It has also been suggested that breaking of seed dormancy in some plants involves an ordered sequence of physiological events, which include an increase in levels of cytokinin that is induced by stratification (van Staden *et al.* 1972, Webb *et al.* 1973). Thus, it can be postulated that seed dormancy of *C. macranthos* might involve at least two indispensable and possibly linked factors, whose effects can be overcome by a cytokinin and chilling prior to germination (Miyoshi and Mii 1998).

2-5 Illumination

In previous studies of members of the genus *Cypripedium* with one exception (Hoshi *et al.* 1994), successful germination was achieved only when seeds were cultured in continuous darkness (van Waes and Debergh 1986b, de Pauw and Remphery 1993, de Pauw *et al.* 1995, Chu and Mudge 1994, St-Arnaud *et al.* 1992, Harvais 1982). Hoshi *et al.* (1994) cultured immature seeds of four Asiatic *Cypripedium* taxa, namely, *C. debile, C. henryi, C. japonicum* var. *formosanum* and *C. tibeticum* under illumination at 400-800 lux from a fluorescent lamp for 16 hours daily. However, no comparison was made with culture in continuous darkness and no replicate were included. It is unclear whether illumination had a stimulatory or an inhibitory effect on germination in their study. In preliminary experiments with seeds of *C. reginae*, we observed an inhibitory effect of light on germination. Seeds cultured at 20℃ under light at approximately 700 lux for 2 weeks after 2 months of pre-chilling at 4℃ were moved to darkness and, ultimately, the inhibition of germination of seeds was overcome. Germination percentages were almost the same as those of seeds that had been cultured in continuous darkness (Miyoshi and Mii, unpublished data). We also found that light had a stimulatory effect after the protocorm stage. This dramatic change in the effects of light during germination of seeds and development of seedlings might be associated in some way with the shift from heterotrophism to autotrophism at the early stages of the life cycle of *Cypripedium* species. Further investigations are needed to elucidate the mechanisms of the inhibitory effect of light on the germination of *Cypripedium* species and they should help to facilitate the efficient establishment of seedling in this genus.

3. Conclusion.

Enhanced germination of immature and/or mature seeds of some species of *Cypripedium* has been reported and some stimulatory factors, such as chilling treatment, application of cytokinins and treatment with solutions of sodium and calcium hypochlorite before sowing, have been described. Recently, in Japan, symptoms of some viral diseases have been observed in *C. macranthos.* However, transmission of plant viruses, reported during culture of immature seeds of orchids (Lawson and Hsu 1995, Brunt *et al.* 1996), can be avoided by the culture of mature seeds. Thus, mature seeds appear to have great potential as a source material for propagation. However, there have been no reports of the successful germination of mature seeds of *C. japonicum,* which may be a good source material for head-tolerant traits in breeding programs for this genus. The establishment of a germination protocol that is reproducibly successful and allows efficient germination of this species will be the focus of further studies.

Summary

Almost all *Cypripedium* species are becoming extinct and/or endangered in Europe, North America and East Asia. Thus propagation of *Cypripedium* species from seed *in vitro* for production, breeding and conservation is important. Previous studies on the factors that had a stimulatory effects on germination of *Cypripedium* spp. were reviewed. Conclusions and further prospects for development of protocols for propagation from seed in this genus were also indicated.

摘　　要

アツモリソウ属（ラン科）のほとんどの種は欧州，北米および東アジアにおいて絶滅危惧種もしくは絶滅危急種になりつつある。本属の種子繁殖の意義について生産，育種，ならびに保全の各分野から論じた。また，現在までに報告されている本属植物の休眠・発芽に関する過去の報告を概観し，本属植物の種子繁殖技術の現状について紹介するとともに，問題点ならびに今後の展望について論じた。

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