IN VITRO ASYMBIOTIC PROPAGATION OF THE VULNERABLE SLIPPER ORCHID *CYPRIPEDIUM CORDIGERUM* D. DON

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ABSTRACT

This paper presents an attempt to establish a protocol for the conservation of *Cypripedium cordigerum* D. Don a vulnerable species of orchid using *in vitro* symbiotic seed germination. The suitability of four orchids seed germination media [Terrestrial orchid medium (BM), modified terrestrial orchid seed germination medium (BM-1), Malmgren modified terrestrial orchid medium (MM), Knudson C medium (KC)] was tested using different photoperiods i.e. 24-h dark or 12-h light per day. The seed capsules were harvested at two different stages of development in order to determine the effect of capsule maturity on seed germination. The maximum percentage germination of seed from intact capsules in the dark was 65.00 ± 0.12% on the BM-1 medium. Release of brownish exudates was stopped by the addition of activated charcoal to the cultures. Seedlings developed in 26.17 ± 0.17 weeks. The current study is the first to report the *ex-situ* conservation of *C. cordigerum*.

Keywords: asymbiotic; *in vitro*; ornamental; terrestrial; vulnerable orchids

Abbreviations: BM – Terrestrial orchid medium; BM-1 – Modified terrestrial orchid medium; MM – Malmgren modified terrestrial orchid medium; KC – Knudson C medium

Introduction

The genus *Cypripedium* includes 56 species and 4 varieties and belongs to the family Orchidaceae (subfamily: Cypripedioideae Lindl.) (Cribb 1997; Wu et al. 2009). It is a horticulturally important genus. The subfamily Cypridedioideae includes the following genera: *Cypripedium*, *Paphiopedilum*, *Phragmipedium* and *Selenipedium*. *In vitro* initiation and establishment of cultures of *Cypripedium* is difficult. Among these genera, *Cypripedium* is reported to be the most difficult (Arditti 2008). The only and most common method for the *in vitro* propagation of species of *Cypripedium* is a symbiotic seed germination. One of the species, *Cypripedium cordigerum,* is a vulnerable species. It is also known as heart-lip lady-slipper orchid. *C. cordigerum* grows at an altitude of 2,500–3,000 m a. s. l. in small areas in terrestrial habitats in Bhutan, Nepal and temperate Indian Himalayas (Bose and Bhattacharjee 1980). This species has beautiful, solitary, white flowers with heart-shaped labellum and plicate leaves (Fig. 1). However, although *C. cordigerum* is well known for its beautiful flowers, its therapeutic utility is unexploited, but its tender leaves are cooked as a vegetable by local rural communities (www.ionopsis.com /edible_orchids.htm).

Currently, *Cypripedium cordigerum* grows sporadically in small groups consisting of 4–6 plants in humus rich soil under partial shade from *Cedrus* trees. Previously, dense populations of this species occurred in habitats in north-western Himalayas (Jalal et al. 2009). Several factors are associated with the decline in the abundance of this species: destruction of its natural habitat due to deforestation for residential areas and agricultural purposes, logging, overgrazing, trampling, climate change and indiscriminate collecting. As a consequence, the rate of decline of this species is increasing. According to the current conservation status, *C. cordigerum* is categorized as vulnerable (VU) and is included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2023). It is categorized as vulnerable species in the Red Data Book of Indian Plants volume 1 (www.iucnredlist.org; Jalal et al. 2009).

This paper proposes that mass propagation of this species, using tissue culture techniques, be used to assist in the conservation of *C. cordigerum*. Currently, there is no information on *in vitro* symbiotic seed germination of *C. cordigerum*. The objective of this study is to establish a protocol for *in vitro* symbiotic seed germination using immature/mature seed, four types of media kept in the dark or under a 12-h photoperiod.

Percentage germination of slipper orchids in both *in vivo* and *in vitro* conditions is very low, particularly so *in vitro* (Pierik et al. 1988). They lose the ability to germinate shortly after harvesting (De Pauw and Remphrey 1993). Although, a single capsule contains millions of seeds, very few germinate in the wild.

The use of *in vitro* culture techniques greatly enhances the ability to propagate, multiply and conserve the genepool of endangered and commercially viable species. *In vitro* symbiotic seed germination enables the conservation of endangered germplasms as it can be used to rescue embryos that would be aborted in natural habitats and shortens the breeding cycle (Knudson 1922; Arditti et al. 1982). In addition, *in vitro* symbiotic seed germination can also increase the number of mature seeds that

Kaur, S.: *In vitro* asymbiotic propagation of the vulnerable slipper orchid *Cypripedium cordigerum* D. Don
European Journal of Environmental Sciences, Vol. 13, No. 2, pp. 90–95
https://doi.org/10.14712/23361964.2023.10

germinate successfully (Lauzer et al. 1994; Whigham et al. 2006). For these reasons, a symbiotic seed germination is utilized here to initiate cultures *in vitro*.

Terrestrial species of orchids from temperate regions are more difficult to germinate *in vitro* than tropical epiphytic species (Oliva and Arditti 1984; Hossain et al. 2013). Among terrestrial species of orchids, Cypripediums have specific microhabitat requirements for germination (Kull 1999). They are a source of material for horticulture and medicine (Zhu 1989). Unlike many other orchids, species of *Cypripedium* have stringent needs for germination. There is little in the literature on their specific requirements (Arditti and Ernst 1993). Several physical and nutritional factors affect seed germination in *Cypripedium* (Zeng et al. 2014). In nature, germination and propagation of species of *Cypripedium* is quite low due to ecological constraints. Currently, there are only a few reports on the a symbiotic seed germination of *Cypripedium* species *in vitro* (De Pauw and Remphrey 1993; Hsu and Lee 2012; Huh et al. 2016) and no information exists on *in vitro* symbiotic seed germination of *Cypripedium cordigerum*. There is a need for more studies on seed germination in *Cypripedium*. For that reason, the present study provides information on the germination of *Cypripedium cordigerum in vitro*, a protocol for a symbiotic seed germination, and identifies the optimum (1) capsule age, (2) medium for germination and (3) photoperiod.

Materials and Methods

Plant collection

A plant of *Cypripedium cordigerum* with capsules growing on a roadside slope (altitude 2,276 m a. s. l.) at Chrabra, Shimla forest hills, Himachal Pradesh, India (Latitude range 31°6′12″N 77°10′20″E) was collected in summer (May–June). The plant was replanted in a pot (diameter 27.5 cm × 22.4 cm) containing soil and kept in a greenhouse under natural light conditions, 70% relative humidity and 22 °C / 15 °C day/night temperatures. The seed from dehisced pods was collected on paper.

Culture medium for *in vitro* seed germination

To initiate the cultures, four media: BM (Terrestrial orchid medium; van Waes 1984), BM-1 (Modified terrestrial orchid medium; van Waes and Debergh 1986), KC (Knudson 1946), MM (Malmgren modified terrestrial orchid medium; Malmgren 1996), were used. The pH of each medium was adjusted before autoclaving using 1N NaOH and 1N HCl. The autoclaved media were kept at 37 °C to check for any contamination. Seed viability was assessed by staining with TTC 1% (2,3,5-triphenyl-2H-tetrazolium chloride) at a pH of 6.5 adjusted using 1N NaOH (van Waes and Debergh 1986; Lauzer et al. 1994). The seed was stained for 40–48 h at 30 °C in the dark. Viable seeds contained ovoid embryos that appeared healthy and were a pink-red in colour. To reduce the release of brownish exudates by growing seedlings, activated charcoal (2 g / l) was added to the BM-1 medium.

Fig. 1 Plant of *Cypripedium cordigerum*.

Surface sterilization of green capsules

Each capsule was first scrubbed with a soft brush in running tap water to remove any debris and rinsed thoroughly in a detergent solution. They were swabbed with ethyl alcohol under sterile (laminar) hood and surface sterilized with an aqueous solution of 0.1% mercuric chloride ($HgCl_2$; Qualigens, Mumbai, India) containing 1–2 drops of Teepol as a wetting agent for 7 min, then rinsed with sterilized distilled water to remove any traces of mercuric chloride on its surface. Thereafter, the capsule was flamed on a burner, slit open longitudinally and the seed scooped out into a petri dish.

Sterilization of seeds from dehisced capsule

The seed from a dehisced capsule was collected on a thin sheet of glazed paper. Inside a laminar air-flow hood, with great care, the seed was poured in 100 ml flask and treated with sodium hypochlorite (4%) (Merck, Mumbai, India) for 7–8 minutes. The solution was then filtered and washed twice with sterilized distilled water. The sterilized seeds were evenly spread thinly over the media in culture tubes with the help of a spatula.

Inoculation and incubation conditions

The inoculations were done under aseptic conditions in a laminar air-flow cabinet. The culture vessels were incubated either in the dark or under a 12-h light photoperiod of 40 $\mu mol^{-2}\ s^{-1}$ light intensity (Fluorescent tubes, Philips India Ltd, India) at 25 ± 2 °C. There were eight replicates of each experiment. To check the reproducibility of the protocol, the experiment was repeated twice. The cultures were sub-cultured as and when required.

Percentage germination

Four weeks after of inoculation, a few seeds were scooped-out of the culture vessel. These were dispersed in a drop of water on a glass slide and observed under a light microscope. Percentage germination was calculated using the following formula:

Germination (%) = (Number of enlarged seeds with swollen embryos/Total number of seeds) × 100. This was recorded weekly in order to determine differences in the stage of development of the cultures, using a stereo zoom microscope (Nikon H600L, Japan).

Observation and statistical analysis

This experiment was of complete randomized design (CRD). For each treatment there were four replicates. The cultures were observed regularly under a binocular microscope (Olympus SZX10, Japan). Photographs were taken using a digital camera, Nikon, Digit Sight, DS, Ril Nikon, and data recorded accordingly. The results were tested using one-way ANOVA and analysed using Tukey's Multiple Comparison test in SPSS (Version 17) software package (SPSS Inc., Chicago, corporation, US).

Results

Effect of capsule age and photoperiod on a symbiotic seed germination

The age of the capsule determined the percentage germination of the seed, with the seed from the immature capsule germinating more readily, 65.05 ± 0.13%, than that from the mature capsule (Table 1). The mature seeds took longer to germinate and the percentage germination was lower, 18.02 ± 0.02% (Table 1).

Seeds started germinating after 7.15 weeks, when embryos started to swell (Table 2) and achlorophyllous spherules ruptured the testa. These grew in size while still attached to the seed coat. During the next 4 weeks, the conical, achlorophyllous protocorms developed (Fig. 2a). A shoot-tip became evident on the apical part of the protocorm and leaf primordia developed after 15.95 ± 0.25 weeks. The protocorms remained achlorophyllous under both light conditions (12-h light / 24-h dark). The chlorophyll developed at 1st leaf stage. Just below the shoot-tip a root primordium developed after 21.88 ± 0.25 weeks (Fig. 2a). The root was off-white in colour and densely hairy (Fig. 2b). Morphogenetic events were more advanced in those cultures kept in the dark than in those kept under a 12-h photoperiod. Near the shoot primordia, a horizontally growing positively phototropic root developed after 26.17 ± 0.17 weeks.

Effect of media on percentage germination and seedling development

The quickest response and highest percentage germination of 65.05 ± 0.13% was recorded in the dark 7.15 ± 0.17 weeks on the modified terrestrial orchid medium (Fig. 3). For the BM and KC media, the percentage germination was lower, with only 30.10 ± 0.10 and 20.75 ± 0.05 of the seeds germinating, respectively. Under a 12-h photoperiod, the percentage germination was lower. The seeds took longer to start germinating and the percentage germination was also lower for all other media (Table 2).

Those cultures that were initially kept in the dark were illuminated for 12-h per on their respective media in order to prevent the protocorms becoming necrotic and dying. The development of seedlings, within 26.17 ± 0.17 weeks, was recorded for the BM-1 medium. The seeds cultured on MM medium did not germinate in the dark or under a12-h photoperiod. The seedlings that developed on the KC medium were quite weak and did not grow, and were transferred to BM-1 medium.

Table 1 Effect of capsule age on the percentage germination of *Cypripedium cordigerum* on the BM-1 medium.

Capsule stage	Germination %
Mature Seed (Dehisced)	18.02 ± 0.02^{a}
Immature (Undehisced)	65.05 ± 0.13^{b}

Values in columns with the same superscript are not significantly different at $p \leq 0.05$ according to Tukey's test.

Table 2 *In vitro* germination of immature seed of *Cypripedium cordigerum* on different media under a 12-h photoperiod.

Media	Germination percentage	Initiation of response (weeks)	Development of (weeks)			Seedlings (weeks)
			Protocorm	1st leaf	1st root	
BM	30.10 ± 0.10^{bd}	11.12 ± 0.14^{bcd}	17.00 ± 0.00^{bcd}	21.03 ± 0.64^{bcd}	24.68 ± 0.09^{bcd}	29.20 ± 0.27^{bcd}
BM-1	65.05 ± 0.13^{acd}	7.15 ± 0.17^{acd}	11.10 ± 0.15^{acd}	15.95 ± 0.25^{acd}	21.88 ± 0.25^{acd}	26.17 ± 0.17^{acd}
MM	0.00^{abc}	0.00^{abc}	0.00^{abc}	0.00^{abc}	0.00^{abc}	0.00^{abc}
KC	20.75 ± 0.05^{bd}	15.12 ± 0.15^{abd}	20.05 ± 0.11^{abd}	25.42 ± 0.21^{abd}	27.05 ± 0.19^{abd}	31.12 ± 0.09^{abd}

BM-1 – terrestrial orchid medium (modified); BM – terrestrial orchid medium; MM – Malmgren modified terrestrial orchid medium; KC – Knudson C medium. In a column, the values followed by the same letter superscripts are not significantly different at $p \leq 0.05$ according to Tukey's test.

Fig. 2 *In vitro* symbiotic seed germination and seedling development of *Cypripedium cordigerum* (a) Different stages in the germination and development, (b) Development of 1st leaf, (c) Seedling formation.

In culture the seedlings produced brownish exudates that inhibited the growth of leaves. In order to promote growth activated charcoal (2%) was added to the BM-1 medium, in which the seedlings produced shoots (Fig. 2c).

Discussion

The success of a symbiotic seed germination depends on the maturity of the seed and a suitable culture medium. In the current study, percentage germination was significantly affected by the maturity (harvesting time) of the capsule, culture medium and photoperiod.

A higher percentage of the seed obtained from green capsules germinated than that from dehisced capsules. Similar responses are reported for other species of *Cypripedium, Paphiopedilum spicerianum* and *P. venustum,* in which immature seed germinated better than mature seed (Kaur and Bhutani 2013, 2016; Zeng et al. 2014). According to Lee et al. (2007) and Ding et al. (2004) there is a positive correlation between metabolic activity of the embryos and percentage germination. Although the earliest stage at which immature seed can germinate was not determined, previous studies indicate that the decline in percentage germination is primarily due to accumulation of inhibitory substances that lead to rapid loss of viability and induces dormancy in the seeds (Stoutamire 1974; Linden 1980; van Waes and Debergh 1986; Miyoshi and Mii 1988; De Pauw and Remphrey 1993; Rasmussen 1995). Lauzer et al. (1994) state that mature seed of *Cypripedium acaule* is extremely difficult to germinate. Zeng et al. (2014) indicate that mature seed has an impermeable testa and the suspensor channel is also closed and consequently, the embryo desiccates, which accounts for the reduction in the percentage germination in species of *Cypripedium*. Harvais (1980) correlate the reduced percentage germination of seeds of *Cypripedium* with the accumulation of suberin in the testa, which make mature seed hydrophobic and prevents absorption water and nutrients by the embryo, which prevents mature seed from germinating in *in vitro* (Lee et al. 2008).

In the current experiment, there were significant differences in percentage germination and time taken by seed to initiate germination on variety of different media. The maximum percentage germination was recorded on the BM-1 medium, followed by the BM and KC media. A comparative analysis of the composition of media indicates that seed of *C. cordigerum* require organic nitrogen. The available organic nitrogen in the different media differed, with the most in BM-1. In this experiment, the inorganic nitrogen content of KC may have reduced percentage germination due to the lower activity of nitrate reductase as earlier suggested by Raghavan and Torrey (1964), van Waes and Debergh (1986) and Malmgren (1992). The results presented corroborate earlier results for *Habenaria macroceratitis* (Stewart and Kane 2006), *Paphiopedilum spicerianum* (Kaur and Bhutani 2013) and *Paphiopedilum venustum* (Kaur and Bhutani 2016) that

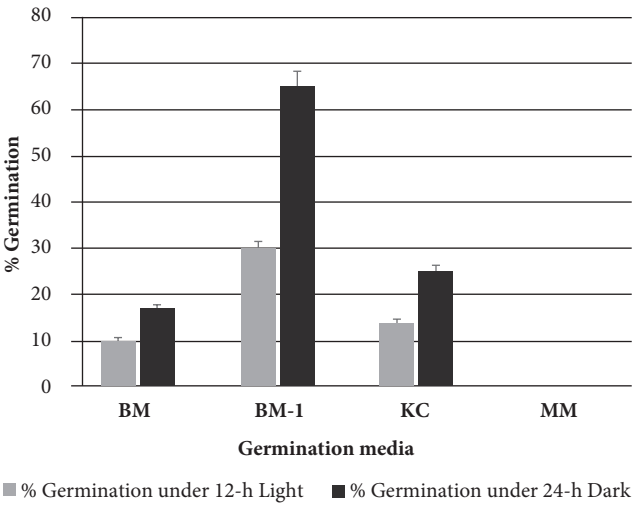

Fig. 3 Percentage germination of *Cypripedium cordigerum* recorded on different media and under different photoperiods.

an organic form of nitrogen induces high levels of germination and growth in cultures. Organic compounds present in culture media promotes seed germination in several species of slipper orchids (Curtis 1947; Stewart and Kane 2006; Dutra et al. 2008; Kaur and Bhutani 2016) and *C. cordigerum* does not appear to be an exception, as its seed failed to germinate in the MM medium, which is also an organic nitrogen-based culture medium, but the MM medium contains the amino acid glycine, whereas, BM-1 medium contains amino acid L-glutamine. Thus, the amino acid glycine could have inhibited the germination of *C. cordigerum* on the MM medium.

In *C. cordigerum*, darkness (24-h) favoured the early onset of seed germination and a high percentage germination. These results are in accord with earlier results on the germination of various species of terrestrial orchids, in which germination was inhibited when illuminated (Arditti et al. 1981; van Waes and Debergh 1986; Pierik et al. 1988; Yamazaki and Miyoshi 2006; Zeng et al. 2012; Kaur and Bhutani 2013). In contrast, germination in the slipper orchid *Paphiopedilum venustum* is higher when illuminated (Kaur and Bhutani 2016). Previous studies indicate that the responses of terrestrial orchids to illumination in terms of germination vary and are species specific (Arditti et al. 1981).

The highest percentage germination for *C. cordigerum* was recorded in the dark. In nature, *C. cordigerum* grows mainly in shaded areas; thus, this appears to be an adaptation, which affects its germination *in vitro*. The findings of this experiment and descriptions in previous studies support the inhibitory effect of illumination and darkness, on percentage germination and seedling development in terrestrial species of orchids and the response to these conditions appear to be an intrinsic trait (genotype) of each species.

In *C. cordigerum*, roots developed after the formation of protocorms similar to that reported earlier for *Cypripedium reginae* (Zeng et al. 2014). Previous studies reveal that the development of roots in species of *Cypriperdium* vary; it is species specific and according to Oliva and Arditti (1984), in the majority of other species of *Cypripedium* there is simultaneous formation of shoots and roots. However, interestingly in *C. acaule* the shoots developed after the formation of roots, whereas it is the reverse in *C. californicum*.

In *C. cordigerum*, the production of chlorophyll occurs after the formation of a protocorm. Stoutamire (1974) states that protocorms lacking chlorophyll are a characteristic feature of species growing in well-drained soils. Since *C. cordigerum* also grows in soil, protocorms lacking chlorophyll appear to be a genetic trait.

In culture, the exudation of phenolic compounds was successfully stopped by the addition of activated charcoal to the medium. Addition of activated charcoal also promoted an increase in the number of shoots. The beneficial effects of activated charcoal is that it adsorbes inhibitory substances (Phenolics) as reported earlier for seedling cultures of *Paphiopedilum* and *Phalaenopsis* (Butcher and Marlow 1989; Hicks and Lynn 2010) *Paphiopedilum spicerianum* (Kaur and Bhutani 2013) and *P. venustum* (Kaur and Bhutani 2016). Pacek-Bieniek et al. (2010) in their study on *Zygostales grandiflora* state that activated charcoal promotes germination, seedling development and growth of aerial roots. There are reports of the supportive effect of activated charcoal on the growth of seedlings of *Cypripedium* spp. (Ernst 1974, 1975; Yan et al. 2006; Bae et al. 2009).

Conclusion

In vitro symbiotic seed culture facilitates the conservation of germplasm of vulnerable species of orchids of floricultural importance. Modified terrestrial orchid medium proved the best in terms of germination in the dark and seedling development under 12-h photoperiod. For the differentiation of protocorms into seedlings, the cultures initially kept in the dark required exposure to a 12-h photoperiod. The results of the present study should facilitate future improvements in the *ex-situ* conservation of this vulnerable species and the restoration of *C. cordigerum* in its natural habitat. Further research is also needed on improving the *in vitro* and *ex vitro* methods for saving the germplasm of this horticulturally important species, especially the role of mycorrhiza in establishing symbiotic cultures, initiating *in vitro* flowering and acclimatization of *in vitro* cultures.

Acknowledgements

Financial assistance, from Department of Science and Technology, Government of India, New Delhi, India to senior author, is gratefully acknowledged. English language assistance from Mrs. Harjit Kaur Pabla is also acknowledged. The author also gratefully for the expert reviewers critical assessment of the manuscript, which improved the quality of this paper.

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