Mass propagation and conservation of Daffodil Orchid (*Ipsea speciosa*): An endangered endemic orchid

DLC Kumari[1], KKS Fernando[2], SDA Wijesundara[3],
[1]Department of Crop Science, Faculty of Agriculture, University of Ruhuna, Mapalana, Kamburupitiya,
[2]Seed Certification and Plant Protection Service, Department of Agriculture, Peradeniya,
[3]Royal Botanic Gardens, Peradeniya,

Abstract

Experiments were carried out to identify the correct maturity stage for in-vitro seed germination and to develop an in-vitro protocol for mass propagation of Ipsea speciosa an endangered orchid species. After hand pollination of the flowers, the pod length, girth and color changes of pods were observed at weekly intervals in order to determine the morphological indicators of maturity. For In-vitro seed germinatio , pods were collected 4,6,8 and 10 weeks after pollination on nine different media viz 1.MS 2. MS (Murasinghe & Shoog) medium 2. MS with PVP (2g/L) 3. MS with charcoal (2g/L) 4. KNC (Knudson C medium) 5.KNC with PVP (2g/L) 6. KNC with charcoal (2g/L) 7. KNC with banana extracts (75g/L) 8. V&W (Vacin & Went medium) 9. V&W with banana extract (75g/L) and coconut water (150ml/L) were tested. Growing rhizome tips and rhizome parts were used as explants in in-vitro vegetative propagation experiments and MS medium with charcoal (2g/L) and without charcoal were used as establishment media. After establishment, different combinations of NAA and BAP were tested to induce shoot proliferation. Three different potting media (1.sand: compost (1:1) 2. Coir dust 3. Compost: top soil (1:1) were tested for acclimatization of in-vitro raised plantlets. Survival rate was observed in weekly intervals.

Results revealed that seeds enclosed within pods collected eight weeks after pollination was the best stage for in-vitro seed germination of I. speciosa. 100% germination was observed on MS medium with PVP and charcoal, KNC with banana extract and V&W with banana extract and coconut water. Eight- week-old pods took 20 days to germinate whereas ten-week -old -pods took 60 days to germinate at a rate of 50%. Growing rhizome tips were identified as the best explant source for culture establishment on MS medium with charcoal. 0.5 mg/L BAP + 5mg/L NAA gave rise to the highest proliferation rate (1: 6). The most suitable potting medium for acclimatization of in-vitro raised plantlets was found to be compost and top soil (1:1) in which 80% survival rate was observed.

Key words: conservation, embryo culture, endangered orchids, mass propagation, tissue culture

Introduction

In Sri Lanka, Orchids (family - orchidaceae) are among prominent flora, which is adapted to a wide range of eco-climatological zones. However their existence have been endangered due to various pressures on the environment imposed by man. Orchids with showy flowers and with medicinal properties encounter an added disadvantage due to over-collection from the wild. Although, legislative measures play an important role the most effective measure to conserve orchids is to make available the species in the required amounts and reintroduce them to their natural habitats.

Ipsea speciosa was identified as an endangered species with medicinal properties. The suggested approach to preserve this orchid requires high capacity multiplication techniques to generate adequate number of plants. In the present study, the feasibility of in-vitro seed germination and in-vitro multiplication were as measures of rapid propagation of *I. Speciosa* for the proposed conservation plan.

Materials and Methods

Mother plants were collected from their natural habitats (Bandarawela, Loolkandura) after obtaining permission from Department of Wildlife Conservation and Forest Department. Information on Natural habitats of *I. Speciosa* were gathered by referring to the revised handbook to the "Flora of Ceylon" and IUCN the world conservation union maps.

(A)Morphological indicators of maturity

Flowers of *Ipsea speciosa*, which were maintained under green house conditions, were hand pollinated. Pod length, girth and visual observations such as color, appearance and texture were recorded at weekly intervals.

(B) Effect of the age of pod on germination

Pods were collected from the mother plant at different intervals (4, 6, 8, and 10 weeks after pollination). They were kept under running tap water for half an hour, follwed by shaking in 10% Clorox solution for 20 minutes inside a laminar flow cabinet. The pods were rinsed well with sterilized distilled water for three times. Then each pod was dipped in absolute ethanol and flamed. Pods were cut lengthwise and the seeds were scraped into the medium.

(C) Effect of media on germination

Table 1:Media tested for *in-vitro* germination

Basal media	Modification of the media
MS	1.MS
	2.MS+PVP (2g /L)
	3. MS + Charcoal (2g /L)
KNC	4. KNC
	5. KNC+ PVP (2g /L)
	6. KNC + Charcoal (2g /L)
	7. KNC + Banana extracts (75g/L)
V& W	8. V& W
	9. V& W + coconut water (100ml/L) +Banana extract (75g/L)

(D) *In- vitro* vegetative propagation of *I. speciosa*

The explants (Growing rhizome tips (1cm height) and rhizome parts (1cm ×0.5cm)) were shaken thoroughly in tap water containing a few drops of detergent (teepol) for 5 minutes. Then the explants were kept under running tap water for half an hour, followed by dipping in 1% (v/v) Thiophanate methyl (Topsin -A Fungicide) solution for half an hour in a laminar flow cabinet. Then explants were washed 2-3 times with distilled water and shaken in 10% Clorox solutions (with 2 drops of Polyoxyethelene sorbitan monolaurate (Tween- 20)), for 20 minutes. The explants were rinsed three times with sterilized distilled water folllwed by shaking in 70 % alcohol solution for 2 minutes. Then rinsed in sterile dinstilled water 3 or 4 times. Surface sterilized rhizome tips/rhizome parts were placed singly in culture bottles. MS medium and Ms medium supplemented with charcoal (2g/1) both in solid and liquid form were tested as establishment media. Cultures were incubated in light at 25± 2^0C. Growth response was scored at weekly intervals up to five weeks. (1= fair growth, 3= good growth, 5= very good growth)

After five weeks, the established cultures were transferred to MS medium with different concentrations of NAA and BAP. (0.5 mg/I BAP with 1,2,3,5mg/ I.NAA).Proliferation (number of plantlets produced) was recorded at two-week intervals up to six weeks.

(E) Hardening of the plantlets

Well- developed plantlets were removed from culture vessels and washed thoroughly with tap water. Dead leaves and roots were removed and plantlets were again washed with lukewarm water to remove agar completely. Then the plantlets were dipped in mild fungicide solution for 5 – 10 minutes. Plantlets were established in single propagators containing sterilized three potting media *viz* (1. sand: compost 1:1, 2. coir dust, 3. compost: top soil1: 1). They were maintained under shade and gradually exposed to normal environmental conditions. Survival percentage was recorded at weekly intervals.

All experiments were arranged as Complete Randomized Design and treatments were replicated four times. Data were analyzed using SAS software package. Growth response of *in-vitro* plantlets was analyzed using Kruskal-Wallis -one-way ANOVA non-parametric test.

Results and Discussion

(A) Morphological Indicators of maturity

Pods turned into dark green from light green with maturity. Increase in the length and girth of pods were observed during first four weeks after pollination (Plate 1,Table 2). Pod filling was observed afterwards and when the pods were fully matured the ridges of the pods became

prominent and turned into a purple color [plate 1 (e)].

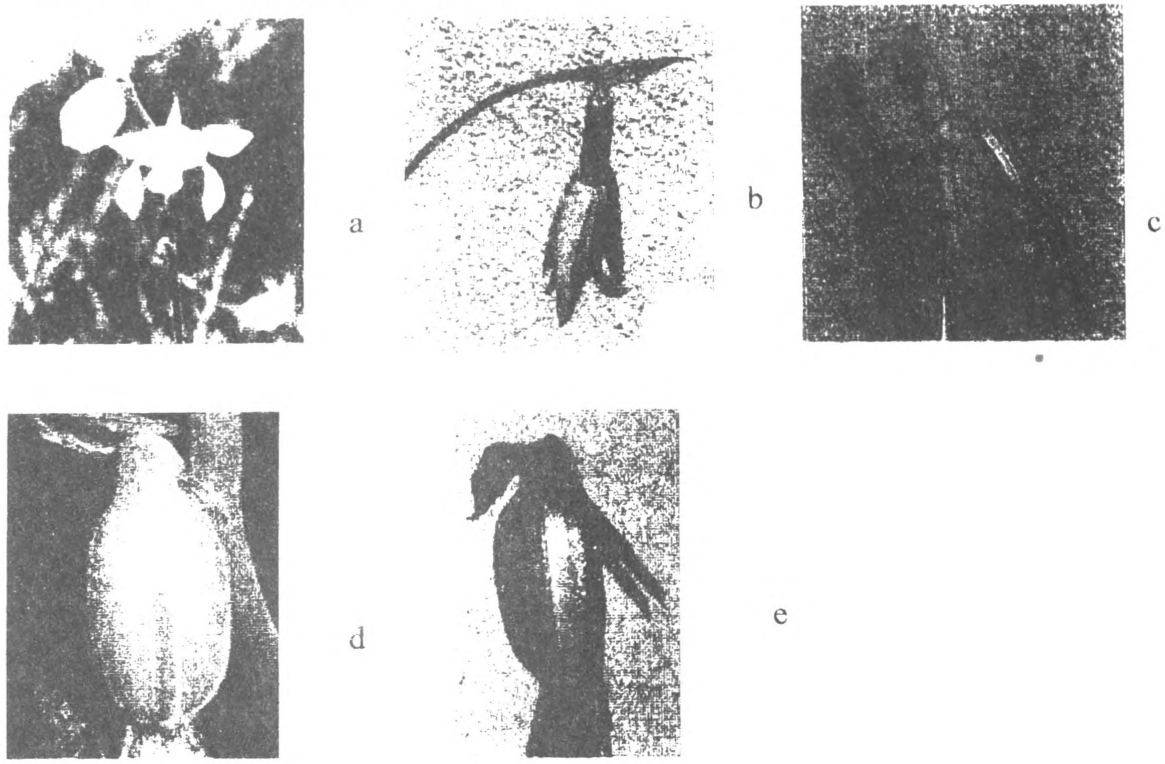


Plate1: Developmental stages of pods of *Ipsea speciosa*
(a=flower; b= pod just after pollination; c=growing pod; d=fully grown pod; e=fully mature pod)

Table2: Growth of pods of *I. speciosa* after pollinatin

Growth parameter	Weeks after pollination									
	1	2	3	4	5	6	7	8	9	10
Mean pod length (cm)	1.0	1.5	2.3	3.4	3.4	3.4	3.4	3.4	3.4	3.4
Mean girth (cm)	1.0	1.8	2.0	2.5	3.0	3.5	4.0	4.0	4.0	4.0

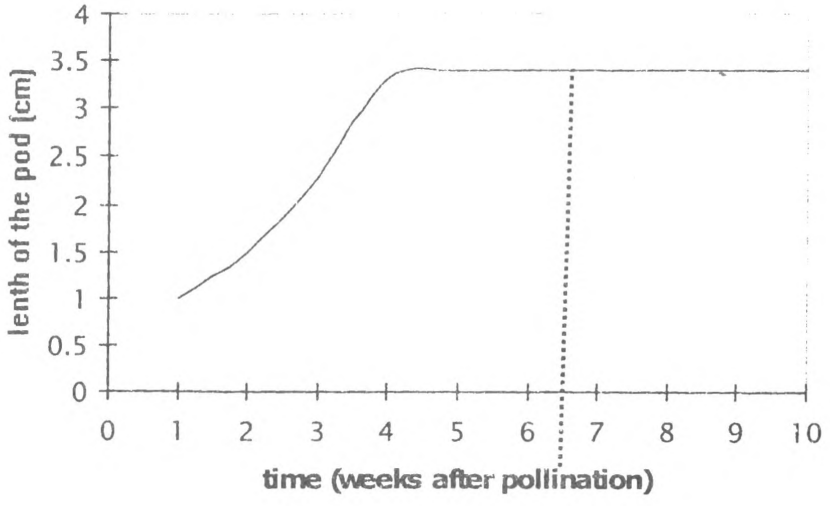


Figure 1:Variation in pod length of *I. Speciosa* at different stages of maturity

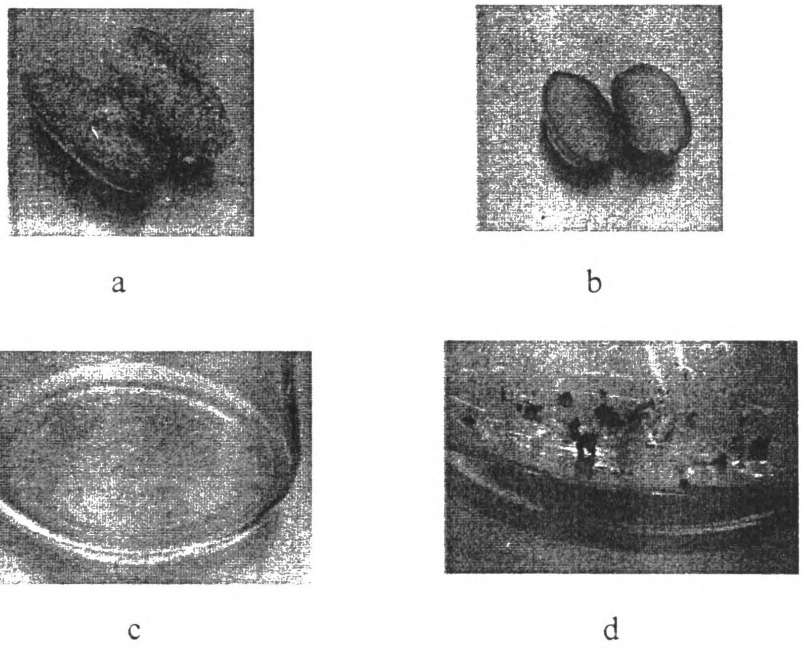
(B) Effect of the age of pod on germination

Eight weeks after pollination the seeds within the pod resembled a white powder. Seeds collected from 8 week-old- pod took 20 days to germinate. The seeds of the immature pods (4 & 6 week-old) gradually turned black and did not germinate (Plate 2-d). Addition of PVP and charcoal into the basal MS and KNC media did not show any improvement. Seeds of mature pods (10- week-old), which appeared as black powder, took 60 days to germinate.(Table 3)

Table3: Germination of seeds excised from pods of different stages of mutury

Age of pod after pollination (weeks)	Germination %	Time taken for Germination (Days)
28	0	30
42	0	30
56	100	20
70	50	60

Observations revealed that the seeds obtained from pods that develop prominent ridges along the valves and stop their growth (diameter and length) respond better. This is useful in selecting the right stage of pod for embryo culture. Vij, (1984) observed similar results for *Rhynchostylis retusa.*



a b c d

Plate2: (a=seeds of mature pod –8week-old; b= seeds of immature pod-6-week-old; c= immature seeds on culture media; d=blackening of immature seeds)

(C) Effect of media on germination

Percentages of germination varied with different media tested (figure2).

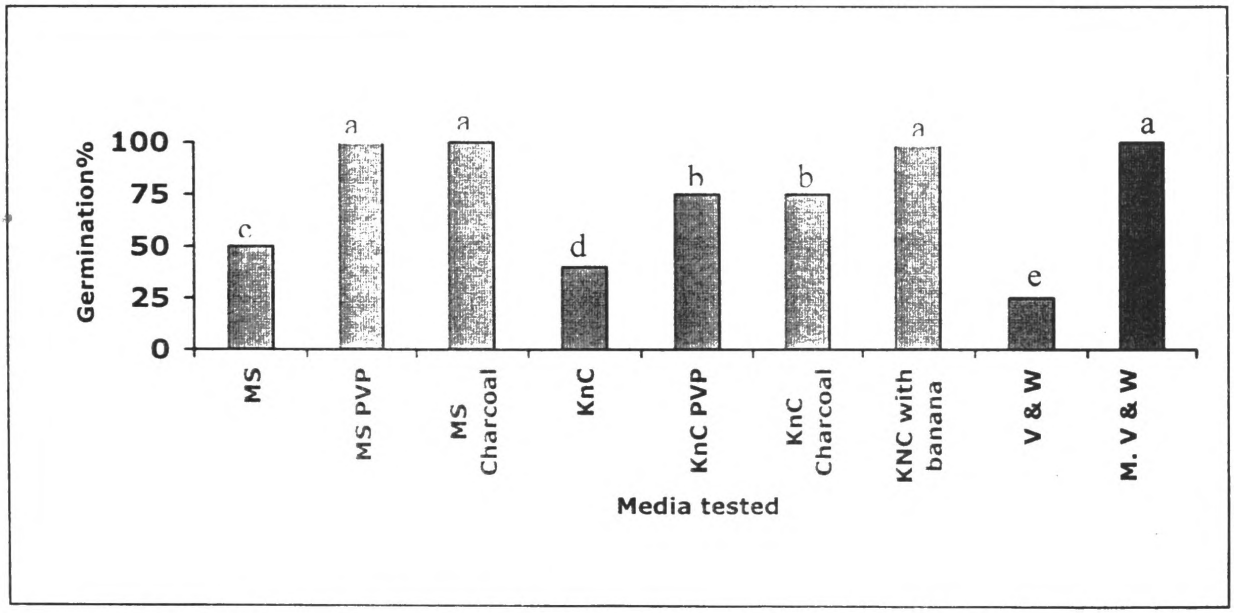


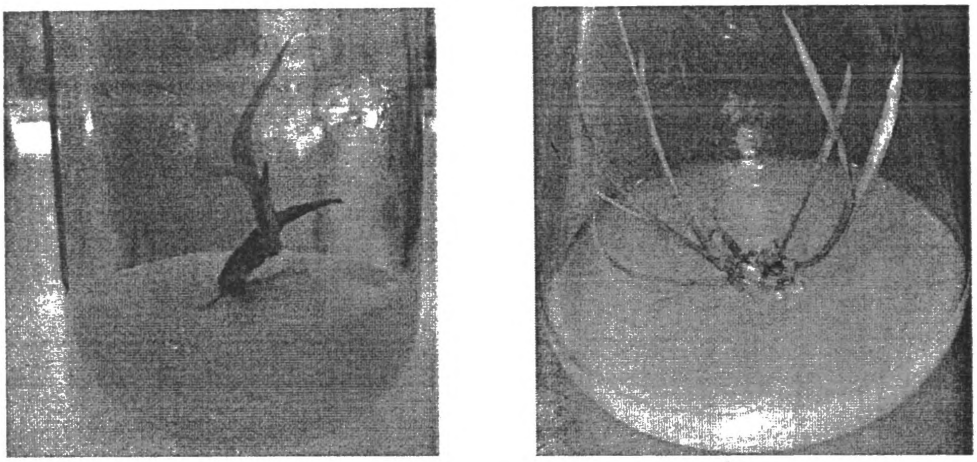
Figure2: In vitro seed germination of I.speciosa in different culture media.

Bars with similar letters are not significantly different.

Germination percentages were significantly higher ($p<0.05$) in MS+PVP, MS+Charcoal, Modified V & W and KNC + Banana extract media than other media tested. 100% germination was recorded when seeds were excised from pods eight weeks after pollination. Bajaj *et.al.*, (1977) achieved 90 percent germination and maximum growth of protocorms in 60 days in *Ipsea malaberica* cultured in liquid nutrient medium supplemented with 0.05 % (w/v) casein hydrolsate. Media containing activated charcoal and banana extracts enhanced germination. Addition of activated charcoal and banana extract did not improve germination of immature seeds. Vij & Sharma, (1996) also reported that selection of right age of the pod has an important role for early and increased seed germination besides other physicochemical factors. Bajaj (1977) tested the growth- promoting properties of different fruit extracts. Banana extract was found to be most effective when compared to extracts of pineapple, papaya and mango. Rao, (1964) reported the synergistic effect of banana extract and coconut water on seed germination and protocorm development of orchids.

(D) *In –vitro* vegetative propagation of *I. speciosa*

It was observed that rhizome parts are not suitable as explants due to high contamination in all media tested. One month after culturing, all cultures were dead.



a b

Plate3: (a = A growing rhizome tip, b= Proliferated plantlets of *Ipsea*)

Growing rhizome tips showed better results when cultured on solid media. When cultured on MS+ charcoal medium, plantlets showed better growth and appearance (According to the Kruscal- Wallis non parametric data analysis).

Ipsea speciosa being a ground orchid, survival rate after culturing was very low both in solid and liquid media tested. For rhizome parts survival rate was 0 for liquid culture. When agitating the explants the endogenous bacteria came out an extremely high contramination rate was observed. 70% survival was observed in solid media tested. According to Krusgali-Wallis none parametic analysis, appearance of growth is high in MS medium supplemented with activated charcoal. Adding activated charcoal to the medium provides dark conditions for root and rhizome development. This will enhance the arial growth of the plant. Bose *et.al.*,1976, observed that activated charcoal appeared to be necessary for producing healthy plantlets and for stimulating shoot and rhizome growth of ground orchids at a level of 0.1-0.3%.

Plantlets were transferred to proliferation media containing different concentrations of NAA and BAP. 5mg/l NAA+ 0.5mg/l BAP gave rise to higher rate of proliferation when compared to other treatments (1:6). A higher rate of survival was observed in plantlets hardened in compost and topsoil when compared to the other two potting media.

Table 4: Survival rate of in-vitro raised plantlets during hardening

Survival % / Media	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Sand: Top soil 1:1	70	60	50	50	50	50
Coir dust	70	60	40	40	30	30
Compost: Top soil 1:1	90	90	90	80	80	80

Conclusions

Correct maturity stage has to be identified for *in-vitro* seed germination of *I. speciosa.* Eight weeks after pollination was the correct maturity stage and eight-week-old seeds took 20 days for *in vitro* seed germination.

MS medium containing charcoal (2g/L) and PVP (2g/L) and V & W medium containing banana extract (75g/L) and coconut water (100ml/L), KNC medium containing banana extract (75g/L) increased the *in vitro* seed germination (100%) of *Ipsea.*

Rhizome tips were found to be suitable as a source of explant for in-vitro vegetative propagation studies.

Acknowledgement

The first author is thankful to Department of Plant Sciences, University of Colombo, for granting her postgraduate research study.

References

Fernando, M., Wijesundara, S. and Fernando, S. (2003) Orchids of Sri Lanka - A conservationist's Companion, A simplified guide to identification (Volume 1) published by IUCN Sri Lanka.

Murashige, T. (1974). Plant propagation through tissue culture. Annual Review. Plant Physiol.25, 135-66

Bose,T.K and Mukherjee,T.P.,(1976) Effect of growth substances on growth, protocorm and plantlet formation in seedlings of Cymbidium giganteum, Orchid Review, 84:107-108

Bajaj Y.P.S. Reinert, J and (1977). Aapplied and Fundamental Aspects of plant Cell, Tissue, and Organ culture (Eds), Springe, Berlin Heidelber New York, Pp 45-65.

Vij, S.P., Sood, A. and Pathak, P., 1989 On the utility of rhizome segments for micropropagating *Eulophia hormusjii* Duth. Journal of orchid society. India 3: 41-45.

Vij, S.P., Sood, A Sharma, M., and Plaha K.K., 1984. Proporagtion of *Rhynochostylis retusa* Bl. (Orchidaceae) by direct organogenesis from leaf segments. Bot. Gaz. 145:210-214

Rao, A.N., (1991). Post-Independence addition to the orchid flora of India with a particular reference to Arunachal Pradesh- a review, J. Orchids Soc. India 5:29-41

Dassanayake, M.D. and Fosberg, E.R., (1981) Flora of Ceylon: a revised handbook, Volume ii, Amerind Publishing Co. Pvt. Ltd., New Delhi