In Vitro Seed Germination of Gastrochilus calceolaris (Buch. -Ham ex J.E.Sm.) D.Don., A Critically Endangered Orchid of Nepal

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ABSTRACT

Gastrochilus calceolaris, an epiphytic orchid, has a great potential for commercial exploitation for beautiful flowers, due to over exploitation the species has been placed as critically endangered in IUCN red list. Orchid requires a combination of different factors for reproduction and has unique physiology of germination. However, the large numbers of seeds are produced but due to the lack of endosperm and few embryo formation, cannot germinate in the nature. The objective of this study was to identify the best medium and organic supplements for seed germination and plantlets development for reintroduction in the nature. Immature seed of this orchid species sowed in Murashige and Skoog (MS) viz; Full strength MS (FMS) and half strength MS (HMS) medium supplemented with coconut water (10%) showed various responses in germination and seedling development. Optimum seed germination was achieved on full strength MS medium supplemented with 10% coconut water. In vitro germinated seed were transferred to full strength of MS medium supplemented with various concentration of NAA and BAP for shoot and root development. Maximum shoot proliferation was obtained in MS medium and root proliferation was better in MS supplemented with 1 mg/l NAA. The present study could be useful for standardizing the protocol for mass propagation of the critically endangered orchid Gastrochilus calceolaris

INTRODUCTION

Orchidaceae belongs to the most diverse plant family known to humankind. Orchids are grown primarily as ornamental purpose because of their persistent flowers and are globally used in traditional herbal medicine (1, 8, 14, 15). Many orchid species are threatened globally by over collection from the natural habitat for horticultural and medicinal purpose (27). Orchid seed lacks endosperm and requires special fungal partner for germination (14, 22, 24, 25). The seeds of the orchids, produced in large numbers in each capsule, are without endosperm. They require specific fungal partner for germination naturally. Due to the reason population of the orchids are gradually declining and need an efficient strategy to save these beautiful members of the plant kingdom. Thus asymbiotic seed germination using micropropagation techniques plays an important role in conservation programs and sustainable use of orchids (6, 17) Gastrochilus calceolaris (Sm.) D. Don is synonymously known as Aerides leopardina Wall. ex Lindl., Saccolabium calceolare (Sm.) Lindl. And Sarcochilus nepalense Spreng. distributed in Nepal, China, India, Thailand and Malaysia. In Nepal, it is distributed to the elevation of 1500 m to 2200 m of temperate region (21, 23). It is famous for its immensely beautiful flower and has an immense potential to serve as a reputed parent in hybridization for producing elite genotypes. The over consumption and harvesting of this species from natural habitat leads to near extinction, so that this species was listed as critically endangered in IUCN red list. (9, 28). Because of the restricted population size and expected medicinal properties of G. calceolaris, it is required to develop an effective conservation strategy to save this plant for future. Plant tissue culture technique provides an opportunity to preserve and commercialize the number of rare and threatened orchid species (7, 10, 17). Hence, the present study was carried out to standardize the protocol for mass propagation and ex situ conservation of G. calceolaris a critically endangered orchid of Nepal.

MATERIALS AND METHODS

1) Sample collection

Immature capsules of Gastrochilus calceolaris were collected from the Dolakha district of Central Nepal at altitude of 1600m with latitude 27.7784° N and longitude 86.1752° E. They were used as explant to produce in vitro seedlings. The plant was identified by the taxonomist Prof. Dr. Keshav Rajbhandari and Prof. Dr. Bijaya Pant, which was cross referenced after with literatures.
2) Sterilization and inoculation
The capsules were cleaned with 1 - 2 drops of Tween 20 and washed with running tap water for at least half an hour. The capsules were then dipped in 1% sodium hypochlorite solution for 15 minutes and submerged in 70% ethanol where they were rapidly set a flame for a few seconds. The capsules were then rinsed three times in sterile water. The surface sterilized capsules were then cut longitudinally with a sterile scalpel and the exposed seeds were removed with forceps and transferred to an agar gel (0.8% per liter) nutrient medium for seed germination. Different strength of MS medium with growth hormones were used to germinate the seeds and germination rate is calculated as below.

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\% \text{ seed germination} = \frac{\text{Number of seed forming spherule}}{\text{Total number of seeds}} \times 100
\]

3) Culture condition
The pH of the medium was adjusted to 5.7 - 5.8 by adding 0.1N NaOH or 0.1N HCl before agar was added. The medium was autoclaved at 15 psi and 121°C for 20 minutes. Cultures were incubated at 24°C ± 1°C provided with 1000 lux illumination by cool white fluorescent light of 16 hours’ light and 8 hours’ dark regime.

4) Protocorms formation
Based on the varying effects of basal media on maximum asymbiotic seed germination, different medium was selected to further examine the effect of plant growth regulators (PGR) on protocorm multiplication and subsequent plantlet development. Fully germinated greenish seeds were cultured in fortified MS medium (12) with the (IAA), (BAP) and kinetin (Himedia Laboratories, Mumbai, India) individually and in several combinations. Each treatment consisted of 6 culture jar with 50 ml medium replicated thrice and in each tube protocorms were inoculated.

5) Shoot and root proliferation
The 16 weeks old protocorms that developed on the MS medium (average of five each protocorms about 0.3 mm to 0.4 mm diameter were sub- cultured on different strength and hormonal combinations of MS medium. Each hormone concentration was tested both alone and supplemented with 10% CW. The sequential growth and proliferation of G. calceolaris was then evaluated. Each culture medium had 4 replicates. After 16 weeks of in vitro grown shoots were transferred in different strength of MS medium with rooting hormones like IBA and NAA for maximum growth of roots. The in vitro growth conditions of light, temperature maintained similar as shoot culture.

6) Statistical analysis
Average value of week was taken for in vitro seed germination. Data of shooting and rooting were presented as mean and standard error. Data were analyzed using Microsoft excel 2016.

RESULTS
The seeds from immature pods of G. calceolaris cultured on full and half strength MS medium, alone and along with or without coconut water, IBA and fungal elicitors, showed various responses. The first visible sign of germination was observed as the swollen yellowish green bulb like protocorms within 8 weeks after showing (Fig 2D).

Among all treated media, the maximum seed germination was observed in MS supplemented with 10% CW. In this condition, the seed germination started at early 6th weeks of seed inoculation. White cottony mass of seeds during the inoculation were found to be lacking embryos which metamorphoses in to yellowish globular structure (Fig 2). Similarly, MS basal medium, MS with 10% CW and MS with fungal elicitors combination media were also found suitable for seed germination respectively 70%, 40% and 20% whereas HMS with 10% CW had the least germination rate i.e., only 2%.
Fig. 3 The rate of seed germination in different combination of medium with MS and HMS with coconut water, hormones and fungal elicitors. The highest percentage of seed germination were found in MS+10%CW water however least percentage was found in HMS+10%CW.

1) Shoot proliferation from protocorms
For the development of shoots of *G. calceolaris* the 16 weeks old protocorms measuring 3 mm - 4 mm in diameter were sub-cultured on FMS and HMS medium supplemented with CW, BAP, NAA. Within four weeks of culture the protocorms in all treatments undergo multiplication and exhibit leaf primordia. The most effective medium for shoot multiplication were FMS medium and FMS supplemented with 10% CW (Fig. 2E). Both shoot number and shoot length were found comparatively higher in FMS and FMS 10% CW medium than other treated medium (Fig. 4). The maximum shoot number and shoot length was 4.7 and 1.8cm and 4.1 and 1.6 cm respectively. However, other media also responded but comparatively less shoot proliferation.

Fig. 4 shoot proliferation in different nutrient media (Best condition FMS followed by FMS+10%CW).

2) Root proliferation medium
Fully grown shoot were transferred to the medium with different rooting hormones for root proliferation. In present study, the FMS medium with 1 mg/l NAA was found most effective than the medium with other rooting hormones. The maximum average root number was 2.8 with the root length 1.93 cm (Fig. 2F and 3). The root proliferation was lower in MS with IBA and HMS with NAA and IBA. However, the least mean root number and length was reported in control FMS medium and HMS (Fig. 5).

Fig. 5 Root proliferation in different nutrient media (Best condition FMS+1NAA).

DISCUSSION
The past decades have magnified the role of *in vitro* techniques in plant conservation efforts. This is largely due to the rapid decline and threats to the world's biodiversity. The importance of *ex situ* conservation is becoming a key element of modern day conservation strategy due to increasing urbanization, population growth and industrialization (13, 19). *Ex situ* conservation of endangered orchid species is essential, as their rates of propagation in natural habitat is very complex. In case of the declining population of *G. calceolaris* from the present study it has been found that even mature seeds of the selected species are found lacking embryo. This condition has created a barrier in the natural propagation of the *G. calceolaris*. In the present study it reported most of the seeds inoculated were without embryo, which nearly took 3 to 4 weeks for the development of embryo *in vitro* (Fig. 2A, 2B). Thus for the *ex situ* conservation for the Nepalese endangered orchid *G. calceolaris* through its mass propagation, it is possible through *in vitro* seed germination.

Among all treated conditions, Full MS and FMS supplemented with 10% CW was found to be the best one, for the germination induction of protocorm shooting whereas FMS supplemented with 1 mg/l NAA was found to be best for the proliferation.

Similar results of the use of elicitors like CW promoted overall germination, protocorm formation and seedling development in *G. calceolaris* were
already reported (18). Others also supported the same enhancement for early and highest germination and protocorm formation in medium with 15% CW rather than basal for *Acampe papillosa* (20) and 20% CW for *Smithsonia maculata* (5).

For the stage of shoot proliferation, among the tested conditions, Basal full-strength MS media showed compelling result with highest shoot no. and length along with MS media supplemented media with 10% CW. The cultured PLBs to develop into seedlings was found to be best in PGR free medium as also supported with the study by Winarto *et al.* (26) but in contrary to the study by Mahendran & Bai (11).

Rooting was supported by MS medium with 1mg/L NAA as auxin. But Mitra medium among PDA, Mitra and MS tested containing BAP (1mg/l-1) in combination with NAA (0.5mg/l-1) supported rooting for *Eulophia dabia* (4). Rooting of *Calanthe densiflora* and *Cymbidium irridoides* was found best when cultured on MS with IBA (2, 16), while most of the *Cymbidiums* needed NAA for rooting (3).

Additional use of the growth additives like Coconut water favored better germination early protocorm induction and seedling development. Different growth regulators and hormones were used for the multiplication of the protocorm but best rate of the multiplication was found better in FMS alone and FMS associated with coconut water. Addition CW (10%) has proved to have a beneficial role in the germination and protocorm induction along with FMS.

Thus propagation protocol for the critically endangered *G. calceolaris* was successfully established and standardized.

**CONCLUSIONS**

The present investigations suggest that in *Gastrochilous calceolaris* can be efficiently proliferated in vitro. Having high horticultural importance, the present protocol for *G. calceolaris* is of much significance being rapid and leading to a large number of clones. Using it would help to minimize the pressure on the natural population of threatened but commercially important medicinal orchids and result in their sustainable utilization.

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**REFERENCES**


Carbohydrate Partitioning between Pseudobulb and Inflorescence during Reproductive Growth of Oncidesa Gower Ramsey ‘Honey Angel’

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ABSTRACT
Oncidesa possesses pseudobulbs as the storage organs which are considered to supply nutrition for the growth of inflorescence. However, since the growth of pseudobulb and inflorescence occurs at the same time, pseudobulb might compete nutrition with inflorescence. The sink-source relationship between pseudobulb and inflorescence is of great interest but is still unclear. The carbohydrate status of various inflorescence developmental stages of Oncidesa Gower Ramsey ‘Honey Angel’ was thus investigated in this study. The pseudobulb contained high content of soluble sugars, at the beginning of inflorescence development. Then it decreased rapidly in pseudobulb when the florets opened. Interestingly, the content of polysaccharides (reserves carbohydrates) in pseudobulbs were lower than soluble sugars at the beginning of inflorescence development stage when the pseudobulb was the largest size. The consumption of soluble sugars and water-soluble polysaccharides (WSPs), and the accumulation of starch might indicate that soluble sugars and WSPs are the temporary form of carbohydrate in pseudobulb. Since inflorescence started to grow rapidly in the late period of pseudobulb enlargement, carbohydrates accumulated as soluble sugars and partial WSPs in pseudobulb were more easily used. Accumulation of starch in the pseudobulb during inflorescence growth might be the carbohydrate source for the growth of new shoots of the next generation.

INTRODUCTION
Oncidesa is one of the most important floral crops for cut flowers. Oncidesa is a sympodial orchid and the growth cycle of each generation was divided into bud stage (S0), plantlet stage (S1), pseudobulb unsheathing stage (S2), and pseudobulb with/without inflorescence stage (S3) (3). The enlarged stems were called pseudobulbs for nutrients storage. The nutrients which were stored by pseudobulb included carbohydrate, inorganic mineral compound, and water (4). The pseudobulb enlarged at S2, and inflorescence rapidly elongated and flowered at S3. The storage nutrients in pseudobulb then supplied the growth of inflorescence and flowering. Although inflorescence growth rapidly after pseudobulb enlargement, inflorescence started to develop at the very early stage of current shoot growth. The nodes and branches of stalk differentiated before S2, and the florets primordia appeared at S2 (6). There was an overlap between pseudobulb enlargement and inflorescence development. For the carbohydrate partitioning at this stage, there was 28.9% of photosynthesis assimilate partitioned to current pseudobulb at S2, and it decreased to 11.0% at S3 when inflorescence was mature. While the photosynthates partitioned to inflorescence increased from 56.5% to 61.8 % (9). Generally, pseudobulb was thought to supply nutrient for the growth of inflorescence. However, pseudobulb competed the carbohydrate with the inflorescence at the early stage of inflorescence development. The objective of this research was to investigate the carbohydrate partitioning between pseudobulb and inflorescence and the change of carbohydrate storage form during the inflorescence development.

MATERIALS AND METHODS
1) Plant materials
Oncidesa Gower Ramsey with different inflorescence developmental stages were collected from grower in Taichung, Taiwan (Fig. 1). The developmental stages of inflorescence were the length of inflorescence at 10 cm (stage 1), 35 cm (stage 2), 65 cm (stage 3), inflorescence well branched (stage 4), and 50% florets open (stage 5). There were 10 replications each developmental stage.

2) Data collection
The length, width, height, and circumference of current pseudobulb, the length, branches number of inflorescences were measured. The current shoot was separated into leaf, pseudobulb, stalk and florets for fresh weight, dry weight measurement, and carbohydrate analysis.

RESULTS
At the early stage of inflorescence development of Oncidesa Gower Ramsey ‘Honey Angel’, the inflorescence was 10 cm (stage 1), and the
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Fig. 1 Inflorescence development of *Oncidusa Gower Ramsey* ‘Honey Angel’. Stage 1-5 represent inflorescence length at 10, 35, 65 cm, inflorescence well branched, 50% florets open, respectively. Bars indicate standard error of the mean (n=10).

The pseudobulb had not expanded to the largest volume. The pseudobulb kept expanding until the length of inflorescence reached 35 cm (Table 1). Then the volume of the pseudobulb slightly decreased and the surface of pseudobulb wrinkled and the circumference of the pseudobulb decreased while inflorescence developing from 35 cm to 50% florets opened (stage 2 to 5). It was due to the decrease of water in the pseudobulb. As the inflorescence growth, the dry weight slightly increased but the water content significantly decreased (Table 1). As the pseudobulb reached its maximum size, the inflorescence had already developed to 35 cm. At this stage, the branches, and florets primordia had been differentiated (6). Obviously, pseudobulb and inflorescence developed simultaneously.

Monosaccharides content slightly increased while inflorescence developed from 10 cm (stage 1) to 35 cm (stage 2) when the pseudobulb reached its maximum size (Fig. 2A). The monosaccharides content was three times higher than that of polysaccharides when the pseudobulb at the largest size (Fig. 2B), then the content of soluble sugar decreased along inflorescence development. The water-soluble polysaccharides (WSPs) also decreased as inflorescence development. Despite the extremely change of the monosaccharides, sucrose content maintained stable in the pseudobulb (Fig. 2A). On the other hand, as other carbohydrate was consumed during the inflorescence growth, starch continued accumulated in pseudobulb (Fig. 2B).

In the inflorescence, concentrations of soluble sugars and polysaccharides decreased because the dilution effect of inflorescence rapidly growth (data not shown). On the other hand, carbohydrates content increased during stage 1 to 5. Sucrose steadily increased during inflorescence growth (Fig. 3A). Polysaccharides showed higher increasing rate after the florets formation (Fig. 3B). While the monosaccharides increased to a certain level at the beginning and rapidly rose when florets opened (Fig. 3A). Divided the inflorescence into stalk and florets. The data showed that rapid increase of monosaccharides occurred in florets (Fig. 3C,3D).

Table 1 Changes of dry weight, water content, circumference of the pseudobulb and inflorescence length of *Oncidusa Gower Ramsey* ‘Honey Angel’ during reproductive growth.

<table>
<thead>
<tr>
<th>Development stage</th>
<th>DW of pseudobulb (g)</th>
<th>Water content of pseudobulb (%)</th>
<th>Pseudobulb circumference (cm)</th>
<th>Inflorescence length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>2.19 b z</td>
<td>93.8 a</td>
<td>9.0 a</td>
<td>11.5 e</td>
</tr>
<tr>
<td>S2</td>
<td>2.49 ab</td>
<td>93.6 a</td>
<td>9.0 a</td>
<td>37.9 d</td>
</tr>
<tr>
<td>S3</td>
<td>2.70 a</td>
<td>93.4 a</td>
<td>9.3 a</td>
<td>67.3 c</td>
</tr>
<tr>
<td>S4</td>
<td>2.85 a</td>
<td>92.1 b</td>
<td>8.7 ab</td>
<td>107.3 b</td>
</tr>
<tr>
<td>S5</td>
<td>2.69 a</td>
<td>91.4 b</td>
<td>8.1 b</td>
<td>113.7 a</td>
</tr>
</tbody>
</table>

z Means within the same column followed by different letters indicated significantly different at $P \leq 0.05$ by the least significance difference test; n = 10.

![Fig. 2 Changes of soluble sugar content (A) and polysaccharides content (B) in current pseudobulb during reproduction growth. Bars on the dots represent standard error of the means, n = 10.](image-url)
DISCUSSION

Pseudobulb played as an important storage organ in orchids. Not only the carbohydrate, pseudobulb also stored large amount of water and inorganic nutrients which makes orchid can survive as an epiphyte plants (4). During the reproductive growth, although there was significant change of the carbohydrate status (Fig. 2), the dry weight of the pseudobulb still increased (Table 1). The major appearance change on pseudobulb was the wrinkle on the surface and significant decrease of fresh weigh, which we can calculate by the change of water content and dry weight (Table 2).

As the long-term carbohydrate reserved form, starch is not soluble in water, and not easily used by plant when it compared to soluble sugars. And as it mentioned above, pseudobulb and inflorescence basically developed at the same time. The similar situation also shown in the bulb of tulip (5). When tulip started accumulated carbohydrate in the bulbs, sucrose was the accumulated form then the degree of polymerization increased to form fructosylsucrose. At the end, the reserved form turned into starch.

Large amount of monosaccharide can be the regulator of water potential of the plant tissue. It is one of the flower open mechanism, flower opened through hydrolyzed the reserves polysaccharides into monosaccharides or the uptake of sucrose (7). The same water potential regulation processes could be found in carnation, daylily, and Dendrobium (1, 2, 8). In this study, large amount of monosaccharide accumulated as the expansion of pseudobulb (Fig. 2A) and also in the opened florets (Fig. 3D), and the carbohydrate reserved form in the pseudobulb turned into starch after the pseudobulb was no longer expanded (Fig. 2B). The increased of the monosaccharides not only because of the temporary need of carbohydrate for inflorescence development, also may be the water potential regulation for storing water in pseudobulb.

CONCLUSION

Oncidessa Gower Ramsey ‘Honey Angel’ accumulated monosaccharides when pseudobulb expanded. Then reserved form turned into starch after the expand of pseudobulb. The reasons that pseudobulb tended to accumulate monosaccharides at the very first time of inflorescence development may be the rapid need of carbohydrate supplement of inflorescence growth and the water potential regulation of the pseudobulb.

REFERENCES