CIBTech Journal of Biotechnology ISSN: 2319–3859 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/cjb.htm
2016 Vol. 5 (3) July-September, pp.54-60/Sil and Kumar De
Research Article

IN VITRO MASS PROPAGATION OF AN ENDANGERED, ORNAMENTALLY AND MEDICINALLY IMPORTANT ORCHID, *COELOGYNE FLACCIDA* LINDL. THROUGH SHOOT TIP CULTURE

Sujit Sil and *Kalyan Kumar De
Department of Botany, Hooghly Mohsin College, Government of West Bengal, Chinsurah -712 101, Hooghly, West Bengal, India
**Author for Correspondence*

ABSTRACT
The present study develops an efficient and reproducible protocol for rapid *in vitro* mass propagation of a critically endangered and floriculturally as well as medicinally most important epiphytic orchid, *Coelogyne flaccida* Lindl. through the culture of small shoot tip explants (0.3 to 0.5mm) derived from in vitro grown seedlings. The shoot tip explants cultured on 0.8% agar solidified Orchimax medium enriched with 2gm l^{-1}tryptone and added with 1gm l^{-1} morphilino ethane sulphonic acids. Orchimax media alone or supplemented with combination of various concentrations of growth regulators: 6-Benzylaminopurine, BAP (0.5 to2.0 mg l^{-1}) and α-naphthalene acetic acid, NAA (0.5 to 1.0 mg l^{-1}) produced shoot and multiple shoots through direct formation of protocorm-like bodies without forming intervening callus tissue. Among the different combinations and concentration of BAP and NAA tested in this study, BAP (1.5 mg l^{-1}) and NAA (0.5 mg l^{-1}) were found to be effective for the shoot multiplication. The well developed in vitro rooted plantlets were hardened successfully in the potting mixture. Nearly 83% of plantlets successfully acclimatized and survived.

Keywords: *Coelogyne flaccida, Shoot Tip, Micropropagation, Orchimax Medium*

INTRODUCTION
Coelogyne flaccida Lindl. is a cool growing, evergreen sympodial epiphytic orchid species. It is one of the prettiest of the coelogynes possessing heavily scented flowers. Biogeographically, it extends from India to China in the tropical to sub-tropical Himalayas at an altitude of 900-2300 m (Clayton, 2002). The pseudobulbs of this orchid bear a pair of linear, lanceolate leaves at the apex. Inflorescence is pendulous recemose type arising from the base of pseudobulb. Long lasting flowers (3-5 cm across) are white with yellow on the middle of the lip, stripped red in the side lobes and spotted red at the base of the middle lobe. The species bloom simultaneously from spring to early summer months. The beautiful flowers of this orchid have high ornamental value as a cut flower which has made it popular. Besides their horticultural importance, the genus *Coelogyne* in general, has some medicinal value. It is mainly used for the treatment of tuberculosis, but different species of *Coelogynae* have some other uses in herbal medicine for example pharmacological studies have shown that the pseudo bulb and leaf of *Coelogyne flaccida* possess an anti-bacterial activity and the paste of its pseudobulbs is applied to the forehead to treat headache and their juice is also administered orally to treat indigestion (Rajbhandari and Bhattarai, 2001; Pyakurel and Gurung, 2008). Biologically active compounds (Phenenthrene derivatives) such as flaccidin and oxaloflaccidin are isolated from whole plants of this species (Majumdar and Maiti, 1989). Another active principle isolated from this orchid is a new type of stilbenoides designated as callosin whose chemical structure was active established as 2, 6 dihydroxy-4, 7 dimethoxy-9, 10 dihydrophenanthrene (Majumdar *et al.,* 1995).
Uncontrolled collection and habitat destruction have led to drastic reduction in number of orchids in India (Pradhan, 1985). *C. flaccida* is also faced with habitat destruction pressures which far exceed its natural regeneration. Beside this, orchids are very slow growing plants which has added another to their being rare and endangered and become threatened in its natural habitats (Bailes, 1985; Wu *et al.,* 2009). The genus *Coelogyne* is included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2012). Therefore, to overcome the danger of extinction of such

horticulturally and medicinally important orchid, it is strongly emphasized to conserve, propagate and multiply the species, through tissue culture techniques and to popularize the species among amateurs, nurserymen and professionals to cultivate the species thus saving its wild populations from getting extinct.

Orchids are propagated in nature by vegetative means as well as through seeds. The rate of vegetative propagation are very slow and seed germination in nature is also very poor i.e. 0.2% -0.5%. In clonal mass propagation, it is immensely important to maintain genetic uniformity of *in vitro* raised progenies. In out breeding taxa like orchids, seed raised progenies are extremely heterozygous. So, to retain genetic integrity of the regenerants, appropriate *in vitro* propagation protocols are required. Orchids are generally propagated in vitro by using various explants such as shoot meristem, leaves, roots, protocorms etc. Among them, the utility of shoot tip containing apical meristem is most effective method for clonal propagation because this method provides opportunities to produce a large number of true-to-type plantlets of interest. Therefore, in the present communication, an attempt was made to establish an efficient and reproducible one step protocol (without any intervening callus tissue formation) for the production and multiplication of large number of genetically uniform plantlets in a short time directly through the formation of protocorm like bodies (PLBs) from in vitro cultured shoot tip used as explants for *C. flaccida* in terms of conservation of such endangered and threatened species and to re-introduce this species in natural habitat.

MATERIALS AND METHODS

The plant materials were collected from their natural habitat of Kalimpong area (1247 m above sea level, Latitude 27.06°, Longitude 88.47°) of Darjeeling hill which is a part of Eastern Himalaya.

For shoot multiplication, the shoot tip as explants of size 0.3 to 0.5 mm were cut aseptically from 250 days old aseptic plantlets raised from *in vitro* asymbiotic seed germination technique. Inside a laminar air-flow cabinet, under aseptic conditions the plantlets were removed from the culture vessels and their leaves and roots were severed off without making any injury. Single shoot tip from the apical portion of individual peudobulb was isolated and inoculated on Orchimax medium enriched in 2gm l^{-1} tryptone (Duchefa Biochemi BV; The Netherlands, Cat. No. 0 0257) in 80 culture tubes (each tube containing 20 ml of medium).

Tryptone provides an additional source of reduced organic nitrogen, vitamins and nutritional agents. 1gm l^{-1} of MES (morphilino ethane sulphonic acids) was also added as a buffer (pH range of 5.5-6.7) in the medium to prevent acidification during culture. Orchimax media alone or supplemented with combination of various concentrations of growth regulators: 6-Benzylaminopurine, BAP (0.5 to2.0 mg l^{-1}) and α-naphthalene acetic acid, NAA (0.5 to 1.0 mg l^{-1}). The final pH of the medium was adjusted to 5.8 before adding agar (0.8%) and sterilized at 15 kps at 121^{o}C for 15 minutes. After every 30 days of culture, either explants or the protocrom- like bodies or regenerated rootless shoots were sub-cultured regularly on the same fresh medium for further growth.

After inoculation, the cultures were kept in control room and exposed to artificial light (fluorescent light) giving 2659 µmol m-1 s-1 culture level with a light/dark cycle of 16/8 h at 25±2°C at 50-60% relative humidity. After regeneration of plantlets with roots from shoot tip explants, they were finally sub-cultured in ½ strength Orchimax macro and micro elements without supplementation of any hormone and sugar. On reaching a height 60-65 mm, the plantlets with three or four well-developed roots were taken out of the culture, washed thoroughly to remove all remnants of agar gel under running tap water and were finally potted in wetted coconut husk for acclimatization. The pots were maintained under mist and 50% shade for 1 month and after that they were moved to standard green house conditions. The whole observation under *in vitro* and *ex vitro* conditions was taken at regular intervals of one week up to the 24 weeks and the obtained results were recorded.

The fresh roots of the regenerated plants were pretreated with the mixture of saturated Paradichlorobenzene (p-DB) and 8-hydroxyquinolene (1:1) for 4 hours at 14-16°C temperature, followed by washing and fixation in acetic-ethanol (1:3) for overnight. The root tips were kept in 45% acetic acid

solution for 1 minute and stained with 2% aceto-orcein stain and 1 (N) HCL (9:1). Finally root tips were squashed in 45% acetic acid and the chromosome number of mitotic metaphase was counted.

RESULTS AND DISCUSSION

Results

Shoot tip (Figure 1 A) consists of two leaf primordia which encompass completely the very minute terminal apical dome or shoot meristem (resident meristem) which is not clearly seen in free-hand section (Figure 1 B). After 4-6 weeks, few (more or less 7-12) immature spherical newly formed protochrom-like bodies (PLBs) of different sizes were formed directly (Figure 1C) without intervening callus tissue from the shoot tip during their growth and development by the influence of different combination and concentration of hormones (i.e. BAP and NAA) under *in vitro* condition (Table 1). During the second subculture, the protocorms further increased in size divided and formed clusters. Rhizoids differentiation was prominent in clusters. Sometimes they could be easily mistaken as roots (Figure 1D). In hormone-free basal Orchimax medium (i.e. control set), very few i.e. 2-3 PLBs were also formed from the shoot tip. After regeneration of PLBs, the explants turned brown in all cases whereas, the newly formed PLBs grew as normal. After 12 weeks of initial culture, numerous young Chlorophyllous PLBs were formed which were microscopically visible prominently with a developing growth appendicle (arrow) or a very small conical shaped vegetative apex at the upper side and many fine white thread like rhizoids appeared at the lower side (Figure 1E). Formation of numerous PLBs in course of time takes place by the protochrome budding which is a characteristic feature of orchid and it leads to the formation of multiple plantlets in course of in vitro development. With passage of time, PLB formation was followed by the development of embryonic shoot with two very young leaves and with tuft of rhizoids below (Figure 1F). Rhizoids are the epidermal hair and penetrate into the medium, it indicates their absorptive function. These are considered akin to root hair. The formation of rhizoids at the early stage is a unique feature of the Orchidaceae. After 16 weeks of initial culture, the embryonic shoots gradually losing their rhizoids were transformed into rooted plantlets (Figure 1G). The average number of regenerants varied with the type of growth regulator(s) added in the nutrient pool. Maximum number of shoots with roots was obtained from the shoot tip, cultured in Orchimax medium + BAP (1.5mg l^{-1})/ BAP (1.5mg l^{-1}) + NAA (0.5mg l^{-1}) forming 68±0.22 and 86±0.19 plantlets, respectively after 16 weeks of initial of culture.

The rooted (arrow) plantlets were finally subcultured into hormone-free ½ strength Orchimax macro and micro elements containing basal medium where it grew normally up to 20 weeks of initial culture (Figure 1H). Root tip cells in the regenerants uniformly revealed a somatic complement of 2n = 40 chromosomes (Figure 1 I) like that of mother plant. Chromosome numbers of the regenerants are cytologically stable and generally remain diploid.

The *in vitro* well developed rooted plantlets of *C. flaccida* were successfully hardened on potting mixture up to 24 weeks of initial culture. Nearly 83% of plantlets survival was recorded.

Discussion

The explants of *Coelogyne flaccida* were ideally procured from *in vitro* grown, contamination free and physiologically uniform and stable plantlets of same age for the present investigation. This was done to avoid the variability of the physiological status of the explants.

In the present investigation, multiple shoots formation were observed without any intervening callus and protocorm-like-body (PLB) formation on all the tested Orchimax basal media alone with BAP or supplemented with various concentrations and combination of BAP and NAA but there were quite differences between and within media in terms of mean number of PLB formation and rooted shoot formation after 6 and 16 weeks of initial culture. This study also revealed that though hormone free Orchimax medium was capable for shoot development in very low number, but it could not elicit for more PLB formation and shoot multiplication even after 16 weeks of culture suggesting that plant hormones are essential to be supplemented in the medium for their further multiplication.

In this experiment, among all the media tested alone with BAP, Orchimax medium with 1.5 mg l^{-1} BAP was found to be most effective in eliciting PLB formation and shoot multiplication which indicates that

Orchimax medium alone with BAP might be suitable for shoot proliferation. In the present cultures, an individual treatment with BAP also proved equally and highly effective to that of combinations of auxin and cytokinin. This result was also supported by previous work of several researchers *on Dendrobium densiflorum* (Luo *et al.,* 2006), *Geodorum densiflorum* (Bhadra and Hossain, 2003), *Cymbidium* and *Cattleya* (Nagarju *et al.,* 2003).

Among the different combinations and concentration of BAP and NAA tested in this study, BAP (1.5 mg l^{-1}) and NAA (0.5 mg l^{-1}) were found to be effective for the shoot multiplication. The obtained result showed that combination of BAP and NAA is also suitable for shoot multiplication and also suggested that the phytohormones - BAP and NAA - both are necessary for its fast growth and development. The previous work of several researchers also showed that the high concentration of BAP and low concentration of NAA was favorable for the induction of multiple shoots. Similar results were obtained by Talukdar *et al.,* (2003) in *Dendrobium* orchid, Sunitibala and Kishor, (2009) in *Dendrobium transparens*, Aeridesodorata (Pant and Gurung, 2005), Cymbidium aloifolium (Rajkarnikar, 2011), *Cymbidium forestii* and *Cymbidium kanran* (Chung *et al.,* 1998), *Dendrobium fimbriatum* (Rajkarnikar and Niraula, 1994), Pant and Thapa, (2012) *Dendrobium primulinum*. Shiau *et al.,* (2005) cultured the axenic nodal segments of in vitro seedling of *Dendrobium candidum* on MS medium with 2 mg l^{-1} BAP and 0.1 mgl^{-1} NAA and they obtained 73.2% shoots within 75 days. Hence, the regeneration potential of explants is markedly influenced by their physiological status (Arditti and Ernst, 1993; Vajrabhay, 1978; Vij and Kaur, 1998; Basker and Narmatha, 2006; Janarthanam and Seshadri, 2008; Kaur and Bhutani, 2010, 2013), chemical stimulus present in the nutrient pool (Kaur *et al.,* 2015) and division activity of the apical meristimatic cells is also influenced by the condition of the cell walls i.e., those cells divide more actively who has less rigid cell walls according to Misra and Bhatnagar, (1995). However, the quality, quantity and nature of growth regulators have foremost effect on the regeneration capacity of the shoot tip. This simple one step protocol has the potential to mass propagate and conserve this rare orchid of medicinal and ornamental importance. Further focus of the current study is on acclimatization of *in vitro* raised cultures of *C. flaccida* and restoring them back in their natural habitat.

Table 1: *In Vitro* Plant Regeneration Response of Shoot Tip Explants of *C. flaccida* via Direct PLB Formation on Orchimax Medium Containing Different Concentration and Combination of BAP and NAA after 6 and 16 Weeks of Initial Culture

Media + Cat. No	Growth Hormone (BAP mg l^{-1})	Concentration of Hormone (NAA mg l^{-1})	Mean Number of Protochrome-Like Bodies after 6 Weeks of Initial Culture (±S.E)	Mean Number of Rooted Plantlets after 16 Weeks of Initial Culture (±S.E)
Orchimax 0 0257	0(BM)	-	2.75±0.27	2.5±0.28
Orchimax 0 0257	0.5	-	6.05±0.16	15±0.18
Orchimax 0 0257	1.0	-	12.80±0.62	49±0.26
Orchimax 0 0257	1.5	-	17.00±0.36	68±0.22
Orchimax 0 0257	2.0	-	13.25±0.40	53±0.16
Orchimax 0 0257	0.5	0.5	7.60±0.22	20±0.15
Orchimax 0 0257	1.0	0.5	13.30±0.64	54±0.18
Orchimax 0 0257	1.5	0.5	20.55±0.62	86±0.19
Orchimax 0 0257	2.0	0.5	15.30±0.64	62±0.36
Orchimax 0 0257	0.5	1.0	6.35±0.39	18±0.15
Orchimax 0 0257	1.0	1.0	11.25±0.33	45±0.15
Orchimax 0 0257	1.5	1.0	14.25±0.22	59±0.21
Orchimax 0 0257	2.0	1.0	10.25±0.55	39±0.31

Culture conditions: Orchimax medium, 25±2°C, 16 weeks of culture, 4 replicates were used in each combination.

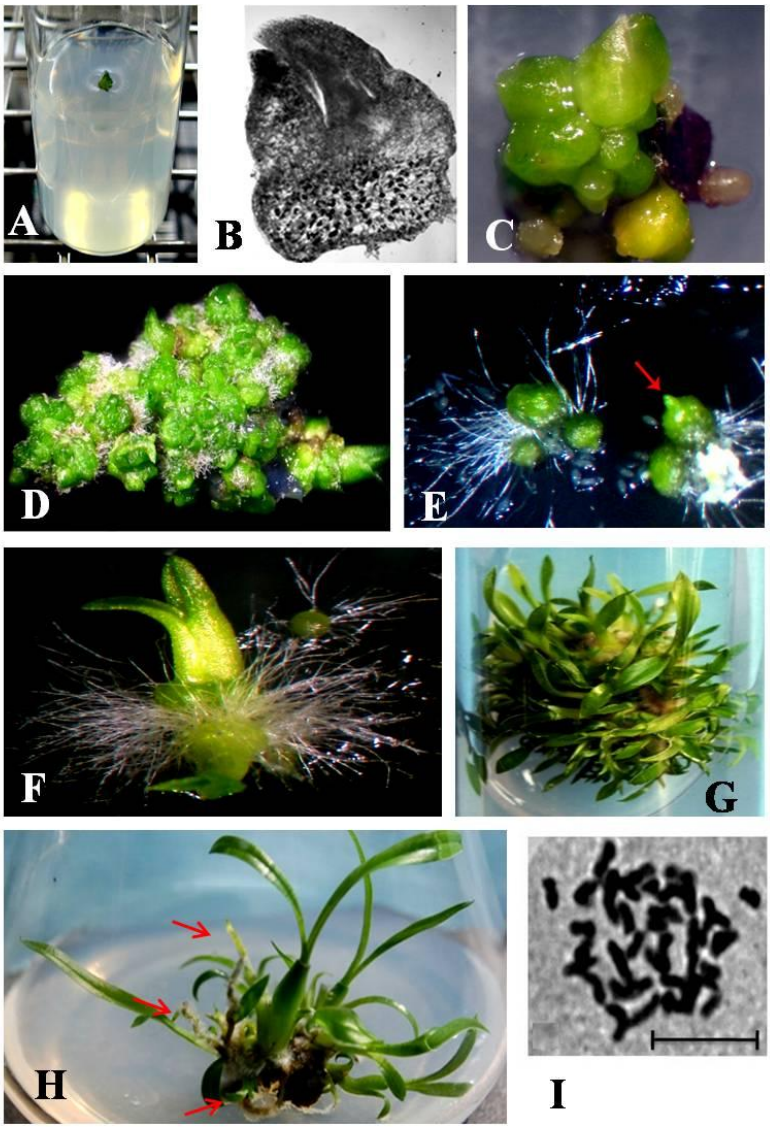

Figure 1: Steps of *in Vitro* Mass Propagation of *Coelogyne Flaccida* Lindl.
A. Excised Shoot Tip Explants on Medium at the Initial Stage;
B. Free Hand Longitudinal Section of Shoot Tip Showing Two Leaf Primordia Encompassing the Apical Dome (Not Visible Clearly due to Very Small Size);
C. More or Less 7-12 Immature Spherical Newly Formed Protochrom-Like Bodies (PLBs) of Different Sizes Formed Directly after 4-6 Weeks of Culture;
D. During the 60 Days of Culture, the Protocorms Further Increased in Numbers Divided and Formed Clusters. White Hair-Like Rhizoids Differentiation was Prominent in Clusters;
E. After 12 Weeks of Initial Culture, Numerous Young Chlorophyllous PLBs Formed which were Microscopically Visible Prominently with a Developing Growth Appendicle (Arrow) or a Very Small Conical Shaped Vegetative Apex at the Upper Side;
F. Development of Embryonic Shoot with Two Very Young Leaves and with Tuft of Rhizoids below;
G-H. After 16 Weeks of Initial Culture, the Embryonic Shoots Gradually Losing their Rhizoids Transformed into Rooted Plantlets;
I. Root Tip Cells in the Regenerants Uniformly Revealed a Somatic Complement of 2n = 40 Chromosomes [Bar =50µm.]

Conclusion

Micropropagation of *Coelogyne flaccida* by shoot tip cultures could be effectively established when shoot tips were placed on the Orchimax medium with BAP (1.5 mg l^{-1}) and NAA (0.5 mg l^{-1}). This protocol is simple, easy to carry out and can provide a large number of PLBs and plantlets for mass propagation in a short period of time.

We expect that this ability will also open up the prospect of using biotechnological approaches for *Coelogyne flaccida* improvement and conservation as well meet the commercial demand of this wild endangered orchid. This protocol could be one of the most suitable alternative tools to minimize the pressure on natural population of this medicinal orchid and their sustainable utilization.

ACKNOWLEDGEMENT

We acknowledge the Department of Botany, Presidency College, Kolkata, West Bengal for providing the excellent laboratory facilities for this type of work.

REFERENCES

Arditti J and Ernst R (1993). *Micropropagation of Orchids*, (John Wiley, New York, USA).

Bailes CP (1985). Orchids in Nepal. The conservation and Development of a Natural Resource. Advisory Report and Recommendations, Royal Botanic Gardens, Kew.

Basker S and Narmatha Bai V (2006). Micropropagation of *Coelogyne stricta* (D.Don) Schltr. via pseudobulb segment cultures. *Tropical and Subtropical Agroecosystems* **6**(1) 31-35.

Bhadra SK and Hossain MM (2003). *In vitro* Germination and Micropropagation of *Geodorum densiflorum* (Lam.) Schltr. an Endangered Orchid Species. *Plant Tissue Culture* **13**(2) 165-177.

Chung JD, Leu JH, Lee S and Kim CK (1998). Effect of medium composition on multiple shooting growth of mericlone from rhizome of shoot tip culture of temperate Cymbidium species. *Biological Abstract* **105**(4) 50.

CITES (2012). Convention on International Trade in Endangered Species of Wild Fauna and Flora CITES (2012) Appendices I, II and III. http://www.cites.org

Clayton D (2002). *The genus Coelogyne, a synopsis*, (Natural History Publication, Kota Kinabalu, Malaysia).

Janarthanam B and Seshadri S (2008). Plantlet regeneration from leaf derived callus of *Vanilla planifolia* Andr. *In vitro Cellular and Developmental Biology- Plant* **44** 84-89.

Kaur S and Bhutani KK (2013). In Vitro Mass Propagation of Ornamentally and medicinally Important *Coelogyne flaccida* Lindl. through Pseudobulb Segments. *Plant Tissue Culture and Biotechnology* **23**(1) 39-47.

Kaur S, Bhandari P and Bhutani KK (2015). Characterization of Bioactive Compounds at Seedling Stage and Optimization of Seed Germination, Culture Multiplication of *Dendrobium nobile* Lindl.-A Study In Vitro. *International Journal of Advanced Research* **3**(4) 1041-1052.

Kaur S and Bhutani KK (2010). Micropropagation of Malaxisacuminata D. Don: A rare orchid of high therapeutic value. *The Open* Access *Journal of Medicinal and Aromatic Plants* **1** 29-33.

Luo JP, Wang Y, Zha XQ and Huang L (2006). Micropropagation of *Dendrobium densiflorum* through protocorm like bodies: effects of plant growth regulators and Lanthanoids. *Plant Cell, Tissue and Organ Culture* **93**(3) 330-340.

Majumdar PL and Maiti DC (1989). Flaccidin and oxaloflaccidin, two phenenthrene derivatives of the orchid *Coelogyne flaccida*. *Phytochemistry* **28** 887-890.

Majumdar PL, Banerjee S, Maiti DC and Sen S (1995). Stilbenoides from the orchids *Agrostophyllum callosum* and *Coelogyne flaccida*. *Phytochemistry* **39**(3) 649-653.

Misra AK and Bhatnagar SP (1995). Direct shoot regeneration from leaf explants of cucumber (*Cucumis sativus L.*). *Phytomorphology* **45** 47-55.

Nagaraju P, Das SP, Bhutia PC and Upadhyaya RC (2003). Effect of media and BAP on protocorms of *Cymbidium* and *Cattleya*. *The Journal of the Orchid Society of India* **17** 67-71.

Pant B and Gurung R (2005). *In vitro* seed germination and seedling development in *Aeridesodorata* Lour. *The Journal of the Orchid Society of India* **19** 51-55.
Pant B and Thapa D (2012). *In vitro* mass propagation of an epiphytic orchid, *Dendrobium primulinum*Lindl.through shoot tip culture. *African Journal of Biotechnology* **11**(42) 9970-9974.
Pradhan GM (1985). *Commercial Flowers*. In Bose TK & Yadav LP (edition) (Naya Prakash Publications, Calcutta, India).
Pyakurel D and Gurung K (2008). *Uses of orchids*. In: *Enumeration of Orchids and Estimation of Current Stock of Traded Orchids in Rolpa District*. A report submitted to District Forest Office, Rolpa, Nepal 6-28.
Rajbhandari KR and Bhattarai S (2001). *Beautiful Orchids of Nepal*, (Kishor Offset Press (P) Ltd. Thamel Kathmandu, Nepal).
Rajkarnikar KM and Niraula R (1994). Tissue culture of Dendrobium fimbriatum for mass production. In *Second National Botanical Conference*, Kathmandu, Nepal 34.
Rajkarnikar KM (2011). Propagation of *Cymbidium aloifolium* (L.) Sw. *In vitro* by Seeds. Bulletin: *Department of Plant Resources, Thapathali, Kathmandu, Nepal* **33** 27-30.
Shiau YJ, Nalawade SM, Hsia CN, Mulabogal U and Tsay HS (2011). *In vitro* propagation of the Chinese medicinal plant, *Dendrobium candidum* Wall. Ex. Lindl., from Axenic nodal segments. *In Vitro Cellular & Developmental Biology - Plant* **41** 666-670.
Sunitibala H and Kishor R (2009). Micropropagation of *Dendrobium transparens* L. from axenic pseudobulb segments. *India Journal of Biotechnology* **8** 448-452.
Talukdar SK, Narsiruddin KM, Yasmin S, Hassan L and Begum R (2003). Shoot proliferation of *Dendrobium* orchid with BAP and NAA. *Journal of Biological Science* **3**(11) 1058-1062.
Vajrabhaya M (1978). Tissue Culture of dormant buds from *Cattleya* back bulbs. *Orchid Review* **86** 256-257.
Vij SP and Kaur S (1998). Micropropagation of therapeutically important orchids: *Malaxis acuminate* D. Don. *The Journal of the Orchid Society of India* **12** 89-93.
Wu CY, Raven PH and Hong DY (2009). *Flora of China 25 (Orchidaceae)*, (Scientific Press, Beijing, China and Missouri Botanical Garden Press, St. Louis, MO, USA).