# Studying regenerative competence of *Satyrium nepalense* D.Don through leaf segments *in vitro*

**Chauhan Shaveta[1] and Sharma Sanjay[2*]**
1. Department of Botany, HRMMV College, Jalandhar-144008, Punjab, INDIA
2. Department of Biotechnology, DAV College, Jalandhar-144008, Punjab, INDIA
*sanjayjns@gmail.com

## *Abstract*

*Satyrium nepalense is an endangered terrestrial orchid extensively collected from wild for its use in conventional system of medicine which has led to deleterious affect on its size and prevalence in nature. The prospects of germinating seeds in vitro were assessed in immature seeds (4 weeks after pollination) and mature seeds from dehisced capsule (8weeks after pollination) on Mitra medium and its combination with or without activated charcoal and growth additives. Activated charcoal, however, proved obligatory for germination.*

*The regeneration response of leaf segments (apical and basal) procured from 12 week old in vitro growing cultures, was tested in yeast extract ($1gl^{-1}$) supplemented Mitra medium. The effect of plant growth regulators on culture initiation and development was also tested. The regeneration response was invariably observed in basal segment and the number of explants responding to regeneration along with their developmental pathway varied depending on the growth stimulus provided in the nutrient pool.*

**Keywords:** *Satyrium nepalense*, regeneration, protocorm like bodies, morphogenesis.

## Introduction

Orchidaceae is one of the largest family among angiosperms with more than 25000 species. They exhibit a wide range of habit and are found growing in all types of habitats throughout the world except in Antarctica. They possess beautiful flowers with long shelf life which make them highly priced in cut flower industry. Beside this they are also known for their medicinal properties in traditional Indian medicine systems like Ayurveda (Ashtvarga),Unani and also in Chinese folklore medicines.They are used for enhancing immunity, building up of strength, stamina and help in rejuvenation due to which it is an important ingredient of Chyawanprash, an ayurvedic health supplement. They are indiscriminately and illegally collected from wild by local people and tribes.

The tubers of terrestrial orchids are highly priced in international market. Dried tubers and powdered ones are used to form Salep which is used as a drink, tonic, aphrodiasic, dietary supplement and as a stabilizer in ice cream production[12]. The orchids exhibit great diversity in flower shapes by exhibiting various types of mimicry and modifications in floral parts to attract the pollinators. They produce large number of dust like microscopic and non endospermic seeds which require specific mycorrhizal endophyte for their germination due to which only less than 1% of seeds are able to germinate in nature. The anthropogenic activities have not only affected their pollination biology but also make them the most vulnerable group of plants on earth.

Orchids undergo distant hybridization in nature and they exhibit great deal of genetic diversity in their descendants.With the discovery of asymbiotic seed germination technique, not only the requirement of fungal partner for seed germination is bypassed but also has added new dimensions to the orchid propagation where we can not only use mature but also immature seeds for germination leading to significantly reduction in the time gap from germination to seedling development. Morel was the pioneer in demonstrating the possibility of using excised shoot-meristem for regenerating complete plantlets of *Cymbidium in vitro*[18] and Wimber gave the formulation for the same[36].

However, the use of excised shoot-tips in monopodial orchids is limited as their excision requires the sacrifice of the entire new-growth and/or the only growing point and endangers the survival of the mother plant. Since then, the regenerative competence of excised leaves, stems, roots, rhizomes, pseudobulbs, inflorescences etc. whose excision is not detrimental to the survival of the mother plant, has been positively tested in a large number of orchids[19].

Though tremendous development has been achieved in orchid micropropagation during last few decades, yet the utility of this technique is still limited due to various problems like very high phenolics exudation from explants, acclimatization of *in vitro* raised plants with high mortality rate during lab to land transfer, somaclonal variations etc.

The genus Satyrium derived its name from Greek word Satyrion (satyr) referring to the aphrodiasic properties of its tubers[32]. Out of about 90 species of this terrestrial leafy erect genus with undivided tubers, only four species are found in Asia (India, Sri Lanka, Nepal, Bhutan and Myanmar) and nearly half are reported from Africa. Two species namely *Satyrium ciliatum* are restricted to few patches in Uttarakhand and Sikkim while *S. nepalense* is found growing in North-West Himalayas and Western Ghats in India. The twin spurred flowers are mostly pollinated by beetles, moths, butterflies and also by carrion flies.

*Satyrium nepalense* is found growing on steep hill slopes or alpine grasslands in Himalayas at an altitude of 1500-3000m

or above flowering from July-September[7].It is commonly known as Ban alu or "Salammisri" due to its oblong tuberous root resembling small size potatoes. It bears pinkish-white flowers, 8-16mm across with two spurs. The sweet tubers are used to obtain Salep or are cooked and consumed.The dried ones are sold as 'Salammisri' and regarded as a stimulant with aphrodiasic properties. They are also reported to be useful in the treatment of malarial fever and dysentery[11]. Its juice is applied externally on cuts and wounds[22].

The local people of Uttarakhand (Western Himalayas) have started using it in ayurvedic preparations as a substitute for another very rare orchid *Eulophia dabia*[13]. It is also used in treatment of chronic nephritis. The species is also found to possess antioxidative and antimicrobial properties against both gram+and gram-bacterias[17,28]. Tubers are extensively harvested for their use in folk medicines and Chinese traditional medicinal system which have dwindled their population in nature.It is included under Appendix II of the CITIES which strictly monitors and controls its trade from wild[12].Our main objective was to develop a methodology for *in vitro* regeneration of this species.

## Material and Methods

Presently the species was collected from Kasauli Hills and Tara Devi Hills (near Shimla) at an altitude of 1800-2500 m above sea level.As the species is a terrestrial orchid, the problem of phenolic exudates was very severe and to check this along with maintaining dark conditions the activated charcoal (AC) was invariably added to the medium.The mature seeds were a symbiotically germinated on Mitra (M) medium with AC under both illuminated and non illuminated conditions. Mitra medium supplemented with yeast extract (YE) was found optimum for seed germination, therefore it was further taken as basal medium for leaf regeneration. For checking their regeneration capabilities, the leaves of about 1 cm in length were selected and excised from 12 weeks old *in vitro* developed seedlings.

The leaf explants were cut into two halves and were tested in YE (1g/l) supplemented M medium and its various combinations with growth adjuncts. The medium was sterilized in autoclave at 121 ºC at 1kg $cm^{-2}$ for 20 minutes and the pH of medium was adjusted to 5.6 before autoclaving. The cultures were maintained at a temperature of 25±2ºC under 12/12-hr(day/night) photoperiod with light intensity of 30 µmol $m^{-2}$ $s^{-1}$.

Frequent subculturing for 2-3 weeks further helps in checking phenolic exudate release from cut ends. Effects of different types of stimulus present in nutrient pool on leaf explants were recorded based on visual observations taken on daily basis and were quantified on the basis of percentages of cultures showing the response and the degree of response of culture. The rate of shoot/PLB multiplication rate was determined in terms of number of shoots (with 2-3 leaves)/Protocorm Like Bodies (with established polarity) available at the end of each passage of specified time.

The organogenetic differentiation under different treatments was also recorded as a function of time in culture. All the experiments were performed under sterile conditions and for each combination, 4 replicates were used and the experiment was repeated thrice. The data was analyzed statistically by using SPSS software and subjected to One-way Analysis of Variance (ANOVA) and significant differences were determined using Duncan's multiple range test with 5% level of significance.

## Results and Discussion

The results are summarized in table 1; Figures A-I. In basal medium(M+YE), explants without any signs of regeneration turned brown and perished within a week. BAP induced the regeneration response in 4.75±0.50 wks either via formation of PLBs (Fig. A) or callus in 75.50±1.73 per cent of explants .The callus was creamish-white and irregularly lobed (Fig. B) initially which developed chlorophyll and subsequently differentiated PLBs (Fig. C). The PLBs differentiated and 3 regenerants were obtained with 2-3 leaves in 15wks which later developed roots in 17wks.

Replacement of BAP with KN on the other hand elicited the response (74.50±0.58 per cent); the basal segment soon differentiated several meristemoids, each of which developed into proliferative PLBs in 2.25±0.50wks (Fig. D) and subsequently yielded 12 regenerants. Complete and healthy plantlets were obtained in 15 wks (Figs. E-G). Both IBA and NAA failed in inducing any regeneration response.

Only auxin which proved stimulatory was IAA with 24.50±0.58 per cent explants showing regeneration in medium supplemented with it (Fig. H-I). The response was weak and delayed with basal explants responding via shoot bud; plantlets were obtained in 24.75±0.96wks. Even auxin NAA in combination with cytokinin BAP proved inhibitory to regeneration response despite of repeated attempts.

The ability of *Cymbidium* leaves to develop PLBs *in vitro* proved much more productive approach for orchids micropropagation in place of using apical shoot-meristem as explant[36]. Success of regeneration using leaf explants depends on many factors like composition of nutrient medium, type and concentration of the growth regulator used, auxin/cytokinin ratio, source and physiological age of the explant and the explant orientation in the culture vessel. The regeneration potential of the foliar explant has so far been tested in many orchid species and hybrids[3,28,29].

Presently the regeneration competence of 12 weeks old *in vitro* raised seedlings leaf about 1 cm in size segmented into apical and basal segments tested in YE ($1gl^{-1}$) supplemented M and its various combinations with growth additives. Frequency and nature of regeneration response however, varied with medium composition.

**Table 1**
***Satyrium nepalense* leaf segments regeneration response *In vitro* on YE supplemented M medium**

| Additive(s) | Percentage of explants responded | Time taken for initiation of response (wks) | No. of meristematic loci invoked per explant | Pathway of regeneration followed | No. of plantlets observed per explant after 20wks of culture | Remarks |
|---|---|---|---|---|---|---|
| M+YE | - | - | - | - | - | No regeneration response |
| M+YE+IAA | 24.50±0.58[a] | 8.50±0.00[c] | 1 | SP-Pl | - | Delayed response |
| M+YE+ IBA | - | - | - | - | - | No regeneration response |
| M+YE+NAA | - | - | - | - | - | No regeneration response |
| M+YE+BAP | 75.50±1.73[b] | 4.75±0.50[b] | 3 | SP/Ca/PLB-Pl | 3 | Creamish-white irregularly lobed callus at basal end |
| M+YE+KN | 74.50±0.58[b] | 2.25±0.50[a] | 6 | PLB-Pl | 12 | PLBs on basal segment |
| MYE+BAP+ NAA | - | - | - | - | - | No regeneration response |

*Pl, Plantlet; PLB, Protocorm like Bodies; Ca, Callus; SP, Shoot primordia; wks, weeks
Entries in column nos. 2 and 3 are mean's: same alphabetical letter in the superscript denotes that the corresponding means are in the same group using Duncan's multiple range test at 5%

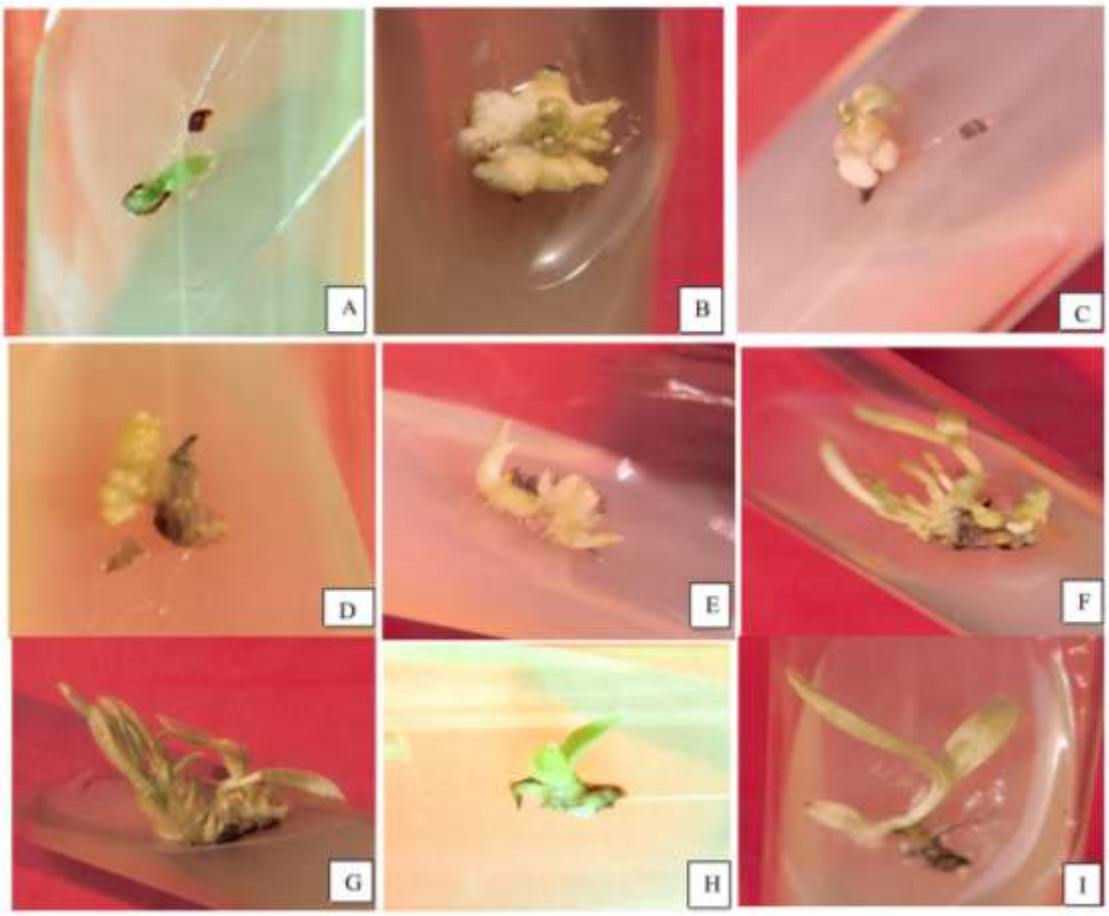


**Fig. 1: Regeneration response of *Satyrium nepalense*, leaf explants *in vitro* (A) Shoot bud mediated regeneration from basal explant (M+YE+BAP), (B) Callus mediated regeneration (M+YE+BAP), (C) Creamish white irregularly lobed differentiating callus on the basal explant (M+YE+BAP), (D) PLB multiplication on basal explant (M+YE+KN), (E-F) PLB mediated plantlet formation (M+YE+KN), (G) Complete plantlet (M+YE+KN), (H-I) Shoot bud mediated plantlet development on basal explant (M+YE+IAA)**

The young leaves proliferate along the surface whereas in mature and older ones, the response is restricted only to the basal regions[34]. The PLBs were found to develop through the repeated mitotic divisions of adventive meristematic cells.

Similarly, in *Acampe praemorsa*[21], *Renanthera imschootiana*[26], *Neofinetia falcata*, *Satyrium nepalense*, *Vanda cristata*, *Vanda testacea*[33], *Ascocenda*, *Aranda*[8] and *Cattleya*[4], regeneration response remained restricted to the leaf base in agreement with the earlier suggestion that in monocots it is generally the leaf base which is meristematic , and upon its isolation and culturing, it can differentiate into new plantlets[38] .

Literature studies indicate that orchid leaves regenerate either directly through shoot buds(*Paphiopedilum philppinense*[5], *Dendrobium* hybrid[35], *Vanda coerulea*[27] and *Renanthera imschootiana*[26]) or indirectly via PLBs (*Tolumnia*[6], *Doritaenopsis* hybrid[23], *Papillionanthe teres*[24], *Saccolabium papillosum*[15], *Vanda coerulea*[27], *Spathoglottis plicata*[31], *Liparis viridiflora*, *Pholidota chinensis*[37], *Phalaenopsis* hybrid[25], *Rhynchostylis retusa*[34] and *Phalaenopsis*[2,30]) and/or callus (*Vanda coerulea*[14], *Ascocenda*[35], *Acampe rigida*[37], *Aerides*, *Luisia*[33], *Dendrobium*[16] and *Paphiopedilum*[1]).

The change in the basal medium and culture conditions may alter the pattern of organogenesis[11]. Presently PGRs were found to be effective in evoking the regenerative response in leaf explants. The response frequency and regeneration pathway however differed with the growth factor (KN/BAP/IAA) in the medium. The efficacy of PGRs in activating proliferative loci in foliar explants and regulating their subsequent development into plantlets is well-documented[2,33] and it was also apparent in the present cultures. Only the basal segments responded and that too along their adaxial surfaces. The explants followed varied regenerative pathways either directly form shoot buds or proliferate to form callus or PLBs, depending upon the medium composition and the quality of PGRs used.

Cytokinins promote regeneration either through formation of PLBs and/or callus (BAP/KN). Effective use of cytokinins for regeneration in leaf explants has also been previously reported[9]. Presently, in *Satyrium nepalense*, KN proved better than BAP which is in line with similar earlier findings in *Aerides maculosum*[20], *Acampe praemorsa*[21], *Acampe rigida*, *Liparis viridiflora* and *Pholidota chinensis*[37]. Addition of IAA proved effective in inducing regeneration response while IBA, NAA proved ineffective. When NAA was combined with BAP, it even showed its inhibitory effect. Inhibitory role of NAA when used individually has been reported in *Neofinetia falcata* and *Rhynchostylis retusa* [33].

## Conclusion

The present data together with that reported earlier suggest that manifestation of the inherent potential of orchid leaves to regenerate *in vitro* is directly related to the source, age, and genetic constitution of the mother explant along with the nutritional recipe employed. The ability of leaves to regenerate all over the surface or only in the apical or basal region varies with the species, the physiological age of the explant, orientation of the explant and the medium composition. A careful selection of the donor leaves and appropriate growth stimulus may provide an ideal method of orchid micropropagation.

Though the protocol for micropropagation through seeds has been reported in this species previously[10], yet regeneration through leaf explants has added more possibilities of conserving this valuable and threatened medicinal orchid and these findings will find its application in the field of medicinal plant conservation, rapid plant propagation to meet pharmaceutical industry demands and also in food supplement production.

## Acknowledgement

The authors are grateful to the Council of Scientific and Industrial Research,New Delhi for financial support and Department of Botany, Panjab University,Chandigarh where all the experimentation work was done.

## References

1. Allenberg H., Nortizen zur Keimung, Meristemkultur and Regeneration Non Erdorchideen, *Die Orchidee*, **27,** 28-31**(1976)**

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

3. Cardoso J.C., Zanello C.A. and Chen J.T., An overview of orchid Protocorm-like bodies, Mass propagation, Biotechnology, Molecular Aspects, and Breeding, *Int. J. Mol. Sci.*, **21(3)**, 985 **(2020)**

4. Champagnat M., Morel G.M. and Mounetou B., La multiplication vegetative des Cattleya a partir de jeunes feuiles aseptiquement *in vitro, Ann. Sci. Nat. Bot. Biol. Veg*. Ser., **12(11)**, 97-114 **(1970)**

5. Chen T.Y., Chen J.T. and Chang W.C., Plant regeneration through direct shoot bud formation from leaf cultures of *Paphiopedilum* orchids, *Plant Cell Tiss. and Org. Cult.*,**76**, 11-15 **(2004)**

6. Chookoh N., Chiu Y.T., Chang C., Hu W.H. and Dai T.E., Micropropagation of Tolumnia orchids through induction of Protocorm-like bodies from leaf segments, *Hort Sci*., **54(7)**, 1230-1236 **(2019)**

7. Deva S. and Nathani H.B., The Orchid Flora Of North West Himalayas, Print and Media Associates, New Delhi, India **(1986)**

8. Fu F.M.L., Studies on the tissue culture of orchids 2, Clonal propagation of *Aranda, Ascocenda* and *Cattleya* by leaf tissue culture, *Orchid Rev*., **87**, 343-346 **(1979)**

9. Gangaprasad A.N., Decruse W.S., Seeni S. and Menon S., Micropropagation and restoration of the endangered Malabar Daffodil orchid *Ipsea malabarica*, *Lindleyana*, **14(1)**, 38-46 **(1999)**

10. Ganeshan M. and Bai V.N., Mass propagation of *Satyrium nepalense* D. Don.-A medicinal orchid via seed culture, *Scientia Horticulturae*, **119(2)**, 203-207 **(2009)**

11. George E.F. and Sherrington P.D., Plant propagation by tissue Culture: Handbook and directory of Commercial Laboratories, Exegetics, Hants, UK **(1984)**

12. Ghorbani A., Gravendeel B., Zarre S. and Boer H., Illegal wild collection and international trade of CITES -listed terrestrial orchid tubers in Iran, *Traffic Bulletin*, **12(2)**, 52-58 **(2014)**

13. Jeevan S.J., Kumar P. and Pangtey Y.P.S., Ethnomedicinal orchids of Uttarakhand, western Himalaya, *Ethnobotanical Leaflets*, **12**,1227-30 **(2008)**

14. Kanika and Vij S.P., Micropropagation of *Vanda coerulea* R.Br. (Orchidaceae) through leaf segment culture, *Ann. Agric. Res. New Series*, **23(2)**,300-303 **(2002)**

15. Kaur S. and Vij S.P., Regeneration potential of *Saccolabium papillosum* leaf segments, *J. Orchid Soc. India*, **14**,67-73 **(2000)**

16. Manorama P., Rao A.N., Goh C.J. and Loh C.S., Leaf callus development in *Aranda* and *Dendrobium*, In Proc. Fifth ASEAN Orchid Congress Seminar, ed., Rao A.N., Parks and Recreation Department, Ministry of National Development, Singapore,102-109 **(1986)**

17. Mishra A.P., Saklani S., Salehi B., Parcha V., Sharifi-Rad M., Milella L., Iriti M.,Sharifi-Rad J. and Srivastava M., *Satyrium nepalense*, a high altitude medicinal orchid of Indian Himalayan region: Chemical profile and biological activities of tuber extracts, *Cellular and Molecular Biology*, **64(8)**, 35-43 **(2018)**

18. Morel G.M., Producing virus free Cymbidiums, *Am. Orchid Soc. Bull*., **29**, 495-497 **(1960)**

19. Morel G.M., Clonal multiplication of orchids, The Orchids: Scientific Studies, ed., Withner C.L., Wiley- Interscience, New York, 169-222 **(1974)**

20. Murthy H.N. and Pyati A.N., Micropropagation of *Aerides maculosum* Lindl. (Orchidaceae), *In Vitro Cell. Dev. Biol.- Plant*, **37**, 223-226 **(2001)**

21. Nayak N.R., Patnaik S. and Rath S.P., Direct shoot regeneration from foliar explants of an epiphytic orchid, *Acampe praemorsa* (Roxb.) Blatter and Mc Cann., *Plant Cell Rep.*, **16**, 583-586 **(1997)**

22. Pant B., Medicinal orchids and their uses: Tissue culture a potential alternative for conservation, *African Journal of Plant Science*, **7(10)**, 448-467 **(2013)**

23. Park S.Y., Yeung E.C., Chakrabarty D. and Paek K.Y., An efficient direct induction of protocorm-like bodies from leaf, subepidermal cells of *Doritaenopsis* hybrid using thin-section culture, *Plant Cell Rep.*, **21**,46-51 **(2002)**

24. Pathak P. and Vij S.P., *In vitro* regeneration of *Papilionanthe teres* (Roxb.) Schltr: Utility of foliar explants, Proc. 7th Asia Pacific Orchid Conference, ed., Ichihashi S., Organizing Committee APOC7, Nagoya, Japan, 226-227 **(2001)**

25. Seeni S. and Latha P.G., Rapid multiplication of *Phalaenopsis* from leaf and root cultured *in vitro*, *J. Orchid Soc. India*, **5(1-2)**, 1-8 **(1991)**

26. Seeni S. and Latha P.G., Foliar regeneration of the endangered red Vanda, *Renanthera imschootiana* Rolfe (Orchidaceae), *Plant Cell Tiss. and Org. Cult*., **29**, 167-172 **(1992)**

27. Seeni S. and Latha P.G., *In vitro* multiplication and eco-rehabilitation of the endangered Blue *Vanda*, *Plant Cell Tiss. and Org. Cult.*, **61**,1-8 **(2000)**

28. Singh D.K., Antioxidant and Antimicrobial activities of medicinally important salep orchid, *Satyrium nepalense*, *Plant Cell Biotechnology and Molecular Biology*, **20(17)**, 752-58 **(2019)**

29. Tanaka M., Micropropagation of *Phalaenopsis* species, Biotechnology in Agriculture and Forestry, High-Tech and Micropropagation IV, ed., Bajaj Y.P.S., Springer-Verlag, Berlin, 20 **(1992)**

30. Tanaka M. and Sakanishi Y., Clonal propagation of *Phalaenopsis* by leaf tissue culture, *Am. Orchid Soc. Bull.*, **46**, 733-737 **(1977)**

31. Teng W.L., Nicholson L. and Teng M.C., Micropropagation of *Spathoglottis plicata*, *Plant Cell Rep*., **16**, 831-835 **(1997)**

32. Teoh E.S., Genus Satyrium to Sunipia, Medicinal Orchids of Asia, 639-658 **(2016)**

33. Vij S.P. and Pathak P., Micropropagation of orchids through leaf segments, *J. Orchid Soc. India*, **4(1, 2)**, 69-88 **(1990)**

34. Vij S.P., Sood A. and Plaha K.K., Propagation of *Rhynchostylis retusa* Bl. (Orchidaceae) by direct organogenesis from leaf segment cultures, *Bot. Gaz.*, **145**, 210-214 **(1984)**

35. Vij S.P., Kaur P., Kondo K., Pathak P. and Gupta A., Regeneration competence of orchid leaves: A study *in vitro*, Plant Genetic Diversity: Exploration, Evaluation, Conservation, eds., Vij S.P., Kondo K., Sharma M.L. and Gupta Ashish, Affiliated East-West Press, New Delhi, India, 289-306 **(2002)**

36. Wimber D.E., Additional observations on clonal multiplication of C*ymbidiums* through culture of shoot meristems, *Cymbidium Soc. News*, **20**, 7-10 **(1965)**

37. Yam T.W. and Weatherhead M.A., Leaf-tip culture of several native orchids of Hong Kong. *Lindleyana*, **6(3)**, 147-150 **(1991)**

38. Zimmer K. and Pieper W., Weitere untersuchungen zur Kultur *in vitro* von Aechmea, *Gartenbauwissenschaft*, **40**, 129-132 **(1975)**.

(Received 15th June 2020, accepted 10th August 2020)