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Artificial pollination and *in vitro* asymbiotic seed germination in garden orchid *Spathoglottis plicata* Blume (Orchidaceae)

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Abstract
Pollination biology of Garden orchid *Spathoglottis plicata* was investigated. Due absence of appropriate pollinator in our area the seed setting was absent or was very low. Artificial pollination (Hand pollination) in *S. plicata* was studied and seed setting was observed by self and cross pollination. Flowers of *S. plicata* were pollinated and subsequent capsule development was carefully monitored. Hand pollination of orchid flowers provides an opportunity to discuss floral morphology and associated reproductive biology. Capsules were harvested 60 days after the pollination prior to capsule dehiscence. *In vitro* asymbiotic germination was 87.3% when seeds were cultured on MS media. The effect of BA and NAA hormones were also observed on seed germination. Seedlings were healthy and stronger when cultured on MS media supplemented with hormones. The cultured seedlings were ready to greenhouse acclimatization after 110 days.

Keywords: *Spathoglottis plicata*, artificial pollination, asymbiotic germination, green house acclimatization.

INTRODUCTION

The Orchidaceae is one of the largest, most diverse, and most important categories of botanically and commercially significant flowering plants with 20,000–30,000 species [1, 2]. Nearly 1,300 species are estimated to occur in India. The floral characteristics of orchids cover an exceptionally wide range of different shape, form, size, and coloration, surpassing flowers of all the other angiosperms. As a result, they are exceedingly valued in flori-trade. Orchids are renowned for their great diversity of pollination systems [3, 4]. *Spathoglottis plicata* or Garden Orchid is a species of terrestrial orchid found in tropical and subtropical Asia. Widespread in tropical Asia from Northern India to the West Pacific across Southeast Asia to Australia in various of the Indian and Malay Islands, Hong-Kong, Southern China and the Pacific Islands of Samoa and New Caledonia.

Orchid flowers are trimerous, possessing three petals and three sepals. The third petal is modified into a labellum or lip typically found oriented toward the bottom of the flower. Potential pollinators use the labellum as a landing platform, directing them to the gynostemium. A distinguishing feature of the Orchidaceae is the gynostemium or column, which is the fusion of the style, stigma, and stamens [2]. The anther cap and pollinia are located at the front of the gynostemium, while the stigma is located directly behind the anther on the underside of the gynostemium (Fig. 1b, c). The pollen grains are joined together into masses called pollinia (Fig. 1d). During a pollination event, pollinators deposit pollinia onto the stigmatic surface (Fig. 1e). A successful pollination event depends on pollen and flower age. *Spathoglottis* flowers remain open for several weeks, while inflorescences continually flower for several months. Pollinating young, fully open flowers is recommended since pollen is most receptive for 1–8 days after flower opening [5, 6, 7]. Likewise, using young flowers less than one week from opening ensures that the stigmatic surface is receptive to pollen. After two weeks, flowers close and pollen becomes brown and unreceptive.

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MATERIALS AND METHODS

Hand pollination procedure

In a fully opened flower, column and anther cap was identified.(Fig. 1b). Anther cap and pollinia was gently removed by the needle. The pollinia were dislodged from the gynostemium by applying slight upward pressure to the bottom of the anther cap (Fig. 1c). The pollinia adhered to the needle on contact. After removal, the pollinia were transferred to the same flower or another flower by gently placing the pollinia onto the stigmatic surface. the hand pollinated flowers were closely monitored for flower senescence, capsule development, and capsule dehiscence. No physical signs are visible indicating *Spathoglottis* capsule maturity, but other species' capsules may turn light green, yellow, or brown. Once capsule development time is estimated by allowing capsules to dehisce. The capsules were harvested one to two weeks before maturity by cutting the capsule from the inflorescence. Harvesting the capsule at this point has ensured that the capsule does not dehisce. Harvested capsules were placed on a clean paper bag and stored at 4–10 ^{o}Cfor a maximum two days, since capsules may dehisce soon after collecting. Storing capsules in the freezer or in plastic bags will cause permanent damage to the capsules, and plastic bags promote fungal and bacterial growth by limiting air exchange.

Asymbiotic media screen

In the present investigation 1/2 MS [8] and full MS with

different concentrations of BA, NAA were examined for their effectiveness in promoting germination and subsequent development of *S. plicata* (Table 1). All media were adjusted to pH 5.8 prior to autoclaving at 20 psi pressure for 20 minutes. The mature capsules before dehiscence were soaked in an aqueous solution of commercial detergent for 30 min. Solid dirt particles adhering to the surface of capsules were removed using a fine brush followed by rinsing with sterile distilled water. The capsules were sterilized with 0.5% Streptomycin to avoid bacterial infection. The capsules were surface-disinfected with 0.1% Mercuric Chloride for 3 minutes, followed by 70% ethanol for 30 sec and rinsed with distilled water before air-drying in a laminar airflow cabinet for 5 min. Green capsules were dissected longitudinally with a sterile surgical blade. Seeds were collected from the capsule with the help of a sterilized spatula and small mass of the aggregated seeds were sown on culture medium containing half strength Murashige and Skoog (MS) and full MS (supplemented with BA &NAA) for *in-vitro* asymbiotic seed germination. Five replicates were used for each germination medium concentrations. Seed germination and protocorm development stage percentages were recorded weekly. Seedling development was scored on a scale of 0 – 5 (Table 3). Asymbiotic seed germination of *S. plicata* maintained at 16/8 (L/D) and 25±5C°. Germination percentages were calculated by dividing the number of seeds in each individual germination and development stage by the total number of viable seeds in the sample. Data were analyzed using Least significant difference test (LSD).

Table 1. Plant growth regulators

BA	0.1	0.5	1	1.5	2	2.5	3	0.1	0.1	0.1	0.5	1	2	2	2
NAA	…	…	…	…	…	…	…	0.1	0.5	1	1	1	0.5	1	2

RESULTS

Artificial pollination

Artificial pollination in *Spathoglottis plicata* was done from December to February at regular interval of time. The flowers started to senesce three days after pollination. Artificial pollination (hand pollination) experiments were conducted during 2008-2011 to increase fruit set. Total 220 flowers were artificially pollinated, out of which 211 capsules developed indicating 95.90 % success rate (Table 2). Self pollination was successful as 77 capsules developed from 82 flowers indicating 93.90 % success rate and 138 flowers were cross pollinated and 134 capsules were successfully developed indicating 97.81 % fruit set. 95.90 % success rate indicate that both types of artificial pollinations (self and cross) are beneficial. Total 80 flowers (20 flowers / year) were left un-pollinated to check natural pollination in nature but only 17 capsules developed indicating only 21.25 % success rate in nature. Flowers are totally dependent on the pollinator for its pollination. Hand pollination greatly enhanced capsule formation.

Time of dehiscence of capsules was calculated as 70 days, the capsules were harvested one to two weeks prior to dehiscence. Harvested capsules were stored for seed germination experiment.

Table 2.Pollination in *Spathoglottis plicata*

Plant Number	Pollination Year	Number of flowers	Self pollinated flower	Cross pollinated flower	Number of capsule developed	Pollination percentage %
1.	2008	24	12	12	24	100%
2.	2009	53	25	28	52	98.11%
3.	2010	78	30	48	75	96.15%
4.	2011	65	15	50	60	92.30%
Total	--	220	82	138	211	95.90%

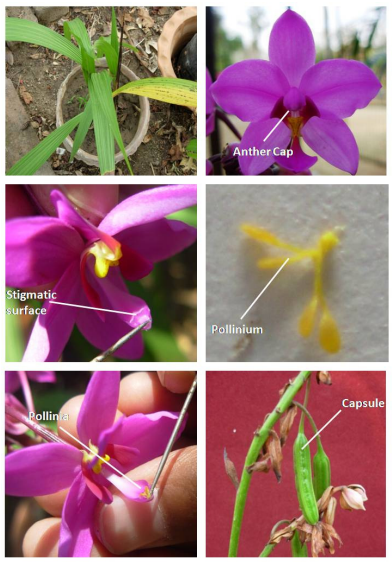

Fig1. Flower morphology of *S. plicata* (a) Habit, (b)Location of anther cap (c) Location of stigmatic surface (d) Removal of Pollinia (e) Transfer of Pollinia onto stigmatic surface (f) Green capsule ready to harvest

Asymbiotic seed germination
Seed Germination

Seeds began to swell after two weeks of inoculation and complete seedling development within 11 weeks on ½ MS. Seed germination was recorded as 87.3 % although seed germination takes place on ½ MS medium but the growth of the seedling was very slow. Hence different concentrations of BA (0.1 mg/l to 3 mg/l) and NAA (0.1 mg/l to 2.0 mg/l) were used. Seeds began to swell after 2 weeks regardless of hormonal concentrations. No significant effect was found on initial seed germination (Stage1) of *S. plicata* (Fig.3). However the seedling showed faster growth on MS medium supplemented with various concentrations of BA alone or in combination with NAA. Those seeds inoculated on hormonal concentration NAA 1g/l and BA 0.1 g/l exhibited both highest final percent germination and most advanced

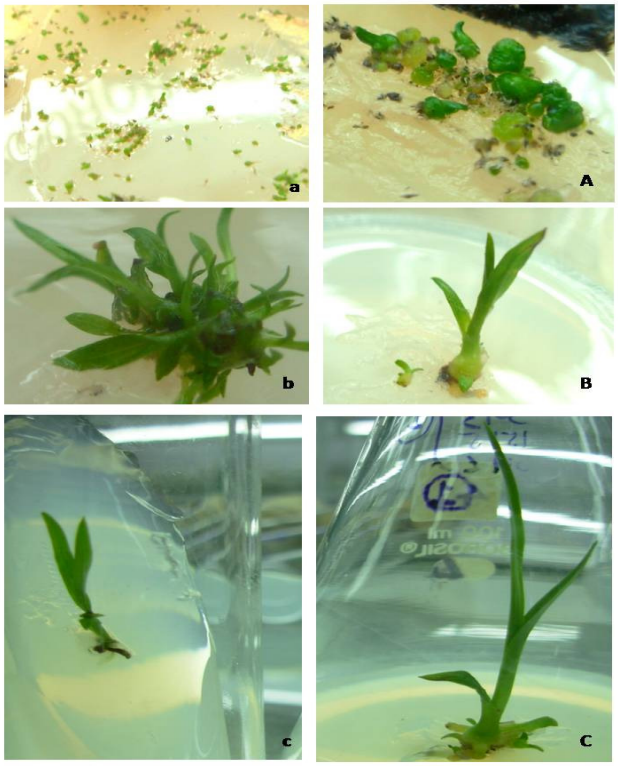

Fig 2. Comparative *In vitro* germination and development of *Spathoglottis* seeds with hormones (Capitalized letters) and without hormones (small letters) (a, A) seed germination (stage 1,2), (b, B) Seedling development (stage 3), (c, C) complete plant (stage 5)

Table 3. Developmental stages of asymbiotically cultured *S. plicata* seeds and seedlings

Stage Description
0 Hyaline embryo, testa intact
1 Embryo swollen, rhizoids present (=germination)
2 Continued embryo enlargement, testa ruptured
3 Emergence of first leaf
4 Elongation of first leaf and further development
5 Complete developed plant

Protocorm developmental stages (Stage 3, 4, 5). Pronounced differences in protocorm morphology were seen in ½ MS and Full MS (with hormones). Those seeds germinated on ½ MS produced protocorm of small size and showed reduced growth of seedlings. Conversely, those seeds germinated on hormones (BA /NAA 0.1/1.0g/l) produced protocorms possessing fully grown roots, shoots, leaves from stage 1 through to stage 5 (Fig.2). Hormones appear to strongly influence on growth of seedling in *S. plicata.* Pseudo- bulb diameter was also influenced by hormones (Fig.5). Leaves produced were long and wide. Infact leaf length, width and number per seedling significantly increased (Fig. 4 & 5). Developmental stages 0, 1, 2,3,4,5 were similar on ½ MS and Full MS with hormone (Fig. 3). The stage 1, 4 and 5 (Fig. 2) are showing the comparative account of 3, 11, 12 weeks respectively. Seedlings developed on half MS were weak, small than on medium supplemented with hormones. Contamination rate of cultures was 5%. Other concentrations of BA/NAA (0.1/0.1, 0.1/0.5) responded in same way but roots were smaller in length. As the higher concentrations of BA/NAA were used (2/0, 2.5/0, 3/0, 1/1, 2/0.5, 2/1, 2/2) seedlings responded very less. In concentration 2.0/0.5 multiple shoots like structures were observed but they were not developed in new seedlings. The percentage of germination and development of seedlings was also low.

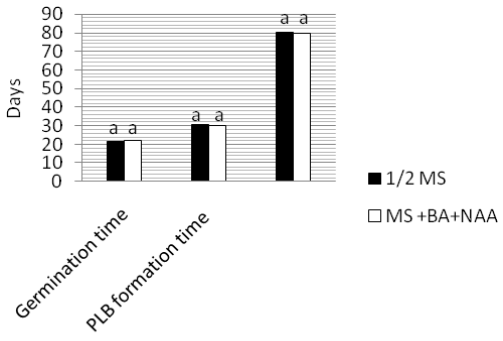

Fig. 3

Fig 3 and Fig 4. Effects of BA & NAA (0.1:1mg/l) on Germination time, PLB formation time seedling development time, number of leaves and number of root in comparison to ½ MS. Histobars with the same letter are not significantly different at α level of significance.

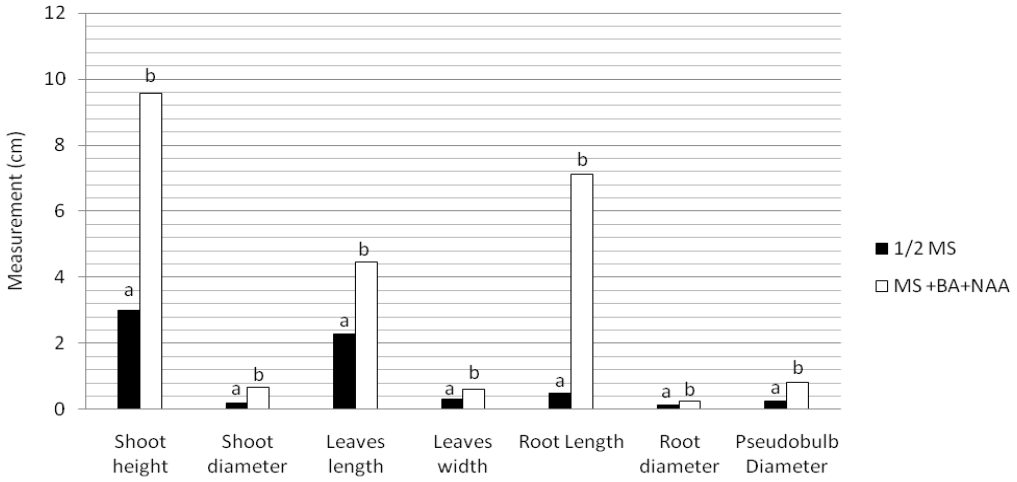

Fig. 5

Fig 5. Effects of BA & NAA (0.1:1mg/l) on Shoot height, shoot diameter, leaves length, leaves width, root length, root diameter and Pseudobulb diameter in comparison to ½ MS. Histobars with the same letter are not significantly different at α level of significance.

DISCUSSION

Spathoglottis plicata is a self-compatible orchid that depends on pollinators to set fruits. Self-compatibility has been widely reported in terrestrial orchids and considered as an adaptation to a poor insect fauna and/ or low insect visitation rates, and likely the first step towards mechanical pollination [9, 10, 11, 12, 13, 14, 15]. Previously breeding experiments in *S. plicata-alba, S. kimballiana* were conducted to check the pollen fertility, pod development [16, 17]. But in this study, natural fruit-set was increased by hand-pollination and developed seeds were used for germination experiments. The numbers of reports on the successful *in vitro* production of terrestrial orchids were observed by several workers [18, 19, 20, 21, 22, 23, 24, 25, 26]. Seed germination of *S. plicata* and *S. kimballiana* on VW medium and liquid medium were studied to check fertile seeds in different ages of capsules after pollination [27]. They observed that 30 days old capsule of *S. plicata* and 40 days old in *S. kimballiana* showed high germination rate and germination index. Successful *in vitro* symbiotic seed germination and artificial pollination has been previously reported in *Spathoglottis kimballiana* [28]. In above Seeds were tested for germination in their report upto stage 2 to check the fertility but in present study majority of seeds were developed upto stage 5 and seedlings were hardened to new plants. Working with *S. plicata* Teng had used the seedling for micropropagation of *S. plicata* but seed germination was not studied [29]. While in these previously published studies both artificial pollination and symbiotic seed germination of *Spathoglottis* species were examined, but researchers failed in developing means of seed germination and early development of seedling with hormones. In present study, we provide an efficient protocol for symbiotic seed germination, as well as artificial pollination in *S. plicata*. We have also explored the effect of BA, NAA on seed germination and seedling development, providing a special emphasis on pseudobulb development.

Previous works on the role of various hormones including BA, NAA has demonstrated inconclusive results. Sometimes enhancing germination, other times showing no effects. This inconsistent response to exogenous hormones has been shown to vary from genus to genus and even species to species [30, 31, 32]. The addition of exogenous cytokinin is of particular interest in symbiotic orchid seed germination because several mycorrhizal fungi have shown to produce cytokinin [33]. Thus we can assume that orchid seed germination and development in the wild may be assisted by their fungal cytokinins. [34, 35]studied the Symbiotic germination and mycorrhiza of *S. plicata* but they did not discussed the exact role of fungus to seed germination respectively. In the present investigation,

BA cytokinin is used and that has enhanced the asymbiotic seed germination of *S. plicata*. However the positive response was seen at lower concentrations of BA (0.1,0.5,1 mg/l) in combination with NAA (0.5,1 mg/l). These results indicate that *S. plicata* seed germination is promoted more at relatively lower concentrations than higher concentrations.

Our current study also reports reduced germination in the presence of higher concentrations of hormones (BA/NAA mg/l, 1/1, 2/0.5, 2/1, 2/2). The current study presents an efficient means to develop a new healthy and strong plant in less time for gardening purpose.

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REFERENCES

[1] Cronquist A.1981. An integrated system of classification of flowering plants. Columbia University Press, Ithaca

[2] Dressler RL. 1981.The orchids: natural history and classification. Harvard University Press, Cambridge

[3] van der Cingel NA .2001. An atlas of orchid pollination: America, Africa, Asia and Australia. A. A. Balkema, Rotterdam.

[4] van der Pijl L & Dodson CH.1966.Orchid flowers: their pollination and evolution. University of Miami Press, Coral Gables, FL.

[5] Proctor HC. 1998. Effect of pollen age on fruit set, fruit weight, and seed set in three orchid species. *Can J Bot ,.*76: 420–427.

[6] Shiau YJ, Sagare AP, Chen UC, Yang SR & Tsay HS.2002. Conservation of *Anoectochilus formosanus* Hayata by artificial cross-pollination and *in vitro* culture of seeds. *Bot Bull Acad Sin,.* 43:123–130

[7] Lo SF, Nalawade SM, Kuo CL, Chen CL & Tsay HS. 2004. Asymbiotic germination of immature seeds, plantlet development and ex-vitro establishmentof plants of *Dendrobium tosaense* Makino, a medicinally orchid. *In vitro Cell Dev. Biol,.* 40:528 – 35.

[8] Murashige T & Skoog F . 1962. A revised medium for rapid growth and bioassay with tobacco cultures. *Physiol Plant,.* 15:473–479

[9] Thien L & Marcks B.1972. The floral biology of *Arethusa bulbosa*, *Calopogon tuberosus*, and *Pogonia ophioglossoides* (Orchidaceae*). Canadian Journal of Botany,.* 50: 2319–2325.

[10] Mehrhoff L. 1983. Pollination in the genus *Isotria* (Orchidaceae). *American Journal of Botany,.* 70:1444–1453.

[11] Beardsell DV, Clements MA, Hutchinson JF & Williams EG.1986. Pollination of *Diuris maculata*R. Br. (Orchidaceae) by floral mimicry of the native legumes *Daviesia* spp. and *Pultenaea scabra* R.Br. *Australian Journal of Botany,. 34*: 165–173.

[12] Sydes MA & Calder DM.1993. Comparative reproductive biology of two sun-orchids; the vulnerable *Thelymitra circumsepta* and the widespread *T. ixioides* (Orchidaceae). *Australian Journal of Botany,.* 41: 577–589.

[13] Sipes S & Tepedino VJ, 1995. Reproductive biology of the rare orchid, *Spiranthes diluvialis*: breeding system, pollination and implications for conservation. *Conservation Biology,.* 9: 929–938.

[14] Clayton S & Aizen M.1996. Effects of pollinia removal and insertion on flower longevity in *Chloraea alpina* (Orchidaceae). *Evolutionary Ecology ,.* 10: 653–660.

[15] Galetto L, Bernardello G & Rivera GL. 1997. Nectar, nectaries, flower visitors, and breeding system in five terrestrial Orchidaceae from Central Argentina. *Journal of Plant Research,.* 110: 393–403.

[16] Soguillon J & Rosario TL. 2007. Developmental characterization of pods of ground orchid *Spathoglottis plicata-alba*. *Philippine Journal of Crop Science,.* 32(2):109-117

[17] Kheawwongjun J & Thammasiri K .2008. Breeding Spathoglottis spp for commercial potted orchids. Proceedings of the International Workshop on Ornamental Plants. Eds Chomchalow N & Chantasmi V. *Acta Horticulturae* 788

[18] Kauth PJ, Vendrame WA & Kane ME . 2006. In vitro seed culture and seedling development of *Calopogon tuberosus. Plant Cell Tiss Org Cult,.* 85:91–102

[19] Stewart SL & Kane ME.2006a. Asymbiotic germination and *in vitro* seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. *Plant Cell Tiss Org Cult,.* 86:147–158

[20] Stewart SL & Kane ME. 2006b. Symbiotic germination of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. *Plant Cell Tiss Org Cult,.* 86:159–167

[21] Stewart SL & Zettler LW 2002. Symbiotic germination of three aquatic rein orchids (*Habenaria repens, H. quinqueseta, H. macroceratitis*) from Florida. *Aquat Bot ,.* 72:25–35

[22] Stewart SL, Zettler LW, Minso J & Brown PM. 2003. Symbiotic germination and reintroduction of *Spiranthes brevilabris* Lindley, an endangered orchid native to Florida. *Selbyana ,.*26:64–70

[23] Zettler LW. 1998a. Propagation of the little club-spur orchid (*Platanthera clavellata*) by symbiotic seed germination and its ecological implications. *Environmental and Experimental Botany,.* 39: 189-195.

[24] Zettler LW. 1998b. Orchid conservation in the 21st century: the value of including mycorrhizal fungi to preserve endangered species. *North American Native Orchid Journal ,.* 4: 261-269.

[25] Zettler LW & McInnis TM.1994. Light enhancement of symbiotic seed germination and development of an endangered terrestrial orchid *(Platanthera integrilabia). Plant Sci ,.*102:133–138

[26] Zettler LW, Poulter SB, McDonald KI & Stewart SL.2007. Conservation-driven propagation *of Epidendrum nocturnum* (Orchidaceae) with a mycorrhizal fungi. *HortScience* ,.42:135–139

[27] Minea M, Piluek C, Menakanit A & Tantiwiwat S. 2004. A Study on Seed Germination and Seedling Development of *Spathoglottis* Bl. Orchids. *Kasetsart J (Nat. Sci.),.* 38:141-156

[28] Kauth PJ, Johnson TR, Stewart SL & Kane ME. 2008. A classroom exercise in hand pollination and *in vitro* asymbiotic orchid seed germination. *Plant Cell Tiss Organ Cult,.* 93:223–230.

[29] Teng WL, Nicholson L & Teng MC.1997. Micropropagation of *Spathoglottis plicata. Plant Cell Reports,.* 16: 831–835

[30] Winther CL.1959. Orchid culture media and nutrient solutions.In: Winther CL (ed) The orchids: scientific studies.Ronald Press, New York, pp 589–599

[31] Arditti J.1967.Factors affecting the germination of orchid seeds. *Bot Rev,.* 33:1–197.

[32] Arditti J & Ernst R. 1984. Physiology of germinating orchid seeds. In Arditti J (ed) Orchid biology: reviews and perspectives, III. Cornell University Press, Ithaca, pp 177–222

[33] Crafts CB & Miller CO.1974. Detection and identification of cytokinins produced by mycorrhizal fungi. *Plant Physiol,.* 54:586–588

[34] Tan TK, Loon WS, Khor E & Loh CS.1998. Infection of *Spathoglottis plicata* (Orchidaceae) seeds by mycorrhizal fungus. *Plant Cell Reports ,.* 18: 14–19

[35] Senthilkumar S, Saravanakumar P, Krishnamurthy KV & Britto SJ. 2001. Morphological and structural features of mycorrhizal roots of *Spathoglottis plicata* and *Dendrobium* species. *Phyta* ,. 5(1): 24-31.