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Research Article

IN VITRO CALLUS INDUCTION AND HIGH FREQUENCY SHOOT REGENERATION FROM LEAF EXPLANTS OF *SOLANUM PUBESCENS* WILLD.

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ABSTRACT

An efficient protocol for the shoot regeneration of *Solanum pubescens* Willd. using leaf derived callus induction has been described. The optimum callusing was observed when leaf explants were cultured on MS medium augmented with 3.0 mg/l 6-benzylaminopurine (BAP) 1.0 mg/l 1-Napthaleneaceticacid (NAA) and 0.5 mg/l Indoleacetic acid (IAA). The couple were subcultured on MS medium supplemented with various concentrations of (BAP 3.0 mg/l), kinetin (KIN 1.2 mg/l) alone or in combination with Gibberellic acid (GA3 0.5 mg/l) for shoot regeneration. The highest shoot regeneration in terms of percent response was found to be 99%. The regenerated shoots were transferred in to half strength MS medium fortified with indole-3-butyric acid (IBA) for root induction. Rooted plantlets were successfully acclimatized. The protocol described here could be used for the mass multiplication and conservation of this plant.

Keywords: *Solanum pubescens; leaf; 1-Napthaleneaceticacid &6-benzylaminopurine*

1. INTRODUCTION

The traditional medicinal uses of plants to treat various diseases in humans have been long explored. Still a large number of plants exist, that have not been experimented for their medicinal uses [1]. Many of these medicinal plants provide relief of symptoms compared to that obtained from allopathic medicines; Solanum is one of the most important and largest genera of the family Solanaceae comprising of about 84 genera and 3000 species [2]. Solanum pubescens belong to the family Solanaceae commonly called as "pajarito" which is a shrub. This plant is widely distributed in tropical region including Southern part of Saudi Arabia and locally known as Millyan and Nakhab [3]. An interesting natural product, O-acetylsolasodine is extracted from Solanum umbelliferum Eschs, has showed significant DNA-damaging activity that may be due to the presence of spiroaminoacetal functional group, steroidal alkaloids are also known for topoisomerase I and II activities and DNA-damaging activity. They are known also to act as inhibitors of P-gp-mediated drug transport and multidrug resistance and therefore may serve as chemosensitizers in combination chemotherapy with the conventional cytotoxic drugs for treating multidrug resistant cancer. These biological properties have drawn our interest to investigate *S. schimperianum Hochst*. (Syn.: *S. polyanthemum Hochst*., *S. careens Dun*., S. yemense Bitter) in search of steroidal alkaloids.

Earlier phytochemical studies on this species revealed the presence of coumarin esculetin, four flavonols; astrgalin, isoquercitin, kaempferol 3-diglucoside or 3-diglucosidekaempferol and rutin, four glycoalkaloids; a-solamargine, b-solamargine, b-solamarine and c-solamarine [4]. A steroidal alkaloid is isolated from Solanum pubescens and in vitro directs multiple shoot induction from Solanum pubescens [5] and Solanum americanum L [6]. In the present investigation, an efficient and reproducible protocol has been developed for rapid multiplication of Solanum pubescens through leaf explants in order to conserve and preserve the germplasm of this season species. 2. Materials and methods

2. MATERIALS AND METHODS

2.1. Collection and authentication of plant materials

The aerial parts of the plant were collected from Sirumalai hills, a part of the Western Ghats regions, Dindigul district, Tamilnadu. The species were authentically identified and compared with herbarium specimen available in BSI, Coimbatore, Tamilnadu and India. Specimen No: BSI/SRC/5/23/2017/Tech/2985. [Fig.1].

2.2. Plant material and surface sterilization

Young branches with leaves of Solanum pubescens were covered in polythene bag and brought to the laboratory within 5 hrs and cultured after sterilization. The young leaves were collected and washed with 1% (v/v) Teepol solution for 10 min followed by washing in running tap water for 5 min. The explants were then surface-disinfected by immersion for 7 min in 0.1% (w/v) aqueous mercuric chloride solution. After three rinses in sterile distilled water, the explants were excised into 1.5 cm x 4 mm in diameter and cultured on callus induction medium.

2.3. Callus induction and shoot regeneration

In a laminar air flow cabinet, sterilized leaf explants (about 1.0 cm in length) were inoculated in culture tubes (22 × 150 mm) containing 25 ml of sterile Murashige and Skoog (MS) [7] medium with 3% (w/v) sucrose 8 g/l agar and pH adjusted to 5.8. Explants were maintained in a growth room in the dark at a temperature of 25°C. After 35 days, callus induction was evaluated, and callus fresh weight and dry weight was determined.

The basal medium was supplemented with different growth regulators in different concentrations and combinations. In the present study, two types of media were employed on the basis of growth regulator used. The first category was callus initiation and induction medium and the second include shoot differentiating medium. The former medium was fortified with various concentrations of (BAP 3.0 mg/l) alone or in combination with 2.0 mg/l (GA3). The latter consisted of different concentrations of N6-benzylaminopurine (BAP 1.0-5.0 mg/l) or GA3 (1.0-3.0 mg/l) alone or in combination with (KIN 0.5-2.5 mg/l), (NAA 0.5-2.5 mg/l). The callus was periodically subcultured on MS medium supplemented with 2.0 mg/l GA3. For shoot regeneration from callus, 50 mg calli were transferred to each culture tube. Data on percentage of calli forming shoots and mean shoot number and length of differentiated shoots were recorded after 35 days of culture. MS medium lacking growth regulators served as the control.

2.4. Shoot elongation, rooting and field transfer

The shoots below 2.5 cm in length were excised and subcultured on MS medium supplemented with 3.0mg/l BAP for shoot elongation. The shoots measuring approximately 3.0 cm in length were harvested from the shoot elongation medium and cultured on half strength MS medium supplemented with various concentrations of indole-3-butyric acid (IBA; 0.5-2.5mg/l) or NAA (0.5-3.0 mg/l)for root induction. Data were recorded for percent rooting, root number and length after 45 days of transfer on rooting medium. Plantlets with well developed roots were removed from culture tubes, washed well to remove the remnants of agar from roots and transplanted to plastic cups (6 cm diameter) containing garden soil and sand (1:1). The plantlets were placed in glasshouse set at 24 ± 2°C, 85-95% relative humidity and irradiance 60 mol m−2s-1) provided by cool white fluorescent tubes. Plants were irrigated with half-strength MS salt solution for 3 weeks and thereafter with water. After 45 days the plants were transferred to larger pots and kept under shade in a net house for another2 weeks before transferring outside under full sun to develop into mature plants.

2.5. Culture conditions

The pH was adjusted to 5.8 with 1.0 N HCl or 1.0 N NaOH before autoclaving the medium at 1.06 kg cm-2 and 121°C for 20 min. The cultures were maintained in a culture room with a 16 h/8 h light/dark photoperiod at 23 ± 2°C unless otherwise mentioned. Light was supplied at intensity of 80 mol m^{-2} s^{-1}supplied by two Philips TL 40W cool-white fluorescent lamps. Each treatment consisted of 20 tubes and all experiments repeated 3 times. The data were presented as mean and its standard deviation (mean ± SD).

3. RESULTS

3.1. Callus initiation and proliferation

Initially explants from leaf, were cultured on MS medium supplemented with different concentration of growth hormones, (auxins and cytokinins) alone or in combination for callusing. All the phytohormones produced callus singly or in combination with variable response. Therefore, MS medium without growth regulators failed to produce any callus. Callus initiation started from the cut ends of the leaf on MS medium supplemented with various concentrations of BAP 1.5mg/l) alone or in combination with 2.0mg/l IAA and 2,4-D used individually 2.0mg/l was optimum from the stage of maximum callus induction. On this medium 98.7% explants produced callus with a mean

fresh weight of 96 mg (Plate-1: a-f; Table-1). The explants showed slight expansion within one week and callus induction was mainly confined to the cut end of the explants. Friable, organogenic light yellowish calli was formed 35days after culture. The calli formed on various induction media were subcultured, multiplied and maintained on MS medium supplemented with 1.5 mg/l BAP. The addition of IAA (1.5mg/l) to 2, 4-D (2.0 mg/l) significantly increased the percent response and fresh weight.

3.2. Plant regeneration

The light yellow friable calli were transferred to MS medium supplemented with various concentrations of BAP (1.0–5.0 mg/l) or GA3 (1.0-3.0 mg/l) alone or in combination with KIN (0.5-2.5 mg/l) for plant regeneration. When callus was subcultured on to different concentrations of BAP (3.0 mg/l) or GA3 (2.0 mg/l) and NAA (1.5 mg/l) alone after a period of 35 days (Plate-II: g, h and i: Table 2). The MS medium supplemented with BAP at concentrations of 1-5 mg/l elevated the shoot numbers to 1.5, 2.8, 3.0, 3.7, 6.5, 3.8, 5.6, and 4.8 per explant. Combining BAP with GA3 further elevated shoot numbers compared to BAP or GA3 alone of the different types and combinations of plant growth regulators employed the highest response was observed in terms of % explants responding (87.8%) The second most successful combination was 4.0 M BAP and 1.0 mg/l KIN (100% explants responding with an average number of 87.8% shoots per explant).Small shoot bud primordial appeared rapidly on friable callus one week after culture in responding cultures.

3.3. Shoot elongation

Multiple shoots were transferred to shoot elongation medium, Elongation medium comparing with MS salts B5 Vitamin and different concentration of GA3 and BAP. The highest shoot regeneration in terms of percent response was found to be 2.0 mg/l GA3 and 1.5 mg/l BAP (98.6 %). The shoots elongated to a mean length of 3.8 cm with minimum 3 nodes in 4 weeks for leaf explant was observed (Plate-II: j: Table 3).

3.4. Rooting and Hardened

Although rooting was observed on half strength MS basal medium, the percent response and number of roots were low. Hence, further experiments were carried out with the half strength MS medium supplemented with NAA or IBA. The elongated shoots measuring a size of 1.0-2.5 cm were transferred to half strength MS medium supplemented with NAA (0.5-2.0 mg/l), IBA (0.5-3.0 mg/l) and IAA (0.5-2.5 mg/l). Comparatively IBA was more effective for root induction than NAA and IAA as the former resulted in optimum rooting frequency (NAA 89.2%+ IAA 75.4%) than the latter half strength MS medium supplemented with 2.0 mg/l IBA(91.3%) was the best for percentage induction (91.3%) and average number of roots per culture. The rooted shoots were success-fully transplanted to thermocol cups containing sand: soil (1:1) and acclimatized 45 days after transplantation of the 80 plants transplanted to soil 72 survived they grew well with irrigation and showed new growth after 6 weeks. (Plate-II k and l: Table- 4).

Table 1: Effect of growth regulators for Callus induction from leaf Explants of *Solanum pubescens*.

PGR (mg/L)			Leaf Explants	
			% of response	Nature of the callus
BAP				
0.5			77.4	Green compact nodular
1.0			84.7	Green compact nodular
1.5			**94.5**	Green compact nodular
2.0			84.6	Green Yellow friable
2.5			86.7	Yellow friable
IAA				
1.0			73.9	Green friable
1.5			78.5	Green friable
2.0			**97.6**	Green Yellow friable
2.5			85.7	Green Yellow friable
3.0			78.5	Green friable
IAA	**BAP**			
2.0	0.5		76.7	Green friable
2.0	1.0		84.8	Green Yellow friable
2.0	1.5		87.6	Green Yellow friable
2.0	**2.0**		**95.5**	Yellow friable
2.0	2.5		77.8	Yellow friable
BAP	**IAA**	**2,4-**D		
1.5	0.5	1.0	76.6	Green friable
1.5	1.0	1.5	81.5	Green Yellow friable
1.5	**1.5**	**2.0**	**98.7**	**Green Yellow friable**
1.5	2.0	2.5	79.8	Green friable
1.5	2.5	3.0	78.9	Green friable

***YF**-Yellow friable: **GC**- Green compact: **GCN**- Green compact nodular: **GYF**- Green Yellow friable: **GF**- Green friable*

Table 2: Multiple shoot induction responses from leaf explant of *Solanum pubescens* grown on MS medium containing cytokinins and auxin, after 25 days.

PGR (mg/L)			leaf Explants		
			% of response	No of shoot bud /explants	Shoots length Mean ± SD
BAP					
1.0			45.5	2.1±0.34	1.7±0.46
2.0			55.4	3.5±0.32	2.6±0.56
3.0			74.7	4.7±0.39	3.7±0.77
4.0			43.8	1.8±0.13	2.3±0.56
5.0			51.5	2.8±0.28	2.8±0.45
GA_3					
1.0			68.5	2.4±0.35	1.6±0.45
1.5			51.2	3.6±0.44	2.7±0.34
2.0			77.4	4.5±0.77	3.8±0.78
2.5			54.2	2.3±0.64	2.4±0.56
3.0			60.5	2.1±0.31	3.2±0.48
BAP		KIN			
3.0		0.5	56.4	2.4±0.67	1.4±0.34
3.0		1.0	57.2	2.3±0.44	1.6±1.32
3.0		1.5	78.8	3.7±0.77	2.8±1.45
3.0		2.0	65.8	3.3±0.64	1.5±0.86
3.0		2.5	73.6	2.6±0.54	1.9±0.77
BAP	**GA_3**	**NAA**			
3.0	2.0	0.5	56.5	1.6±0.48	1.6±0.44
3.0	2.0	1.0	52.3	2.7±0.68	2.3±0.56
3.0	**2.0**	**1.5**	**87.8**	**3.9±1.07**	**3.5±0.74**
3.0	2.0	2.0	76.5	2.8±0.53	2.2±0.68
3.0	2.0	2.5	73.8	2.5±0.34	3.2±0.37

Table 3: Effect of BAP and GA_3 on shoot elongation from *S. pubescens* on ½ MS medium after 35 days.

PGR (mg/l)		% response	No. of shoots per Explant Mean ± SD	Shoot length (cm) Mean ± SD
BAP	GA_3			
0.5	78.5	0.5	3.0±2.0	1.8±1.18
1.0	85.2	1.0	4.6±1.28	2.6±1.14
1.5	**98.6**	**1.5**	**7.3±1.24**	**3.8±0.95**
2.0	96.7	2.0	6.9±2.42	3.0±1.15
2.5	93.3	2.5	6.2±2.12	2.8±0.34

Table 4: Effect of different forms and concentrations of auxins on rooting in *Solanum pubesencs*

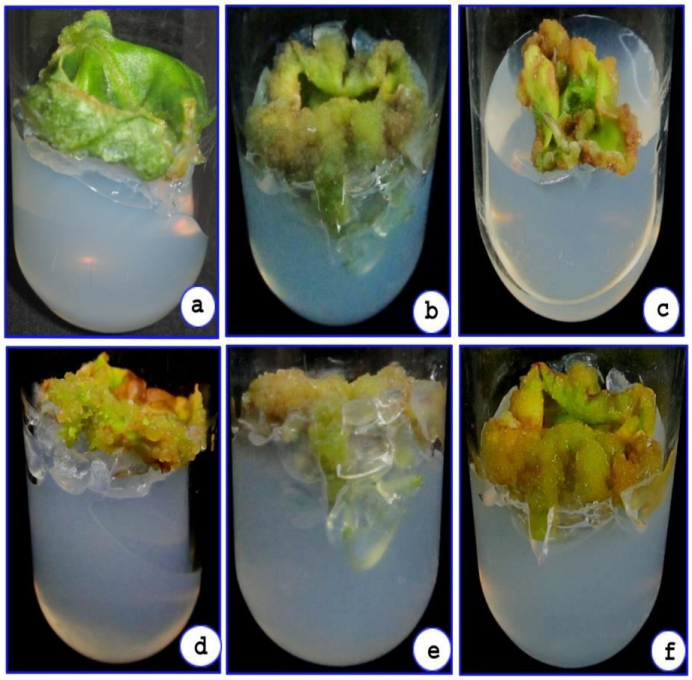
PGR (mg/l)	Leaf explant		
	% Response	Mean root numbers Mean ± SD	Mean root length (cm) Mean ± SD
NAA			
0.5	67.4	2.3±1.16	1.6±1.13
1.0	76.8	4.5±1.22	1.9±1.17
1.5	83.9	4.8±1.12	2.8±1.24
2.0	89.2	5.1±1.24	3.4±1.28
2.5	76.8	4.3±1.23	2.5±1.08
IBA			
1.0	64.7	2.1±1.26	2.0±0.26
2.0	78.5	3.4±1.34	2.7±0.43
3.0	91.3	5.4±1.42	3.6±0.65
4.0	80.3	4.6±1.16	2.8±0.54
5.0	71.4	3.5±0.91	2.4±0.49
IAA			
0.5	46.3	1.3±1.48	1.4±1.03
1.0	57.6	2.2±1.51	1.1±1.23
1.5	64.5	2.6±1.45	2.2±1.24
2.0	75.4	3.3±1.61	2.6±0.95
2.5	53.3	1.3±1.33	1.5±0.56

Fig.1: Habit of *Solanum pubescens*

4. DISCUSSION

The aim of the present investigation was to obtain high frequency shoot organogenesis from callus. There are some reports available on micropropagation of another species *Torenia fournieri* [8]. However, there is no report available on micropropagation of *S. pubescens*. Therefore, arapid micropropagation system is important for the speedy multiplication and conservation of this summer season plant.

Plate-1



*In vitro ca*llus induction: development stages from leaf explants

Fig.2: Plate 1: a & b) Green compact nodular callus on MS + BAP 1.5 mg/l, c & d) Yellow friable Callus on MS + IAA 2.0 mg/l +BAP 2.0 mg/l, e & f) Green yellow friable **Callus on MS+ BAP 1.5 mg/l + IAA 1.5 mg/l + 2, 4-D 2.0 mg/l.**

Plate-II

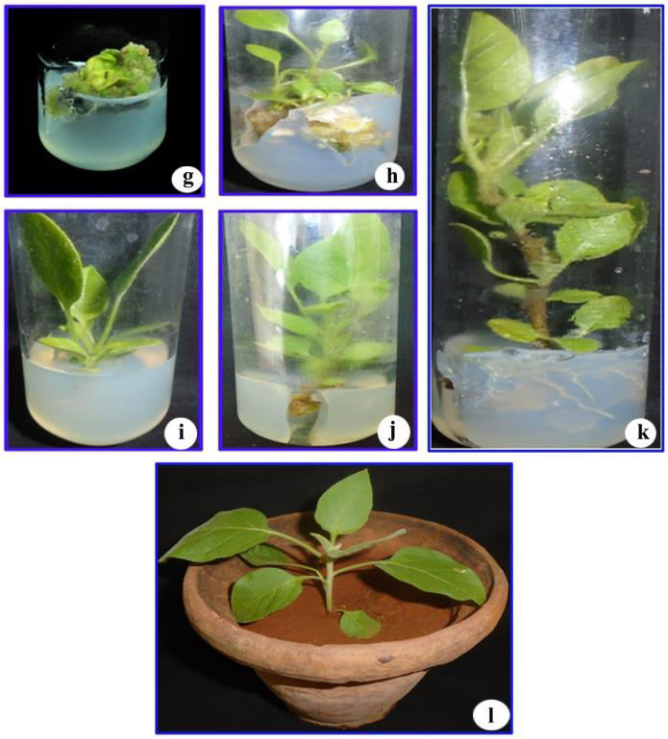


Fig. 3: (g) Callus Induction, (h) Multiple Shoot Induction, (i) Shoot Induction, (j) Shoot Elongation, (k) Root induction and (l) Hardened

We have used several explants for callus induction as a preliminary study. How-ever, the satisfactory result was obtained with internodal explants only. Therefore, only the internodal explants were used for the present study. Similar results on superiority of intermodal explantson callus induction and shoot organogenesis have been reported in other systems like *Hypencum bupleuroides*. Use of 1.5 M BAP along with either 0.5 M NAA or IAA resulted in excellent multiplication and rooting; inclusion of 0.1 M GA3to the medium containing 1.5 M BAP and NAA gave best performance. It has been reported that inclusion of BAP and GA3 in the medium enhanced shoot regeneration including shoot length in *Saussure aobvallata* and *Aconitum bolfouri* [9, 10]. Successfully optimized *in vitro* protocols required gradual acclimatization of the regenerated plantlets in the controlled environmental conditions as reviewed by the several workers [11,12].The development of long roots at low auxin levels and short roots with an increase in concentration of auxin has also been reported earlier [13]. In our experiment, addition of 3% CW produced a maximum of 87.8 Shoots of stunted growth with 100% shoot regeneration ability. Increase in concentration to 5% of CW promotes elongated shoots (0.72 cm) compared to BP (Plate-II Fig. K). Likewise, enhanced shoot development using CW has been in many Dendrobium [14-19] and in *Cymbidium pendulum* [20].

Molnar et al., [21] stated that CW was the most complex combination of compounds, contains a number of amino acids, organic acids, nucleic acids, several vitamins, sugars and sugar alcohols, plant hormones (auxins, cytokinins), minerals, and other unidentified substances and none of which alone is totally responsible for growth promoting qualities. In this study, without addition of any plant growth regulators, protocorm produced multiple shoots and roots more or less equally to cytokinins and auxins. These results suggest that complex natural supplements can efficiently support shoot induction, multiplication and multiple root formation in *Dendrobium longicornu* [22] and in a species of *Dendrobium* [23], individual NAA was reported to produce multiple numbers of shoots than cytokinins and stimulates seedling growth in *C. punctulata* 24. Multiple root inductions on shoot multiples have also been reported in *Dendrobium chrysanthum* and in *Dendrobium* sp [25].

Only few works were undertaken on the micropropagation of Medicinal plants. Of these, major works were on the micropropagation of medicinal plants such as *Bacopa monnieri* [26, 27] and *Acorus calamus*

[28,29]. For *in vitro* clonal propagation, the common explants used are the nodal segments. In the present study also, the explant selected was the leaf explant from where direct organogenesis is achieved, similar result was obtained in *N. indica* [30] also, while floral buds of *N. cristatum* produced friable callus from which organogenesis was achieved [31,32]. In this study, even though growth regulator-free MS medium was able to induce bud break and shoot formation, the number of shoots formed from single explant was found to be less in number. BAP was the single cytokinin used for the multiple shoot formation. Several reports point out the capacity of BAP for bud proliferation and multiple shoot formation in many plants such as *B. monnieri* and *Avicennia marina* (33). Averages of 65 Shoots were obtained from 2-week-old cultures in medium with 1.0 mg/L BAP while 45 shoots were obtained from per piece in 38 days of culture in *Passiflora caerulae* L. [34]. Two auxins (IBA and NAA) were tried for the root induction, both showed favorable results. It is reported earlier that the auxins at lower concentration facilitate better root formation [35]. Similarly, the increase in the rooting percentage and the better rooting in the medium containing IBA were reported in *Alnus glutinosa* [36]. Earlier reports indicate that NAA also induces callus tissue; hence, establishment of plants in the field is hindered by the interfering callus tissue [37, 38].

5. CONCLUSION

The present study describes an efficient *in vitro* regeneration of *Solanum pubescens*. Promising plant regeneration from leaf explants was influenced markedly by combinations of BAP, GA_3 and Kinetin. All the *in vitro* regenerated shoot swere rooted successfully for direct and indirect methods from *Solanum pubescens*. This study aims to develop a standard protocol to initiate multiple shoot culture at a standardized media and hormonal concentration of plant that maybe beneficial for *in vitro* large scale propagation of the plant this could reduce the time, energy, labor and cost of production of plantlets. This ensures large scale propagation of the targeted plants, which is important for the sustainable supply of plant materials to the pharmaceutical industries and for conservation of elite germplasm in nature resources. In the present investigation on *Solanum pubescens* by using leaf explants for producing of Plant Regeneration were observed.

Conflict of interest: the authors declare no conflict of interest.

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7. REFERENCES

1. Choi EM, Hwang JK. *J Ethnopharm,* 2003, **89:**171-175.
2. Yasin JN, Flora of Pakistan, Pakistan. Agricultural Research Council, Islamabad, Pakistan.1985; 168.
3. Chaudhary SA. Flora of the Kingdom of Saudi Arabia. Ministry of Agriculture and Water, Kingdom of Saudi Arabia. 2001; 2 (II).
4. Coune C, Denoel A. *Plants Med. Phytotherapy Research*, 1975, **9:**14-20.
5. Ayyadurai V, Ramar K. *International Journal of Research-Granthaalayah*, 2016, 4; **1:**23-28.
6. Ramar K, Prakash TA, Ayyadurai V, *Annals of Plant Sciences*, 2014, 3; **1:**582-587.
7. Murashige T, Skoog F. *Physiologia Plantarum,* 1962, **15:** 473-497.
8. Tanimota S, Harada H. *Journal of Plant Physiology*, 2013, **115:**11-18.
9. Pandey H, Nandi SK, Kumar A, Palni UT, Chandra B, Palni LMS. *Journal of Horticulture Science & Biotechnology*, 2004, **79:**34-41.
10. Joshi M, Dhar U. *Plant Cell Reports*, 2003, **21:**933-939.
11. Hazarika BN. *Current Science*. 2003, **85:**704-1712.
12. Chandra S, Bandopadhyay R, Kumar V, Chandra R. *Biotechnology Letters*, 2010, **32:**1199-1205.
13. Ozean G, Barshchi M, Firek S, Draper J. *Plant Cell Reports*, 1992, **11:**44-47.
14. Kitsaki CK, Zygouraki S, Ziobora M, Kintzios S. *Plant Cell Reports,* 2004, **23:**284-290.
15. Roy J, Banerjee N. *Scientia Horticulturae*, 2003, **97:** 333-340.
16. Sheelavanthmath SS, Murthy HN, Hema BP, Hahn EJ, Paek KY. *Scientia Horticulturae*, 2005, **106:**395-401.
17. Lo S, Nalawade S, Kuo C, Chen C, Tsay H. *In vitro Cell. Developmental Biology,* 2004, **40:**528-535.
18. Vyas S, Guha S, Kapoor P, Usha RI. *Scientia Horticulturae*, 2010, **123:**551-557.
19. Nambiar N, Tee CS, Maziah M, *Plant Omics*, 2012, **5:**10-18.
20. Kaur S, Bhutani KK, *HortScience. Prague,* 2012, **39:** 47-52.
21. Molnar Z, Virag E, Ordog V, *Acta Biologica Szegediensis*, 2011, **55:**123-127.

22. Dohling S, Kumaria S, Tandon P. *AoB Plants,* 2012, 2012, pls032.
23. Parvin MS, Haque ME, Akhter MF, Khaldun ABM. *Bangladesh Journal of Agricultural Research*, 2009, **34:** 411- 416.
24. Sharma SK, Tandon P, in: S.P. Vij (Ed.), Biology, Conservation and Culture of Orchids Influence of growth regulators on asymbiotic germination and early development of *Coelogyne punctulata* Lindl, Affiliated East West Press, New Delhi, India. 1986: 441- 451.
25. Vij SP, Pathak P. *Journal of Orchid Society of India*, 1989; **3:**25-28.
26. Kaur J, Nautiyal K, Pant M. *International Journal of Current Microbiology and Applied Sciences*, 2013, **2:**131-138.
27. Anu AK, Babu N, John CZ, Peter KV. *J Plant Biotechnol Biochem.* 2001, **10:**53-55.
28. Verma S, Singh N. *Eurasian Journal of Agricultural & Environmental Sciences*, 2012, **12:**1514-1521.
29. Sharma S, Kamal B, Rathi N, Chauhan S, Jadon V, Vats N, et al. *Wettst. African Journal of Biotechnology*, 2010, 9; **83:**18-22.
30. Jenks MA, Kane ME, McConnell DB. *Plant Cell Tiss Organ Cult,* 2000, **63:**1-8.
31. Niranjan MH, Sudarashana MS. *Phytomorphology,* 2000, **50:**343-344.
32. Murashige T, Skoog F. *Physiologia Plantarum*, 1962, **15:**473-97.
33. Al-Bahrany AM, Al-Khayri JM. *Plant Cell Tiss Organ Culture*, 2003; **72:**87-93.
34. Gattuso S, Severin C, Salinas A. *Journal of Tropical Medicine*, 2003; **4:**249-56.
35. Pandey P, Mehta R, Upadhyay R. *Asian Journal of Pharmaceutical and Clinical Research*, 2013, **6:**115-118.
36. Jose SC, Romero L, Janeiro LV. *Silva Fennica*, 2012, **46:** 643-54.
37. Biondi S, Thorpe TA. *Botanical Gazette*, 1982, **143:** 20-25.
38. Kapai VY, Kapoor P, Rao IU. *International Journal of Biological Technology*, 2010, **1:**1-14.