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RESEARCH ARTICLE

DIRECT ORGANOGENESIS OF A CRITICALLY ENDANGERED MEDICINAL LIANA, *COSCINIUM FENESTRATUM* (GAERTN.) COLEBR. (MENISPERMACEAE)

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ABSTRACT

An efficient and reproducible regeneration protocol *via* direct organogenesis has been established using nodal explants of *Coscinium fenestratum*. Nodal explants were inoculated on MS medium supplemented with BAP and TDZ at 2.0 and 1.0mg/L respectively were produced high frequency of shoots (91.01%). The elongated shoots subcultured on half strength MS medium supplemented with IBA alone at 0.6mg/L produced high frequency of roots (97.42%). Rooted plantlets were acclimatized and established in garden soil, sand and vermicompost in the ratio 1:1:1 with good survival rate under greenhouse conditions. This protocol can be efficiently used for mass multiplication and conservation of this critically endangered medicinal liana in its native habitat.

Keywords: *Coscinium fenestratum,* MS medium, BAP, TDZ, IBA.

1. INTRODUCTION

Plants with medicinal properties have been known for thousands of years and have been used as traditional medicines by the people to treat disease. Traditional medicine has remained as the most affordable and easily accessible source of treatment in the primary healthcare system of developing countries including India. Medicinal plants have a great importance due to their potential uses in pharmaceutical industries. Plant tissue culture is well known biotechnological tool for the rapid propagation of medicinal plants for the purpose of commercialization and conservation (1). The species which are failed in successful propagation through seeds or lacking vegetative reproduction can be multiplied through *in vitro* regeneration by employing this technique. Greater demand for these plants especially for the purpose of food and medicine is one of the causes of their rapid depletion from primary habitats. Micropropagation offers a great potential for large scale multiplication of such useful species and subsequent exploitation and also the application of this technology provides materials required for the isolation of drugs by the pharmaceutical industries without depleting natural plant resources.

The plant, *Coscinium fenestratum* (Gaertn.) Colebr. (Menispermaceae) is widely used as a medicinal plant in many Southeast Asian countries, mainly Oorali tribes of Idukki district and Kaadar tribes of Thrissur district for fever, muscle pain, abdominal pain, inflammation, ulcers, wounds, jaundice, burns, skin diseases, snake bite poisoning and diabetic food supplements (2). It is a main source of berberine in southern part of India (3,4). It is estimated that about 114 tons of woody stem bark of this species is extracted annually from the Western Ghats. Due to these wide medicinal importances, this species is severely exploited from its wilds in the western part of Tamil Nadu, India (5). In light of this fact, the present study was aimed to carry out the *in vitro* regeneration studies in the endangered medicinal liana, *C. fenestratum*.

Fig. 1. Habit of the study species, *Coscinium fenestratum.*

2. MATERIALS AND METHODS

2.1. Plant material and surface sterilization

Healthy and immature nodal segments of the study species, *C. fenestratum* were collected from Velliangiri hills, Coimbatore district, Tamil Nadu, India is used as explant (Fig. 1). These segments were washed under running tap water followed by treatment with a surfactant, tween 20 (5% w/v) for 5 min. To eliminate the fungal contamination, the explants were treated with carbendazim (5% w/v) fungicide also for 5 min followed by 2 or 3 rinses in sterile double distilled

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water. To eliminate bacterial contamination, explants were also treated with antibiotics (5% w/v) (Ampicillin and Rifampicin) for 5 min followed by three rinses in sterile double distilled water. Furthermore, surface sterilization was carried out by dipping the explants in 0.1% mercuric chloride ($HgCl_2$) for 3 minutes followed by 3-4 rinses in sterilized double distilled water to remove traces of $HgCl_2$.

2.2. Establishment of culture and shoot regeneration

For the shoot induction, nodal segments were inoculated vertically on MS medium (6) containing different concentrations and combinations of growth regulators such as BAP (ranging from 0.5 – 3.0 mg/L), TDZ (0.5 mg/L and 1mg/L), 2,4-D (0.5mg/L) and Kn (0.5mg/L). These cultures were incubated at 25±2°C temperature under light. The percentage of explants responding, number of shoots per explant and length of the shoots can be recorded after 40 days of culture.

2.3. Rooting of in vitro derived shoots

The proliferated shoots of 2-3cm length will be excised from the culture and transferred to half strength MS medium supplemented with various concentrations of auxin (IBA and IAA ranging from 0.2 to 1.6 mg/L and NAA ranging from 0.5 to 3.0 mg/L) for root formation. After two weeks, the rooting attributes *viz.,* the per cent shoots responding for rooting, number of roots per shoot and root length will be measured.

2.4. Percentage of response

The percentage of response of explants can be calculated as per the following formula:

$$\text{Percent response} = \frac{\text{Number of explants responded}}{\text{Number of explants cultured}} \times 100$$

2.5. Hardening of plantlets in the greenhouse

Rooted shoots will be thoroughly washed to remove the adhering gel followed by dipping in 1% (w/v) fungicide, Bavistin solution for 10-15min to remove the fungal contamination. The plantlets will be transplanted to various types of sterilized potting media as detailed below:

Hardening medium composition	**Proportion of components (v/v)**
Garden soil + sand + vermicompost	1:1:1
Red soil + sand + vermicompost	1:1:1
Vermicompost + red soil	1:1
Red soil + sand	1:1
Decomposed coir waste + perlite + vermicompost	1:1:1

Twenty five plantlets per potting mixture will be tested in the green house and growth rate can be calculated after 30 days of hardening. After acclimatization, the plantlets will be exposed to natural environmental conditions.

2.6. Observations and data analysis

The cultures were regularly subcultured on fresh medium after 4-5 week interval. The experiments were repeated thrice with twenty five replicates per treatment. All the values were expressed as mean ± standard deviation (SD) of two determinations and subjected to one-way analysis of variance (ANOVA) followed by *post hoc* Duncan's multiple range test using SPSS software (version 9, SPSS Inc., Chicago, USA). $p < 0.05$ was chosen as the criterion for statistical significance.

3. RESULTS

3.1. Shoot regeneration

The response of nodal explants for direct shooting was varied according to the combinations and concentrations of the growth regulators, BAP, TDZ, 2,4-D and Kn in the MS medium. The MS medium containing the growth regulators, BAP and TDZ at 2.0 and 1.0mg/L respectively exhibiting high degree of shooting characters like per cent explants response to shoot initiation (91.01%) and number of shoots per explant (3.87 shoots/explant) and shoot length (5.92cm) (Table 1, Fig. 2(b-g)). Due to better response of node for direct shooting, the node derived shoots were taken for further studies on rooting and hardening to derive the elite plantlets.

3.2. Rooting of in vitro derived shoots

The rooting attributes such as percentage of shoot produced roots, number of roots produced per shoot and root length have been significantly varied depending on the concentration of individual supplementation of auxins like IBA, IAA and NAA in half strength MS medium for the study species, *C. fenestratum* are exhibited in Tables 2. For the node derived shoots, the highest percentage of root formation (97.42%), greater number of roots per shoot (4.78roots/shoot) and length (4.17cm) were observed in half strength MS medium supplemented with IBA alone at 0.6mg/L (Table 2, Fig. 2h). However, the rooting percentage was significantly reduced at the lower concentrations of IAA and NAA.

Table 1. Effect of different concentrations and combinations of growth regulators in the MS medium on direct shooting (shoot initiation, shoot number and shoot length) from the nodal explant, *Coscinium fenestratum.*

Growth regulator (mg/L)				Culture response (%)	Number of shoots/explant*	Shoot length (cm)
BAP	TDZ	2,4-D	Kn			
0.5	0.5	0.0	0.0	33.10	0.81^{d}±0.09	2.24^{d}±0.31
1.0	0.5	0.0	0.0	55.26	1.51^{c}±0.26	3.41^{c}±0.41
1.5	0.5	0.0	0.0	59.15	1.76^{c}±0.20	4.02^{b}±0.62
2.0	0.5	0.0	0.0	61.98	1.87^{b}±0.28	4.05^{b}±0.17
2.5	0.5	0.0	0.0	64.12	1.98^{b}±0.32	4.23^{b}±0.62
3.0	0.5	0.0	0.0	75.11	2.15^{b}±0.47	5.07^{b}±0.49
0.5	1.0	0.0	0.0	78.46	2.55^{b}±0.41	5.11^{ab}±0.36
1.0	1.0	0.0	0.0	80.91	2.95^{a}±0.11	5.62^{a}±0.43
1.5	1.0	0.0	0.0	83.67	3.28^{a}±0.39	5.79^{a}±0.31
2.0	1.0	0.0	0.0	91.01	3.87^{a}±0.51	5.92^{a}±0.56
2.5	1.0	0.0	0.0	85.23	3.57^{a}±0.58	5.63^{a}±0.83
3.0	1.0	0.0	0.0	82.34	3.21^{a}±0.53	5.51^{a}±0.71
0.5	0.0	0.5	0.0	30.65	0.79^{d}±0.11	1.27^{e}±0.11
1.0	0.0	0.5	0.0	42.89	1.07^{cd}±0.25	2.35^{d}±0.12
1.5	0.0	0.5	0.0	46.67	1.23^{c}±0.31	3.56^{c}±0.22
2.0	0.0	0.5	0.0	52.24	1.35^{c}±0.43	3.97^{c}±0.31
2.5	0.0	0.5	0.0	56.28	1.62^{c}±0.31	3.82^{c}±0.27
3.0	0.0	0.5	0.0	48.12	1.26^{c}±0.57	2.14^{s}±0.22
0.5	0.0	0.0	0.5	24.65	0.72^{d}±0.15	1.78^{e}±0.15
1.0	0.0	0.0	0.5	29.80	0.77^{d}±0.63	1.82^{e}±0.25
1.5	0.0	0.0	0.5	35.17	1.00^{cd}±0.05	1.96^{de}±0.35
2.0	0.0	0.0	0.5	43.04	0.97^{cd}±0.61	2.01^{de}±0.26
2.5	0.0	0.0	0.5	47.24	1.01^{cd}±0.33	2.13^{d}±0.43
3.0	0.0	0.0	0.5	31.00	0.85^{d}±0.18	2.01^{de}±0.31

*Values are presented as the mean ± standard deviation (SD) of three independent experiments.
Values not sharing a common letter in a column are significantly different (p< 0.05).

Table 2. Effect of different concentrations and combinations of growth regulators in the half strength MS medium on rooting percentage, root number and root length after subculturing of node derived shoots of the species, *Coscinium fenestratum.*

Growth regulator (mg/L)			Culture response (%)	Number of roots / shoot*	Root length (cm)*
IBA	IAA	NAA			
0.2	0.0	0.0	76.12	3.42^{b}±0.05	3.44^{b}±0.11
0.4	0.0	0.0	90.11	4.45^{a}±0.23	3.95^{ab}±0.12
0.6	0.0	0.0	97.42	4.78^{a}±0.18	4.17^{a}±0.13
0.8	0.0	0.0	89.14	4.14^{ab}±0.14	4.09^{a}±0.12
1.0	0.0	0.0	81.12	3.97^{ab}±0.18	3.92^{ab}±0.14
1.2	0.0	0.0	72.21	3.56^{b}±0.43	3.89^{ab}±0.23
1.4	0.0	0.0	68.47	3.23^{bc}±0.26	3.67^{b}±0.38
1.6	0.0	0.0	55.09	2.22^{c}±0.78	3.01^{bc}±0.87
0.0	0.2	0.0	25.12	0.54^{e}±0.23	1.25^{d}±0.34
0.0	0.4	0.0	39.16	1.12^{cd}±0.36	2.05^{de}±0.16
0.0	0.6	0.0	55.01	2.21^{c}±0.37	1.82^{d}±0.37
0.0	0.8	0.0	61.23	3.58^{b}±0.43	2.59^{c}±0.24
0.0	1.0	0.0	62.25	3.71^{b}±0.15	3.06^{b}±0.21
0.0	1.2	0.0	55.09	2.38^{c}±0.14	2.75^{c}±0.25
0.0	1.4	0.0	51.97	2.21^{c}±0.54	2.53^{cd}±0.42
0.0	1.6	0.0	35.11	0.91^{d}±0.32	0.84^{e}±0.11

0.0	0.0	0.5	38.85	$0.76^{d}\pm0.41$	$0.65^{e}\pm0.22$
0.0	0.0	1.0	43.31	$0.72^{d}\pm0.32$	$2.21^{c}\pm0.18$
0.0	0.0	1.5	54.16	$2.43^{c}\pm0.41$	$2.62^{c}\pm0.13$
0.0	0.0	2.0	63.42	$3.51^{b}\pm0.27$	$2.91^{bc}\pm0.28$
0.0	0.0	2.5	57.12	$2.63^{c}\pm0.11$	$2.56^{c}\pm0.17$
0.0	0.0	3.0	45.05	$1.15^{cd}\pm0.16$	$1.06^{de}\pm0.11$

*Values are presented as the mean ± standard deviation (SD) of three independent experiments.
Values not sharing a common letter in a column are significantly different ($p< 0.05$).

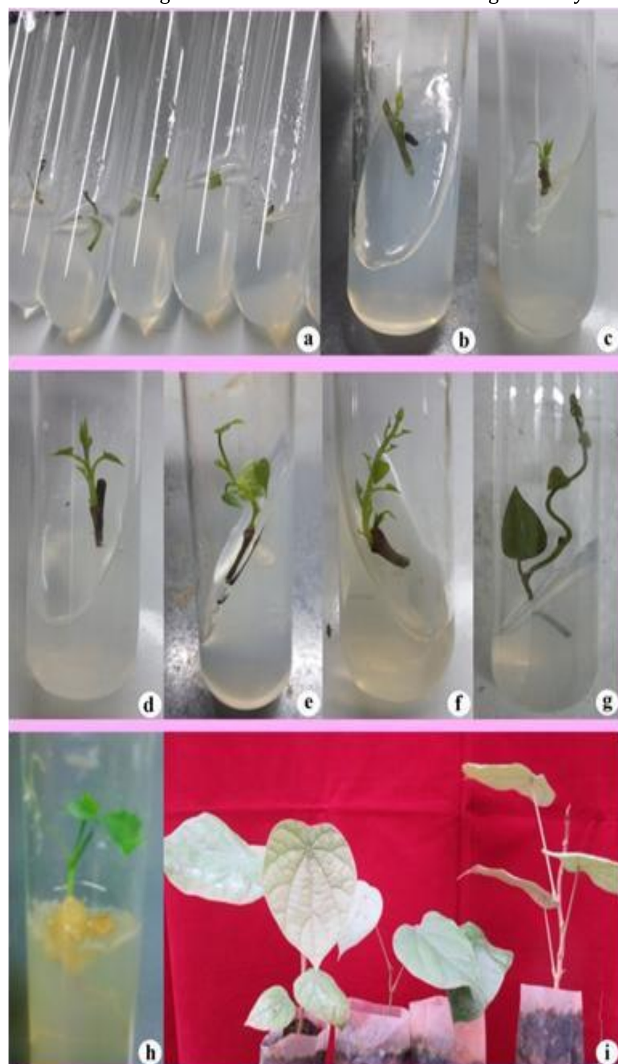

Fig. 2. Stages in *in vitro* regeneration of nodal explant of *Coscinium fenestratum* through direct organogenesis.

a) - Inoculation of nodal explant on MS medium supplemented with BAP and TDZ at 2.0mg/L and 1.0mg/L respectively.
b) & c) - Initial stage of shoot formation on MS medium supplemented with BAP and TDZ at 2.0mg/L and 1.0mg/L respectively.
d), e), f) & g) - Effective shoot formation on MS medium fortified with BAP and TDZ at 2.0mg/L and 1.0mg/L respectively.
h) - Root induction of *in vitro* derived shoots on MS medium containing IBA alone at 0.6mg/L.
i) - An acclimatized potted plantlets in the hardening medium composed by garden soil, sand and vermicompost in the ratio of 1:1:1 by volume.

3.3. Acclimatization

Hardening experiments were conducted for the study species by using various hardening media to determine the survivability rate of plantlets. For the node derived (88%) *in vitro* regenerated plantlets, the survivability rate was significantly higher in the hardening medium composed by garden soil, sand and vermicompost in the ratio of 1:1:1 by volume followed by the hardening medium consisting of red soil, sand and vermicompost in the ratio of 1:1:1 by volume for node derived (78%) *in vitro* regenerated plantlets (Table 3, Fig. 2i).

Table 3. Effect of different composition of hardening medium on survivability rate of node derived plantlets of the species, *Coscinium fenestratum.*

Hardening medium (v/v)	Number of plantlets transferred	Number of plantlets survived	Survivability (%)
Garden soil + sand + vermicompost (1:1:1)	50	44	88
Red soil + sand + vermicompost (1:1:1)	50	39	78
Vermicompost + red soil (1:1)	50	25	50
Red soil + sand (1:1)	50	22	44
Decomposed coir waste + perlite + vermicompost (1:1:1)	50	35	70

4. DISCUSSION

Generally, *in vitro* regeneration techniques have been found to be effective in circumventing cross ability barriers encountered in conventional methods. A wide range of Menispermaceae plants have now been successfully propagated using *in vitro* techniques (*Tinospora cordifolia* (7,8), *Tinospora formanii* (9) and *Cissampelos pareira* (10). This technique facilitates the introduction of successfully produced *in vitro* plantlets into the suitable micro-sites in natural communities to enhance the population of valuable plant species which have failed/less efficient in natural reproduction processes. The supplementation of growth hormones like auxins and cytokinins individually or in combinations with MS medium at different concentrations is having varied response with respect to callus formation and organogenesis

in many plant species (11). In the present study, the *in vitro* regeneration responses of the study species, *Coscinium fenestratum* are discussed below.

The subculturing of node for shoot formation of the species, *Coscinium fenestratum* onto the MS medium containing BAP (2.0mg/L) + TDZ (1.0mg/L) was found to be most effective. Cytokinins are one of the most important hormones for shoot proliferation in many plant species (12). Further, a wider survey of literature suggests that BAP is the most reliable and effective cytokinin (13). Kyozuka (14) reported that cytokinins can induce activation of meristems and cause shoot proliferation. Thomas and Gangaprasad (15) already reported the requirement of more quantity of BAP for effective shoot formation in the species, *Enicotema axillare.* Amin *et al.* (16) explained that in many cases, the growth regulators like many kinds of cytokinins served as better available source of nitrogen for organogenesis particularly shoot formation. Similarly, in *Vitex negundo,* Sahoo and Chand (17) reported BAP as the most effective growth hormone for shoot bud induction. Multiple shoots were formed from epicotyl explants of *Coscinium ferestratum* on MS medium supplemented with cytokinins (18,19). Huetteman and Preece (20) reported that Thidiazuron is a potent cytokinin hormone used for the growth of woody plants in tissue culture.

The root induction during the subculturing of node derived shoots was significantly higher in the half strength MS medium with IBA at 0.6mg/L. It indicates that the growth regulator, auxin is most essential for rooting attribute. Similar trend of results have been reported in other Menispermaceae member, *Tinospora cordifolia* (8). Tanimoto (21) have already reported the importance of auxins in the root formation during the subculturing of secondary explants. Similar kind of observation on the low level requirement of auxins for better rooting was reported in many species (22-24).

Hardening is a crucial step prior to transplantation of plant to soil. The well developed plantlets of the study species were acclimatized in various potting media. The node derived plantlets were responded well in the medium containing garden soil, sand and vermicompost. It may be due to the presence of suitable physical and chemical conditions of respective potting media for the survivability of these study species. The same hardening medium compositions were used by Jamuna and Paulsamy (25,26) for the growth of the medicinal plant, *Hypochaeris radicata.* These specific hardening media was also recommended for the micropropagation of epicotyl explants of the study species, *Coscinium fenestratum* for better survival in the field and they were morphologically similar to the mother plants (19).

From the present study, it can be concluded that the auxin and cytokinin interact synergistically to control the balance of cell division and differentiation. The protocol developed can be used to regenerate the species massively and hence for commercial purpose.

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REFERENCES

1. Kitto, S.L. 1997. Commercial micropropagation. *Horticultural Science* **32**: 1012-1014.
2. Ravishankar, V.R., P.S. Rajesh, H.M. Kim, 2013. Medicinal use of *Coscinium fenestratum* (Gaertn.) Colebr.: an short review. *Oriental Pharmacy and Experimental Medicine* **13**(1): 1-9.
3. Chitra K, Sujatha K, Polisetti H, Karri S, Reddy CU (2011) Standardization of *Coscinium fenestratum* with reference to berberine by high performance thin layer chromatography. Res J Pharm Biol Chem Sci 2:226–229.
4. Arawwawala, L.D.A.M. and W.A.N. Wickramaarachchi, 2012. Berberine content in *Coscinium fenestratum* (Gaertn.) Colebr grown in Sri Lanka. *Pharmacologia* **3**(12): 679-682.
5. Tushar, K.V., Satheesh George, A.B. Remashree and Indira Balachandran, 2008. *Coscinium fenestratum* (Gaertn.) Colebr. – A review on this rare, critically endangered and highly traded medicinal species. *Journal of Plant Sciences* **3**(2): 133-145.
6. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco cultures. *Physiologia Plantarum***15**(3): 473-497.
7. Patel, M.P. and K.B. Ishnava, 2014. *In vitro* study of *Tinospora cordifolia* (Wild.) Miers (Menispermaceae) – a multipurpose plant, by using different plant bark extracts for secondary metabolite production. *Journal of*

Herbs, Spices and Medicinal Plants **20**(4): 341-349. DOI: 10.1080/10496475.2013.876482.

8. Sivakumar, V., M.S. Dhana Rajan, A. Mohamed Sadiq and M. Jayanthi, 2014. *In vitro* micropropagation of *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms – An important Medicinal Plant. *Journal of Pharmacognosy and Phytochemistry* **3**(2): 5-10.
9. Sheema, D.P., M. Najla, E.S. Swetha, P.S. Udayan and K.K. Elyas, 2017. Callus induction and organogenesis from *Tinospora formanii* Udayan and Pradeep: A rare endemic plant. *An International Journal of Tropical Plant Research* **4**(1): 71-76.
10. Gokul, H. and D.H. Tejavathi, 1997. *In vitro culture and production of alkaloids in Cissampelos pareira* L. *(Menispermaceae).* Symposium, Emerging trends in plant tissue culture and molecular biology, Hyderabad. 266-272.
11. Tripathi, L. and J.N. Tripathi, 2003. Role of biotechnology in medicinal plants. *Tropical Journal of Pharmaceutical Research* **2**(2): 243-253.
12. Jaya, S. and V. Ranjana, 2012. Effect of different culture media on shoot induction of *Mallotus philippenesis* (Lam) M Arg. *Indian Journal of Fundamental and Applied Life Sciences* **2**(3): 59-64.
13. Rajesh, P., C. Ramesh, C.K. Ugam, M. Maneesh and S. Navin, 2008. *In vitro* clonal propagation of bael (*Aegle marmelos* Corr.) CV. CISH-B1 through enhanced axillary branching. *Physiology and Molecular Biology of Plants* **14**(4): 337-346.
14. Kyozuka, J. 2007. Control of shoot and root meristem function by cytokinin. *Current Opinion in Plant Biology* **10**(5): 442–446.
15. Thomas, S. and A. Gangaprasad, 2005. Rapid clonal multiplication through *in vitro* axillary shoot proliferation of *Enicostema axillare* (Lam.) Raynal – A rare medicinal plant. *Indian Journal of Botanical Research.* **1**(1): 115-120.
16. Amin, M.N., M.M. Rahman and M.S. Manik, (2003). *In vitro* clonal propagation of *Paederia foetida* L. - A medicinal plant of Bangladesh. *Bangladesh Association for Plant Tissue Culture & Biotechnology* **13**(2): 117-123.
17. Sahoo, Y. and P.K. Chand, 1998. Micropropagation of *Vitex negundo* L., a woody aromatic medicinal plant shrub, through high frequency axillary shoot proliferation. *Plant Cell Reports* **18**(3-4):301-307.
18. Warakagoda, P.S., S. Subasinghe and M.T.K. Gunasekare, 2017. *In vitro* clonal propagation of *Coscinium fenestratum* (Gertn.) Colebr. (Weniwel) through nodal explants. *Journal of the National Science Foundation of Sri Lanka.* **45**(2): 133-141
19. Senarath, W.T.P.S.K., 2010. *In vitro* propagation of *Coscinium fenestratum* (Gaertn.) Colebr. (Menispermaceae) – an endangered medicinal plant. *Journal of the National Science Foundation of Sri Lanka.* **38**(4): 219-233.
20. Huetteman, C.A. and J.E. Preece, 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell, Tissue and Organ Culture* **33**: 105-119.
21. Tanimoto, E., 2005. Regulation of root growth by Plant Hormones – Roles for Auxin and Gibberellin. *Critical Reviews in Plant Sciences* **24**(4): 249-265.
22. Ananthi, P., B.D. Ranjitha Kumari and A. Ramachandran, 2011. *In vitro* propagation of *Rorippa indica* L. from nodal and shoot tip explants. *International Journal of Biotechnology Molecular Biology Research* **2**(3): 51-55.
23. Hoang Loc, N. and H. Van Kiet, 2011. Micropropagation of *Solanum hainanense* Hance. *Annals of Biological Research* **2**(2): 394-398.
24. Verma, O.P. 2012. Standardization of auxin concentration for root induction in *Chrysanthemum morifolium. Advances in Applied Science Research* **3**(3):1449-1453.
25. Jamuna, S. and S. Paulsamy, 2014a. *In vitro* propagation and evaluation of antioxidant properties of the medicinal plant species, *Hypochaeris radicata* L. (Asteraceae) distributed in Nilgiris, the Western Ghats. *Asian Journal of Biomedical and Pharmaceutical Sciences* **4**(31): 29-37.
26. Jamuna, S. and S. Paulsamy, 2014b. Standardization protocol for the micropropagation of seldomly present medicinal herb, *Hypochaeris radicata* L. *via* indirect organogenesis. *Researc0068 in Biotechnology* **5**(3): 1-9.