Research Article

Influence of Various Carbon Sources and Organic Additives on *In Vitro* Growth and Morphogenesis of *Leptadenia reticulata* (Wight & Arn), A Valuable Medicinal Plant of India

Sudipta KM[1]*, Kumara Swamy M[2], Anuradha M[1, 2, 3]
[1]Department of Biotechnology, Acharya Nagarjuna University, Nagarjunanagar, Guntur, India.
[2]Padmashree Institute of Management and Sciences, Kommagatta, Bangalore, India.
[3]Rishi Foundation, #234, 10th C main, 1st Block, Jayanagar, Bangalore, India.
***Corresponding author's E-mail:** su_sudeepta@yahoo.co.in

Accepted on: 30-06-2013; **Finalized on:** 31-07-2013.

ABSTRACT

Leptadenia reticulata is a source of several bioactive and medicinal compounds. Unrestricted exploitation of its natural resource has included this species as one among the threatened and endangered species in India. For conservation and sustainable utilization through tissue culture seems to be a promising alternative to overcome the above said situation. The present work was carried out to study the effect of various carbon sources and natural additives on *in vitro* growth and morphogenesis of *L. reticulata*. Among carbon sources, maximum number of multiple shoots (5.20±0.21cm) and shoot length (5.1±0.16cm) was observed on Murashige and Skoog (MS) media supplemented with 2% sucrose. The maximum chlorophyll content (1.76±0.40mg/g tissue) was observed on MS media supplemented with 2% sucrose followed by 2% table sugar (1.42±0.5mg/ml). Highest protein content (16.3±0.24mg/ml) and total carbohydrate content (15.7±0.30mg/ml) was observed on media containing 3% sucrose. Among organic additives, 10% coconut water in MS media resulted highest number of multiple shoots (6.20±0.10), shoot length (4.72±0.06cm). Other parameters like chlorophyll content (1.7±0.02), total protein content (22.4±0.08) and total carbohydrate content (22.5±0.01) were also found to be superior on 10% coconut water supplemented media. This is the first report on the use of cheaper carbon source and organic additives in tissue culture studies of *L. reticulata*. This study has derived a low cost protocol by using cheaper substitutes to medium for *in vitro* multiplication of *L. reticulata* on a large scale which is vital for tissue culture industries.

Keywords: Coconut water, *L. reticulata,* Organic additives, Shoot proliferation, Table sugar.

INTRODUCTION

Leptadenia reticulata (Retz) wight & Arn. belongs to family Asclepiadaceae is an important medicinal plant with synonyms in Indian language as Jivanti, Dori, Swarn Jivanti etc. This plant species is distributed in tropical and subtropical parts of Asia, Africa, Burma, Srilanka, Philippines and Madagascar. In India it is found in Gujarat, Punjab, Himalayan ranges, konkon, Nilgiris and Southern part of India. Because of its multiple uses in curing several diseases such as hematopoesis, emaciation, cough, dyspnoea, fever, burning sensation, night blindness *L. reticulata* draws the attention for utilizing it as drugs by pharmaceutical industries. It is regarded as good cure for tuberculosis and effectively used for several ear and nose problems. Methanolic extract of this plant is having antibacterial activity and is used for the treatment of skin infection and wounds.[1] Anjaria[2] tried Leptaden tablets (a formulation from *Leptadenia reticulata*) on some clinical cases and reported its beneficial use as galactogogue for increasing milk.

Commercial exploitation and conventional propagation is hampered due to its poor seed viability, low rate of germination and seasonal availability. Till date, there are only few reports on the micro propagation of *Leptadenia reticulata*[3-5] has been reported. The cost of components in tissue culture medium has been the main concern for commercial laboratories which add to the cost of plant produced.[6] Many researchers have substituted various components in to the media such as gelling agents, additive compounds and sucrose, as objective to minimize the cost of the media. Various factors are considered for the growth and multiplication of shoots *in vitro.* Exogenous carbon sources serve as very important parameter in the medium as main energy source and osmotic agents to support the growth of plant tissues.[7,8] There have been various opinions on the advantageous effects of carbon sources (sucrose, fructose, glucose, table sugar etc.) on the growth of plants *in vitro*. In general, most of the tissue culture studies are performed using sucrose as the sole carbon source due to its efficient uptake across the plasma membrane.[9] However it is also reported by many researchers that different carbon sources such as glucose fructose and maltose have varying effect on tissue morphogenesis and may exhibit positive result.[10-13] Use of fructose in the medium results in hyper-hydricity which leads to low chlorophyll contents and abnormal nitrogen and sugar metabolism.[14] Table sugar is used as an alternative low cost medium component for *in vitro* micropropagation of potato.[15]

The rate of *in vitro* multiplication can be increased by supplementing various natural additives. *In vitro* growth and regeneration of the plant can be improved by adding a small amount of organic nutrients to the medium.[16] Coconut milk, the extract of white solid endosperm of mature coconut is also used for inducing growth and morphogenesis without the need of a defined formulation.[17,18] Use of other natural supplements like

tomato juice, orange juice, malt extract, yeast extract, casein hydrolysate in the media for promoting multiplication and development of *in vitro* cultures have been demonstrated by many authors.[19-22]

From the above report, it is evident that various carbon sources and organic additives affect the growth of *in vitro* cultured plants differently. Modification or replacement of culture media composition with a suitable alternative is very important parameter to reduce the cost of plant and facilitate superior quality of plants. Therefore the present work was undertaken to evaluate the effects of various carbon sources and organic additives on the growth, multiplication and various physiological parameters of *L. reticulata* which may helpful in reducing of cost of production media for micropropagation. Since this kind of study has not been reported so far in this plant, here we made an attempt to reduce the cost of the tissue culture medium by substituting low cost substitutes as natural additives and carbon sources which definitely contribute towards the reduction of cost in plant production by tissue culture industries.

MATERIALS AND METHODS

Nodal segments and shoot tips were collected from healthy, actively growing *Leptadenia reticulata* plants maintained in herbal garden of Padmashree Institute of Management and Sciences, Bangalore. The collected plant materials were washed thoroughly under running tap water and rinsed in few drops of Teepol (Glaxo India Ltd, Mumbai, India) for 5 min. They were surface sterilized with 0.1% (w/v) $HgCl_2$ for 10 min and washed thoroughly with sterile distilled water. The whole process of surface sterilization was performed in aseptic condition in a laminar air flow chamber. For axillary bud initiation, the explants were implanted on Murashige and Skoog (MS)[23] medium supplemented with 0.25 mg/L 6-benzylaminopurine (BA) and kinetin (KN) as described in our earlier study.[24] The pH of the medium was adjusted to 5.7 prior to autoclaving at 121ºC for 20 min. Cultures were maintained at a temperature of 25±2ºC under 16 h light/ 8 h dark photoperiod and sub cultured every 4 weeks. Multiple shoots regenerated on this medium were used for further studies. Established cultures were subjected to sub culturing in same media.

Uniform proliferated shoots (4-5 cm in length) resulted from direct organogenesis were transferred to MS basal medium supplemented with 0.25 mg/L BA and kinetin (KN). The media was further supplemented with different carbon sources viz., sucrose, glucose, fructose, commercially available table sugar (1, 2 and 3%) and sugarcane juice (10, 20 and 30%). The media was also supplemented with 5, 10 and 20% organic growth supplements viz., coconut water, banana homogenate, tomato juice, carrot juice, papaya juice. Data were recorded for the following parameters such as percent frequency of response, number of multiple shoots, shoot length (cm), callus intervention. The total chlorophyll content of the regenerated plantlets was measured by the method[25] and was expressed in mg/g tissue. The total protein content of the regenerated plantlets was measured using standard Lowry's method[26] and was expressed in mg/ml. The total carbohydrate content of the regenerated plantlets was also measured using Anthrone method explained by Sadasivam and Manickam[27] and was expressed in mg/ml. The experiments were set up in completely randomized design with each treatments replicated thrice. Data obtained after 30 days of culture were subjected to analysis of variance and Duncan's multiple range test (DMRT) using SAS software.

RESULTS AND DISCUSSION

Effect of carbon source on multiplication

Growth of *in vitro* cultures of *Leptadenia reticulata* was strongly influenced by the different carbon sources at various concentrations (Table 1). All the carbon sources other than sucrose and table sugar showed inferior response. When sucrose and table sugar were supplemented in MS media, there were no significant differences observed in terms of growth response and multiple shoot proliferation. Sucrose at 2% level showed the best result (72%). However no significant difference was also noticed in term of response while the media is supplimented with 2% table sugar. The highest number of multiple shoots without basal callus was observed on the media supplemented with 2% sucrose (5.20±0.21) followed by 2% table sugar (4.90±0.46). The lowest percentage of response (10%) and minimum multiple shoot number (1.30±0.26) were recorded in MS medium supplemented with 1% glucose.

Though highest number of multiple shoots (5.52±0.24) were generated by media supplemented with 3% sucrose basal callus was induced which is not preferred in case of direct regeneration (Figure 1). A marked difference with decline rate of multiplication was observed when the culture was maintained with fructose, glucose and sugar cane juice. Similar results are reported in *Pinus sylvestris* that the addition of fructose to the medium results in hyperhydricity which leads to low cellulose and chlorophyll contents, less ethylene production and abnormal nitrogen and sugar metabolism.[8,14] Shoots induced on MS medium supplemented with 2% table sugar evidenced maximum shoot length (5.3 ± 0.18cm) as compared to any other carbon sources. The use of 2% sucrose showed 5.1 ± 0.16cm shoot length which is second highest. However, at higher concentrations of carbon sources in the medium resulted callus proliferation than shoot differentiation. The decrease in shoot multiplication at higher concentration of carbon sources may be due to the inhibition of organogenesis and induction of callus proliferation. Similar results are reported in *Pogostemon cablin.*[28]

Effect of various carbon sources were also studied for physiological changes in the plant (Table 2). The higher chlorophyll content (1.76±0.4mg/g tissue) was observed when 2% sucrose was used in MS medium. The highest

total protein content (16.3 ± 0.24mg/ml) and carbohydrate content (15.7 ± 0.30mg/ml) was obtained in the media supplemented with 3% sucrose. This could be attributed to sugar accumulation at higher concentration. However the use of table sugars in the media showed comparably good result (Table 2).

Table 1: Effect of carbon source on morphogenesis of nodal explants of *L. reticulata.*

MS+0.25mg/l BAP + Carbon Sources	Concentration	Percent frequency of response	No of multiple shoots± S.E.	Shoot length± S.E.	Callus
Sucrose	1%	61±0.82^{b}	3.86±0.23^{abc}	4.6±0.14^{abc}	-
	2%	72±0.45^{a}	5.20±0.21^{abc}	5.1±0.16^{a}	-
	3%	51±0.32^{c}	5.52±0.24^{a}	4.6±0.21^{abc}	+
Fructose	1%	32±0.14^{fg}	2.11±0.34^{bc}	3.4±0.23^{de}	-
	2%	44±0.46^{cd}	2.16±0.42^{bc}	3.6±0.18^{de}	+
	3%	38±0.54^{def}	1.64±0.38^{bc}	3.5±0.17^{de}	+
Glucose	1%	10±0.61^{h}	1.30±0.26^{c}	3.1±0.18^{e}	-
	2%	34±0.23^{efg}	2.23±0.56^{bc}	3.2±0.34^{de}	+
	3%	28±0.20^{g}	2.36±0.67^{bc}	3.4±0.36^{de}	+
Table Sugar	1%	64±0.96^{ab}	3.52±0.66^{ab}	4.6±0.28^{abc}	-
	2%	70±0.45^{a}	4.90±0.46^{a}	5.3±0.18^{a}	_
	3%	68±0.31^{ab}	4.16±0.24^{abc}	4.9±0.21^{ab}	+
Sugar cane Juice	1%	46±0.42^{cd}	3.18±0.42^{abc}	3.2±0.32^{de}	-
	2%	52±0.76^{c}	3.26±0.51^{abc}	4.1±0.24^{bcd}	-
	3%	42±0.17^{de}	2.41±0.38^{bc}	3.8±0.38^{cde}	+
Control		16±0.22^{h}	1.0^{d}	1.2^{f}	-

F value 211.33* at α = 0.05;** The Values within each column represent, Mean ± SE followed by same letters in superscript are not significantly different from each other (P< 0.0001). Data analyzed by GLM procedure with Duncan's multiple range test (DMRT) using SAS®.

Table 2: Effect of carbon sources on the physiology of *in vitro* grown plants of *L. reticulata.*

Carbon sources	Concentration	Chlorophyll content (mg/g) ±SE	Total protein content (mg/ml) ± SE	Carbohydrate content (mg/ml) ± SE
Sucrose	1%	0.62±0.40^{d}	11.6±0.32^{ab}	12.6±0.21^{abcd}
	2%	1.76±0.40^{a}	13.5±0.26^{ab}	15.7±0.36^{a}
	3%	0.80±0.54^{c}	16.3±0.24^{a}	15.7±0.30^{a}
Fructose	1%	0.63±0.54^{d}	10.4±0.22^{ab}	9.9±0.22^{cd}
	2%	0.64±0.56^{d}	11.2±0.25^{ab}	11.3±0.28^{abcd}
	3%	0.62±0.49^{d}	14.3±0.32^{ab}	11.5±0.21^{abcd}
Glucose	1%	0.63±0.41^{d}	10.6±0.28^{ab}	10.6±0.33^{bcd}
	2%	0.68±0.48^{d}	11.9±0.21^{ab}	11.8±0.26^{abcd}
	3%	0.62±0.41^{d}	11.6±0.18^{ab}	13.1±0.36^{abc}
Table sugar	1%	0.86±0.38^{c}	11.5±0.32^{ab}	12.5±0.26^{abcd}
	2%	1.42±0.50^{b}	14.4±0.35^{ab}	14.7±0.20^{ab}
	3%	1.32±0.48^{b}	15.1±0.32^{ab}	15.0±0.17^{ab}
Sugarcane juice	1%	0.63±0.45^{d}	9.1±0.25$_{b}$	8.2±0.13^{d}
	2%	0.67±0.50^{d}	10.8±0.28^{ab}	9.6±0.21^{cd}
	3%	0.79±0.52^{c}	12.3±0.26^{ab}	10.8±0.33^{bcd}
Control	-	0.50±0.38^{e}	6.8±0.49^{c}	5.6±0.12^{e}

F value 190.66* at α = 0.05;** The Values within each column represent, Mean ± SE followed by same letters in superscript are not significantly different from each other (P< 0.0001). Data analyzed by GLM procedure with Duncan's multiple range test (DMRT) using SAS®.

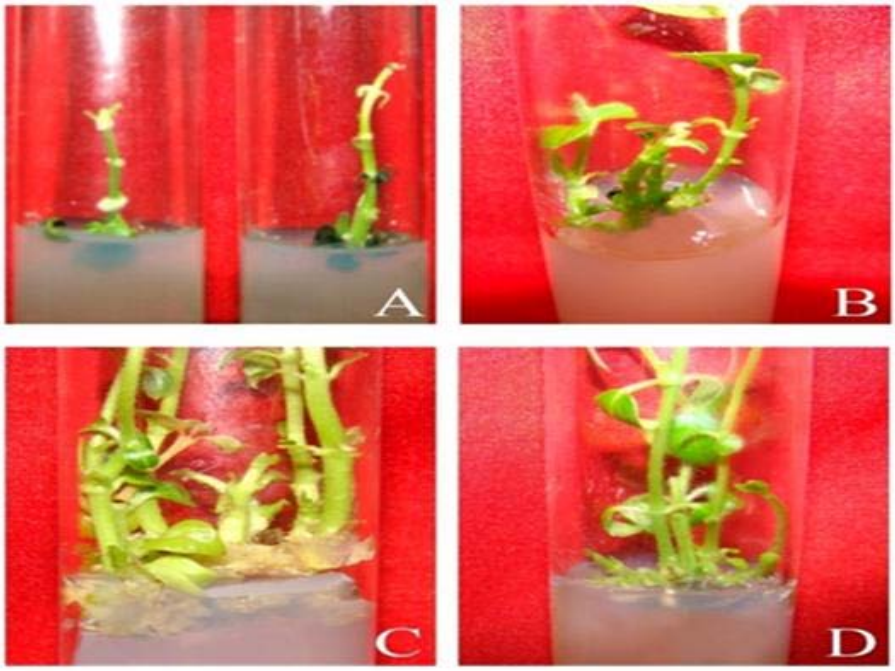

Figure 1: Effect of various concentrations of carbon sources on multiplication of *Leptadenia reticulata.* (A) Control; (B) 2% Sucrose; (C) 3% Sucrose; (D) 2% Table Sugar.

Table 3: Effect of natural extracts on multiple shoot induction in *L. reticulata.*

MS + Natural additives (%)		Shoot length (cm) Mean ± S.E	No. of multiple Shoots per explants	Chlorophyll content (mg/g tissue) Mean ± S.E	Total Protein content (mg/ml) Mean ± S.E	Total Carbohydrate content (mg/ml) Mean ± S.E
Coconut water	5	3.26±0.04^{ab}	3.76±0.16^{ab}	1.0±0.04^{cdb}	16.2±0.06^{cb}	16±0.02^{ab}
	10	4.72±0.06 ^{a}	6.20±0.10^{a}	1.7±0.02^{ab}	22.4±0.08^{ab}	22.5±0.01^{a}
	20	4.46±0.04^{a}	4.12±0.13^{cab}	1.5±0.10^{cab}	23.0±0.11^{a}	18.2±0.03^{ab}
Tomato Juice	5	2.08±0.10^{cb}	3.42±0.06^{cdeb}	1.1±0.04^{cdb}	6.2±0.14^{ef}	12.2±0.06^{d}
	10	2.12±0.10^{cb}	2.00±0.08^{cde}	1.4±0.12^{cadb}	8.6±0.06^{f}	12.6±0.12d^{c}
	20	2.04±0.12^{cb}	1.76±0.04^{de}	1.7±0.08^{ab}	9.3±0.02^{ecfd}	13.3±0.11^{dcb}
Banana juice	5	2.16±0.16^{cb}	1.30±0.04^{cdeb}	1.6±0.07^{cab}	11.8±0.13^{ecfd}	9.4±0.17^{dc}
	10	2.24±0.03^{cb}	2.80±0.09^{de}	1.5±0.03^{cab}	12.2±0.08^{ecfd}	11.5±0.12^{dc}
	20	2.33±0.05^{cb}	1.12±0.10^{e}	1.4±0.12^{cadb}	9.8±0.11^{ecfd}	11.4±0.11^{dc}
Carrot juice	5	3.02±0.09^{cab}	1.00±0.09^{cdeb}	1.4±0.16^{cadb}	8.0±0.12^{f}	10.2±0.18^{dc}
	10	3.41±0.10^{ab}	1.14±0.06^{cde}	1.8±0.14^{ab}	9.8±0.09^{efd}	12.1±0.08^{dc}
	20	3.46±0.07^{ab}	1.20±0.08^{cdeb}	2.2±0.26^{a}	9.5±0.04^{ecfd}	12.7±0.16^{dcb}
Papaya juice	5	2.21±0.08^{cb}	1.7 ±0.05^{cde}	0.8±0.14^{cd}	11.5±0.08^{ecfd}	9.4±0.08^{dc}
	10	2.45±0.06^{cb}	3.1±0.06^{cdb}	1.1±0.06^{cdb}	14.3±0.13^{cd}	10.8±0.08^{dc}
	20	2.42±0.09^{cb}	2.31±0.08^{cdeb}	1.6±0.08^{cab}	12.6±0.18^{ecd}	12.1±0.07^{cb}
Control		1.24±0.16^{c}	1.00±0.12^{de}	0.6±0.04^{d}	6.4±0.16^{ef}	6.1±0.12^{d}

F value 284.86 * at α = 0.05;** The Values within each column represent, Mean ± SE followed by same letters in superscript are not significantly different from each other (P< 0.0001). Data analyzed by GLM procedure with Duncan's multiple range test (DMRT) using SAS®.

Though sucrose has been reported to be the best source of carbon and energy[10], in our study, the results of commercial table sugars and sucrose in the media have shown comparable results. Hence there is a prospect of using table sugar instead of sucrose for *L. reticulata* tissue culture. Similarly promontory role of table sugar has also been reported.[15,28,29] The cost of banana by tissue culture method was reduced up to 90% by replacing the analytical grade sucrose with normal commercial sugar.[30] The cost of media for micropropagation of *L. reticulata* could be reduced significantly by the use of table sugar as a cheaper alternative for analytical grade sucrose.

Effect of natural extract on multiplication

Among the different natural supplements tested, coconut water found to be most suitable when compared to other organic additives used in the media. In 10% coconut water enriched medium, the highest number of shoot per explant (6.20±0.10) was observed and also there was an accelerated developmental process leading to healthy plantlets. Similarly use of 10% coconut water was found to be most effective in increasing frequency of response and multiplication in many plants like *Cynbidium pendulum*[31], *Phalaenopsis violacea*[32] and *Paphiopedillum villosum.*[33] Other parameters like mean shoot length (4.72±0.06), chlorophyll content (1.7±0.02), protein content (22.4±0.08) and carbohydrate content (22.5±0.01) were also found to be superior when 10% coconut water was supplemented in the multiplication media. Similarly the use of coconut water in increasing the number of shoot, shoot length and the number of nodes has also been reported.[34] Though other supplements (banana homogenate, tomato juice and papaya juice) exhibited positive effect, the response percentage and other physiological parameters were significantly lesser (Table 3). The increased morphogenetic effect of coconut water when compared to other organic supplements can be attributed for its chemical composition (glucose, fructose, different amino acids and minerals).[35] The presence of diphenyl urea in coconut water acts as cytokinin and induces the growth and cell division as reported by.[32,36] Agampodi and Jayawardena[37] also reported that the effective growth and regeneration in plants of coconut water supplemented media is due to the natural content of cytokinin and auxin. We observed that higher concentration of coconut water (20%) reduced the percentage of response, number of multiple shoot, shoot length and other physiological parameters (Table 3).

This observation is supported by the findings of[37] who stated that use of coconut water at 20% to 30% showed inhibitory effect on shoot regeneration. Our observation is further supported by the study of Baque[38] in Calanthe hybrids produced abnormal plants with retardation in growth and morphological characteristic at higher concentration of coconut water. To the best of our knowledge, use of natural supplements for micropropagation of *Leptadenia reticulata* is probably the first report so far.

Therefore it is speculated that coconut water at 10% could be used as a potential organic additive in the culture medium for propagation of *L. reticulata* which can be comparable with other synthetic growth hormones. So use of coconut water can practically reduce the cost of media for commercial production of *L. reticulata*.

CONCLUSION

It is concluded from the above study that carbon source plays important role in growth and morphogenesis of *L. reticulata*. As various media components such as nutrients and growth regulators adds to the cost of production of plants by tissue culture methods. In the present study a cost effective protocol was derived by replacing the analytical grade media components with cheaper alternatives. The table sugar and natural additives proved to be efficient in induction of multiple shoot. This cheaper substitute may economically feasible for large scale production of micropropagated plant by tissue culture industry.

Acknowledgement: The authors sincerely thank to National Medicinal Plant Board (NMPB), Govt. of India, New Delhi for providing financial support and we also gratefully acknowledge Padmashree Institute of Management and Sciences, Bangalore for providing facilities to carry out the research work.

REFERENCES

1. Sivarajan VV, Balachandran I, Ayurvedic drugs and their plant sources, Oxford IBH Co.pvt Ltd, 1994.
2. Anjaria JV, Gupta I, Studies on lactogenic property of *Leptadenia reticulata* and leptaden tablet in goats, sheep, cows and buffaloes, The Indian veterinary Journal, 44, 1967, 967- 974.
3. Singh RP, Vinod A, Shekhawat NS, Micropropagation of *Leptadenia reticulata*: A Medicinal Plant, *In Vitro* Cellular Devlopmental Biology –Plant, 39(2), 2003, 180-185.
4. Hariharan M, Sebastian DP, Benjamin S, Prashy P, Somatic embryogenisis in *Leptadenia reticulata* Wight& Arn. A medicinal plant, Phytomorphology, 52(2-3), 2002, 155-160.
5. Parabia MF, Gami B, Kothari LI, Mohan JSS, Parabia HM, Effect of plant growth regulators on *in vitro* morphogenesis of *L. reticulata* from nodal explants, Current Science, 92, 2007, 1290-1293.
6. Babbar SB, Jain R, Walia N, Guar gum as a gelling agent for plant tissue culture media, *In vitro* Cellular Devlopmental Biology Plant, 41, 2005, 258-261.
7. Lipavska H, Konradova H, Somatic embryogenesis in conifers: The role of carbohydrate metabolism, In Vitro Cellular developmental Biology-Plant, 40, 2004, 23-30.
8. Ill-Wan S, Korban SS, Effect of media, carbon sources and cytokinins on shoot organogenesis in the Christmas tree, Scot pine (pinus sylvesteris), Journal of Horticultural Science Biotech, 73, 1998, 822-827.
9. Fuents SRL, Calheiros MBP, Manetti-Filho J, Vieira LGE, The effect of silver nitrate and different carbohydrate sources on somatic embryogenesis in *Coffea canephora*, Plant Cell Tissue Organ Culture, 60, 2000, 5-13.
10. Lemos EEP, Baker DA, Shoot regeneration in response to carbon source on internodal explants of Annona muricata L., Plant Growth Regulation, 25 (2), 1998, 105-112.
11. SKREBSKY EC, NICOLOSO FT, FERRÃO GE, Sucrosee período de cultivo *in vitro* na aclimatizaçao *ex vitro* de ginseng brasileiro (*Pfaffia glomerata* Spreng.Pedersen), Ciencia Rural, Santa Maria, 34 (5), 2004, 1471-1477.
12. Pati PK, Rath SP, Sharma M, *In vitro* propagation of rose a review, Biotechnology Advances Ottawa, 24, 2006, 94-114.

13. Dobránszki J, Silva JAT, Micropropagation of apple: a review, Scientia Horticulturae Biotechnology Advances, 28(4), 2010, 462-488.

14. Bouza L, Jaques M, Maziere M, Arnaud Y, *In vitro* propagation of *Prunus tenella* Batsch. cv. 'Firehill': Control of vitrification increase of the multiplication rate and growth by chilling, Scientia Hort, 52, 1992, 143-155.

15. Demo P, Table sugar as an alternative low cost medium component for in vitro micropropagation of potato (*Solanum tuberosum L.),* African journal biotechnology, 7, 2008, 2578-2584.

16. Molnar Z, Virag E, Ordog V, Natural substances in tissue culture media of higher plants, Acta Biologica Szegediensis, 55(1), 2011, 123-127.

17. Thorpe TA, Stasolla C, Yeung EC, de Klerk G-J, Roberts A, George EF, The Components of Plant Tissue Culture Media II: Organic Additions, Osmotic and pH Effects, and Support Systems, Plant Propagation by Tissue Culture, The Background. Springer-Verlag, Dordrecht, 3(1), 2008, 115-173.

18. Neumann KH, Kumar A, Imani J, Plant Cell and Tissue Culture A tool in Biotechnology Basics and Application. Springer-Verlag, Berlin Heidelberg, 2009, 9-10.

19. Carimi F, De Pasquale F, Puglia AM, *In vitro* rescue of zygotic em-bryos of sour orange, *Citrus aurantium* L., and their detection based on RFLP analysis, Plant Breeding, 117, 1998, 261-266.

20. Rahman ARMM, Islam MO, Prodhan AKMA, Ichihashi S, Effects of complex organic extracts on plantlet regeneration from PLBs and plantlet growth in the Doritaenopsis orchid, Japan Agriculture Research Quarterly, 38, 2004, 55-59.

21. Aktar S, Nasiruddin KM, Khaldun ABM, Organogenesis of Dendrobium orchid using traditional media and organic extracts, Jour of Agri Rural Develop, 5, 2007, 30-35.

22. George EF, de Klerk GJ, The Components of Plant Tissue Culture Media I: Macro- and Micro-Nutrients. In George EF, Hall MA, de Klerk G-J, eds., Plant Propagation by Tissue Culture, The Background. Springer-Verlag, Dordrecht, 3(1), 2008, 65-113.

23. Murashige T, Skoog F, A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant., 15, 1962, 473-497.

24. Sudipta KM, Kumara Swamy M, Balasubramanya S, Anuradha M, cost effective approach for *in vitro* propagation of *Leptadenia reticulata* (wight & arn.) – a threatened plant of medicinal importance, Journal of Phytology, 3(2), 2011,72-79.

25. Yadava UL, A rapid and a non destructive method to determine chlorophyll in intact leaves, Hort Sci., 21, 1986, 1449-1450.

26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, protein measurement with folin- phenol reagent, J Biol Chem, 193, 1951, 265-275.

27. Sadasivam S, A Manickom, biochemical methods for agricultural science, 1st Edn, New Age International Pub. (P) Limited, New Dehi, 1992, 11-12.

28. Kumaraswami M, Sudipta KM, Balasubramanya S, Anuradha M, Effect of different carbon sources on in vitro morphogenetic response of patchouli (*Pogostemon cablin* Benth.), J of Phytology, 2(8), 2010, 11-17.

29. Saeed BA, in vitro selection and evaluation of the genetic purity of regenerated banana plants [Desertation], Department of Biotechnology/ University of Karachi, 2006, 117.

30. Zapata A, Cost reduction in tissue culture of banana. (Special leaflet), Int.Atom Energy Labs. Agric. and Biotech. Lab, Austria 2001.

31. Kaur S, Bhutani KK, Organic growth supplement stimulants for in vitro multiplication of Cymbidium pendulum (Roxb.) Sw Hort Sci (Prague), 39, 2012, 47–52.

32. Gnasekaran P, Xavier R, Uma Rani S, Sreeramanan S, A study on the use of organic additives on the protocorm like bodies (PLBs) growth of Phalaenopsis violacea orchid, J Phytol, 2(1), 2010, 029-033.

33. Long B, Niemiera AX, Cheng ZY, Long CL, In vitro propagation of four threatened Paphiopedillum species (Orchidaceae), Plant Cell Tissue Org Cult, 101, 2010, 151-162.

34. Nasib A, Ali K, Khan S, An optimized and improved method for the in vitro propagation of kiwi fruit (*Actinidia deliciosa*) using coconut water, Pak J Bot, 40(6), 2008, 2355-2360.

35. Santoso UK, Kubo TOta, Tadokoro T, Mackawa A, Nutrient composition of kopyor coconuts, Food Chemistry, 57(2), 1996, 299-304.

36. Texeira da Silva JA, Chan MT, Sanjaya ML, Tanaka M, Priming abiotic factors for optimal hybrid Cymbidium (Orchidaceae) PLB and callus induction, plantlet formation, and their subsequent cytogenetic stability analysis, Sci Hortic-Amsterdam, 109(4), 2006, 368-378.

37. Agampodi VA, Jayawardena B, Effect of coconut (*Cocos nucifera* L.) water extracts on adventitious root development in vegetative propagation of *Dracaena purplecompacta* L, Acta Physiol Plant, 31, 2009, 279-284.

38. Baque MA, Shin YK, Elshmari T, Lee EJ, Paek KY, Effect of light quality, sucrose and coconut water concentration on the micropropagation of Calanthe hybrids 'Bukduseong' x 'Hyesung' and 'Chunkwang' x 'Hyesung'), Aust J Crop Sci, 5(10), 2011, 1247-1254.

\

Source of Support: Nil, **Conflict of Interest:** None.