Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

Advances in Applied Science Research, 2012, 3 (3):1727-1732

ISSN: 0976-8610
CODEN (USA): AASRFC

An *in vitro* plant regeneration system for conservation of the leguminous tree *Albizzia chinensis* (Osbeck) Merr.

Ananya Borthakur[1], Suresh C Das[1], Mohan C Kalita[2] and Priyabrata Sen[3]

[1]*Plant Improvement Division, Tea Research Association, Tocklai Experimental Station, Jorhat, Assam, India*
[2]*Department of Biotechnology, Gauhati University, Guwahati, Assam, India*
[3]*Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat, Assam, India*

ABSTRACT

With a view to overcome the hurdles of a conventional method of propagation of the leguminous tree, Albizzia chinensis (Osbeck) Merr., in vitro approaches became necessary for selection of genotypes. A study was undertaken to determine the amenability of apical buds from in vitro seedlings for direct shoot regeneration. To achieve optimal conditions for shoot proliferation, the explants from 7-days-old seedlings were cultured on Murashige and Skoog (MS) basal medium supplemented with 6- benzylaminopurine (BAP) and kinetin solely or in combinations of both at different concentrations. The highest regeneration frequency was obtained with 1 mg l^{-1} BAP in which 76.67% of the apical buds responded with an average of 4-6 shoots per explant and shoot length of 2.10 ± 0.05 cms. Kinetin was found to be ineffective for shoot proliferation whether incorporated singly or in combination with BAP. Rooting of the microshoots was tried in growth regulator-free as well as indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) fortified half strength MS media. Highest rooting (58.33%) of the in vitro regenerated shoots was induced in half strength MS media incorporated with 1 mg l^{-1} IAA with an average of 2 roots per shoot. Soilrite was the best for acclimatization and establishment of the rooted plantlets, as compared to the other potting mixtures. The plantlets showed a survival percentage of 43.33%.

Keywords: Seedlings, cytokinins, kinetin, shoot bud, acclimatization.

INTRODUCTION

Albizzia chinensis (Osbeck) Merr., a member of the family Fabaceae, occurs naturally in India, Myanmar, Thailand, China and Java. It is a native of mixed deciduous forest in humid tropical and subtropical monsoon climates. It is grown extensively in tea plantations to provide shade and improving fertility of the soil [1]. It is also used for reforestation of degraded lands where organic manure is insufficient, produces fuel and timber and is an excellent fodder for cattle [2]. *A. chinensis* is planted for slope stabilization and in Bangladesh, the Garo agroforestry system incorporates *A. chinensis* for shade, weed growth suppression and ecological sustainability (http://www. worldagroforestrycentre.org). Herbal medicines are now being increasingly used for treatment of different human disorders [3] and *A. chinensis* plays an important role as a medicinal plant. The bark of this tree is used for treatment of scabies and skin diseases [4]. The multifarious nature of this tree has led to its overexploitation for use as fuel, fodder and timber [2]. But no much effort has been made for conservation and genetic improvement of this species.

Presently, the selected genotypes of *A. chinensis* carrying desirable traits trees are raised from seeds. But propagation by seeds is faced with the problems of attacks by thrips which prevent flower opening and young pods being damaged by beetles and larvae of various bruchids. A conventional method of vegetative propagation for establishment of clonal banks and large scale multiplication of selected materials could not be developed for *A. chinensis*, even after a series of systematic experimentation using stem and root cuttings [5, 6]. *In vitro* regeneration systems are viable alternatives to conventional vegetative propagation methods [7]. The *in vitro* regeneration of plants from cells or tissues is useful for large scale production of selected genotypes and commercialization of clonal

plants. Such *in vitro* systems provide a source of genetically homogenous cells and tissues and are able to regenerate shoots which can be rapidly and efficiently propagated either by organogenesis or somatic embryogenesis. Genetic transformation is a viable technique for making targeted single trait improvement in clonally propagated plants [8]. The regeneration of plants from cells or tissues is the initial requirement for introduction of genetic variation by genetic transformation without which no genetic improvement of any species is possible.

There are reports on the success of direct *in vitro* shoot regeneration without callus induction in leguminous trees like *Albizzia odoratissima* [9, 10], *Albizzia chinensis* [11], *Bauhinia variegata* and *Parkinsonia aculeata* [12], *Albizzia falcataria* [13], *Dalbergia sissoo* [14], *Acacia seyal* [15], *Pterocarpus marsupium* [16] and *Acacia chundra* [17].

Induction of callus and *in vitro* plant regeneration of *A. chinensis* has been reported from cotyledonary segments [18]. But successful soil establishment of the rooted plantlets could not be achieved due to premature defoliation of leaflets. Direct shoot regeneration of *A. chinensis* was reported from tissues of the transitional area between the hypocotyl and radical from *in vitro* grown seedlings [9]. But this work also does not support any evidence of hardening of the rooted plantlets. Here we present for the first time a report on the direct organogenesis and *in vitro* shoot regeneration of *A. chinensis* from apical buds of *in vitro* seedlings without the formation of an intervening callus phase, acclimatization and establishment of the plantlets in soil. The *in vitro* regeneration system developed in this study can be used for effective propagation of selected genotypes and can be an ideal source of homogenous material for regeneration of genetically modified plants.

The aim of the present study is to investigate whether the apical buds from *in vitro*-raised seedlings of *A. chinensis* can be utilized as reliable sources for mass propagation of this species throughout the year in order to meet its increasing demand and also for commercialization of selected genotypes. The *in vitro* regeneration system developed in this study was efficient for direct shoot regeneration, rooting and acclimatization of plantlets.

MATERIALS AND METHODS

Source of plant material and preparation of explants

Dried, mature seeds were collected from the tea gardens of Barbheta Division of Tea Research Association. The seeds were washed thoroughly with running tap water and few drops of a commercial detergent 'Nocidet' (National Organic Chemical Industries Limited, Mumbai, Maharashtra, India) for 5 min and then rinsed three times with distilled water. This was followed by surface sterilization of the seeds with 0.1% (w/v) solution of mercuric chloride for 10 min with gentle shaking. The seeds were then subsequently rinsed three times with sterile distilled water and germinated aseptically. For multiple shoot proliferation, the apical buds from 7-d-old *in vitro* seedlings were excised and cultured horizontally on shoot regeneration medium.

Culture medium for germination and optimal proliferation of shoots

Germination of seeds was carried out on half strength Murashige and Skoog (MS) medium [19] containing half strength of the MS macro and micro salts. The vitamins, sucrose and myoinositol were as per the original compositions of MS media. Plant growth regulators were not used for seed germination. For multiple shoot proliferation, MS basal media was used containing various concentrations of either BAP (0.25, 0.5, 0.75, 1.0, 1.25 mg l^{-1}) or kinetin at the same concentrations singly or in combination of both. MS medium without growth regulators was used as control. The pH of the medium was adjusted to 5.6 with 1N NaOH or 1N HCl and the medium was solidified with 0.8% agar (Himedia, Mumbai, India). The medium was sterilized by autoclaving for 20 min at 121ºC. Each treatment was tried with 20 explants and repeated thrice. The proliferation was evaluated 30 days after the beginning of the experiment and the percentage of explants producing shoots, the number of shoots per explant and length of shoots were recorded.

Culture conditions

The cultures were maintained at 25 ± 2°C under 16 h light photoperiod with a photosynthetic photon flux density of 37.40 µmol m^{-2} sec^{-1} provided by Photosynthetically Active Radiation (PAR) sources and cool white fluorescent tubes. Regular subculturing of the cultures to fresh medium was carried out at 4 weeks interval.

Effect of different auxins and their concentrations on *in vitro* rooting of the microshoots

Actively growing shoots with two or three leaves were used for induction of *in vitro* rooting. The rooting medium was composed of half strength MS medium with and without auxins like IAA or IBA at concentrations of 1.25, 1 and 0.75 mg l^{-1}. Medium sterilization and culture conditions were carried out as described previously for shoot proliferation experiments. Twenty microshoots were used for each treatment. After 30 days of culture, the rooting percentage, number of roots and length of roots per rooted shoot were recorded and statistically evaluated.

Hardening and pot establishment of the plantlets

Complete plantlets with well developed shoot and root systems were transferred to a hormone free minimal media of pH 5.2 supplemented with sucrose 7.5 g l^{-1} and solidified with 10 g l^{-1} agar [20]. The flasks with rooted plantlets were kept outside the culture chamber and under room temperature (30°C) for one week. The plantlets were then removed from the culture flasks, washed with tap water to remove all agar particles adhering to the roots and transferred to small earthen pots filled with soil, soilrite (Allied Scientific Products, Kolkata, West Bengal, India) or mixtures of sand: soil (1:1 and 1:2). The pots were covered with transparent polythene bags to keep the air surrounding the plantlets saturated with moisture and the plantlets were kept in the dark for 10 days under room temperature. Thereafter the plantlets were exposed to diffused light conditions. The plantlets were irrigated with tap water and the polyethene bags were completely removed after 3 weeks.

Experimental design and statistical analysis

All the experiments were conducted in a completely randomized block design (CRD) with twenty explants per treatment and each treatment was repeated thrice. Data on percentage of explants regenerating shoots, number of shoots per explants, and length of the shoots were recorded at 30 days interval in shoot proliferation experiments. Rooting experiments were also evaluated after 30 days interval and data were recorded on number of rooted microshoots, the number of roots per shoot and length of the roots. Hardening of plantlets was evaluated after 30 days of transfer to pots and percentage of survival of plantlets was recorded for different potting mixtures. The data were subjected to one-way analysis of variance (ANOVA) with 5% significance level to analyze the influence of different treatments. The mean comparisons were carried out by Duncan's Multiple Range Test [21]. The Least significant difference test (LSD) was used to study differences between different treatments.

RESULTS AND DISCUSSION

Germination of seeds and effect of cytokinin concentration for *in vitro* shoot regeneration

The seeds exhibited 90% germination after 2 days of inoculation on half strength MS medium. The seedlings obtained a height of 7-8 cms after 7 days with 2 to 4 leaves (Figure 1a). The apical buds were excised from the 7 day old seedlings and cultured vertically on MS media supplemented with different concentrations of BAP and kinetin.

In the present study, after 15 days of culture, several shoot bud primordia developed from the basal region of the apical bud but no induction of any callus was observed. Shoot proliferation was more pronounced in MS media with 1 mg l^{-1} BAP. Kinetin failed to induce any positive effect in shoot bud induction whether incorporated alone or in combination with BAP. The response of apical bud explants in MS medium supplemented with BAP and kinetin at different concentrations is represented in table 1. No response was observed in MS medium without plant growth regulators. Significantly highest response (76.67%) with 4-6 shoots/explant ($P<0.05$) by ANOVA was shown in MS basal media fortified with 1 mg l^{-1} BAP. The shoots elongated up to 1-3 cms in height (Figures 1 b and 1c) within 30 days of culture and average shoot length recorded was 2.10 ± 0.05 cms in this medium. On the other hand, only 3-4 elongated shoots of length 10 mm to 30 mm was obtained from cotyledonary explants of *Albizzia chinensis* after 42 days of culture in MS medium incorporated with 4 mg l^{-1} BAP [18]. Multiple shoot bud initiation of *Albizzia chinensis* was observed from the transition region between hypocotyl and radicle in MS medium supplemented with BAP 1 mg l^{-1} after 40-45 days of culture [11]. On the contrary, in this study, shoot buds were induced in MS medium containing 1 mg l^{-1} BAP within 30 days of culture.Media composition used in tissue culture studies accounts to a large proportion of any *in vitro* mass propagation protocol [22]. The incorporation of BAP in the medium has been found to induce a promoting effect on multiple shoot proliferation of apical buds of other leguminous trees like *Albizzia odoratissima* [9], *Acacia seyal* [15] and *Acacia chundra* [17]. In the present study, a decrease in rate of shoot proliferation was observed at increased concentration of BAP. An inhibitory effect on shoot multiplication at higher concentrations of BAP has also been found by other workers [23, 24, 25, 26] which agree with our observations. A decline in the shoot regeneration frequency was also recorded at low concentrations of BAP. No response could be obtained in MS medium supplemented with only kinetin. When the BAP 1 mg l^{-1} was combined with different concentrations of kinetin, no enhancement in shoot proliferation was obtained. Stunted growth of shoots was also observed with kinetin added media. Thus, kinetin was antagonistic to shoot proliferation in our studies. The inefficacy of kinetin for shoot proliferation rate was also observed in *Petroselinum crispum* [27], banana [28] and *Aloe vera* [29] which are in agreement with our findings.

The apical buds from the microshoots when isolated and subcultured in 1 mg l^{-1} BAP fortified MS medium, also proliferated in the same manner like the mother apical bud producing an average of 5-6 shoots per apical bud within 30 days. Thus it was possible regenerate an average of 25-30 shoots from one mother apical bud within a period of 60 days. In contrast, an average of 19 ± 1.4 shoots were obtained from cotyledonary explants after the second subculture which was carried out in MS medium containing a combination of 1 mg l^{-1} BAP and 0.5 mg l^{-1} IAA [18].

Table 1 Effect of cytokinins on shoot multiplication from apical buds of *Albizzia chinensis* after 30 days of culture

Growth regulators (mg/l)		MS		
BAP	Kinetin	Percentage of explants regenerating shoots (mean ± SE)	No. of shoots per explants (mean ± SE)	Length of shoots (cm) (mean ± SE)
-	-	-	-	-
1.25	-	43.33 ± 7.6b	2.1 ± 0.5b	1.82 ± 0.14b
1.00	-	76.67 ± 5.8a	5.2 ± 0.6a	2.10 ± 0.05a
0.75	-	38.33 ± 2.9bc	1.3 ± 0.5c	1.47 ± 0.05cd
0.50	-	35.00 ± 5.0c	1.3 ± 0.4c	1.40 ± 0.10cd
0.25	-	-	-	-
1.00	1.00	31.67 ± 2.9c	1.2 ± 0.4c	1.27 ± 0.06d
1.00	0.75	36.67 ± 2.9bc	1.3 ± 0.4c	1.30 ± 0.12d
1.00	0.50	43.33 ± 2.9b	2.0 ± 0.6bc	1.45 ± 0.05cd
1.00	0.25	43.33 ± 2.9b	2.0 ± 0.6bc	1.57 ± 0.27c

The values represented within the columns are the mean±SE of three replicated experiments with 20 explants per treatment. The values followed by different letters are significantly different at P<0.05.

Rooting of the regenerated shoots

The regenerated microshoots (5-6 cm), with 2-3 leaves, when excised and cultured in plant growth regulator free half strength MS medium as well as in the medium supplemented with IBA and IAA exhibited root induction (Figure 1d). Root induction was achieved from the base of the microshoots after 30 days of culture. The best rooting response of 58.33% after 30 days of transfer could be obtained in half strength MS medium fortified with IAA 1 mg l^{-1} (P<0.05) with an average of 1-3 roots per shoot and the length of the roots ranged from 1.97 to 3.27 cms (Table 2). At the same concentration IBA showed a reduced percentage of rooting. *In vitro* rooting is generally promoted by the presence of an auxin in the medium. In the earlier reports also, rooting of this species was obtained in MS (half) medium incorporated with 2.5 mg l^{-1} IBA [11] and in MS medium supplemented with 2.0 mg l^{-1} IBA [18]. However, in our studies IAA was found to be superior than IBA for root induction. IAA was found to be better than other auxins in *Hedeoma multiflorum* [30] and *Cardiospermum halicacabum* [31]. In our studies, addition of IAA at higher concentration lowered the percentage of rooting. Our results are in agreement with those obtained in *Psoralea corylifolia* where high concentration of IAA was found to reduce the percentage of rooting and elongation [32].

Table 2 Effect of half strength MS media supplemented with different concentrations of auxins on root induction from microshoots of *A. chinensis* after 30 days of culture

Auxins		Percentage of rooting (%)	No. of roots	Length of roots (cm)
IAA	IBA	(mean ± SE)	per explants (mean ± SE)	(mean ± SE)
-	-	16.67± 5.8^{c}	2.0 ± 0.0^{a}	1.91 ± 0.52^{a}
-	1.25	46.67± 7.6^{ab}	2.0 ± 0.0^{a}	1.92 ± 0.38^{a}
-	1.0	46.67± 7.6^{ab}	2.0 ± 0.0^{a}	2.01 ± 0.56^{a}
-	0.75	35.00± 5.0^{b}	1.0 ± 0.0^{b}	1.85 ± 0.30^{a}
1.25	-	46.67±7.6^{ab}	2.0 ± 0.0^{a}	1.98 ± 0.30^{a}
1.0	-	58.33±7.6^{a}	2.0 ± 1.0^{a}	2.62 ± 0.65^{a}
0.75	-	46.67±7.6^{ab}	2.0 ± 0.0^{a}	1.87 ± 0.32^{a}

The values represented within the columns are the mean±SE of three replicated experiments with 20 explants per treatment. The values followed by different letters are significantly different at P<0.05.

Hardening of the rooted plantlets

The hardening success of the plantlets was highest in soilrite (43.33%), as compared to the other potting mixtures (P<0.05) (Figure 1e). The percentage of survival of platlets was found to be minimum in soil alone (Table 3). The plantlets exhibited normal growth as compared to the *in vivo* grown seedlings. The earlier reports on *in vitro* studies of *A. chinensis* did not support any evidence of successful transfer of plantlets to soil [11, 18].

Table 3 Hardening of *in vitro* derived plantlets of *A. chinensis* after 30 days of transfer to potting mixtures

Potting mixture	**Response (%)**
Soil	8.33 ± 2.89^{d}
Sand:soil (1:1)	13.33 ± 2.89^{c}
Sand:soil (2:1)	20.00 ± 0.0^{b}
Soilrite	43.33 ± 2.89^{a}

The values represented within the columns are the mean±SE of three replicated experiments with 20 explants per treatment. The values followed by different letters are significantly different at P<0.05.

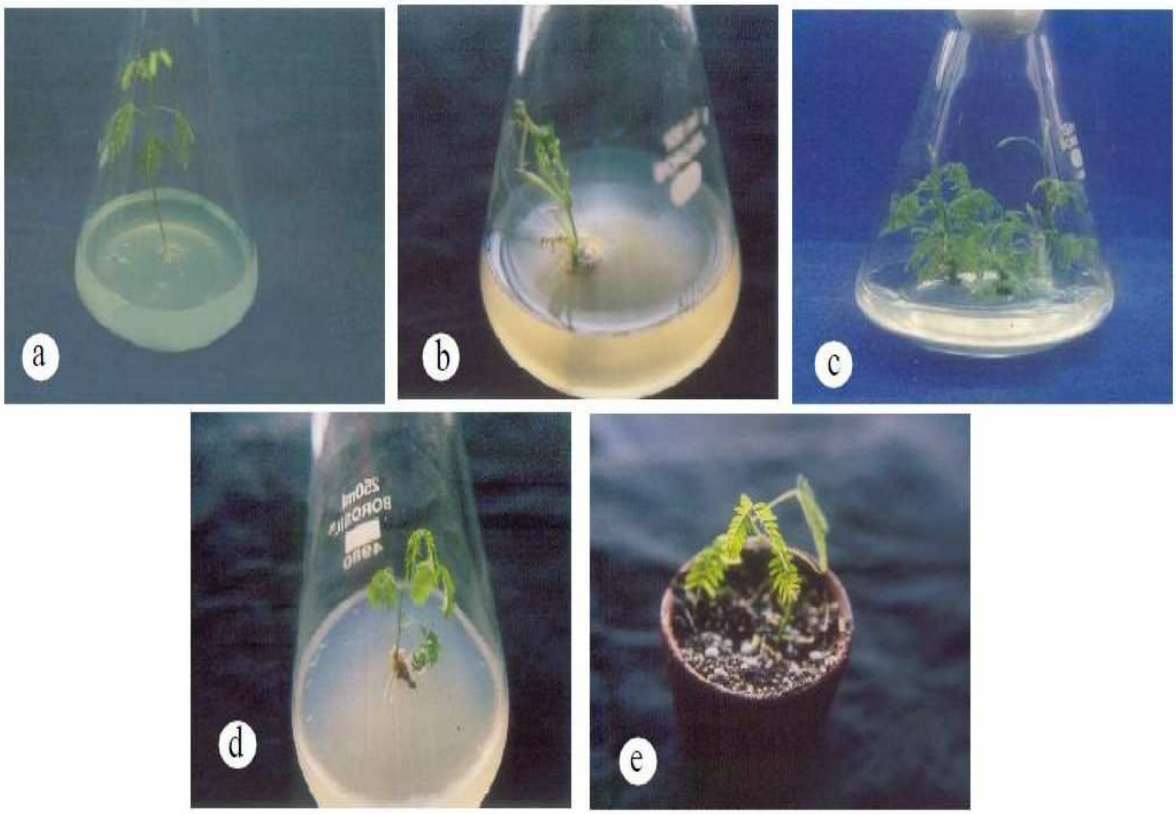

Fig. 1: *In vitro* regeneration of shoots from apical buds of *A. chinensis*

(a) *In vitro* grown seedling
(b) *In vitro* regenerated shoots from apical buds after 21 days of culture
(c) *In vitro* regenerated shoots from apical buds after 30 days of culture
(d) *In vitro* rooting of the microshoots
(e) Plant established in pot

CONCLUSION

Tissue culture studies on *A.chinensis* are very scarce. The protocol developed in this study for *in vitro* propagation of *A. chinensis* was simple, highly efficient, rapid and reproducible. It would be a promising step in developing technologies for the clonal propagation and genetic transformation of this species to generate plants with desirable traits like uniform shade canopy, disease and pest resistance, etc. The protocol was optimized by manipulations of different concentrations of cytokinins for shoot regeneration. BAP was highly influential in promoting multiple shoot proliferation. In the rooting stage, IAA proved to be the better auxin than IBA for *in vitro* rooting. Rooted plantlets were best acclimatized in soilrite. However, there is scope in improvement of the technique for better multiplication of shoots and acclimatization of the *in vitro* derived plantlets.

Acknowledgements
The authors are grateful to the Director, Tea Research Association, Tocklai Experimental Station, Jorhat, for his kind permission to carry out the work in the Tissue Culture Laboratory of Plant Improvement Division and publish the results in the form of a paper. They are also thankful to the scientists of the Tissue Culture team of the Division for their help and suggestions in different stages of the work. The authors thank Dr. S. Singh and Dr. S. Acharya of the Agricultural Biotechnology Department of Assam Agricultural University for going through the manuscript and providing valuable suggestions.

REFERENCES

[1] D.N. Barua; Science and practice in tea culture. Swapna Printing Works Private Limited, Calcutta, India, **1989**.
[2] C. S. Dhanai, A.K. Uniyal, N.P. Todaria, *Silvae Genet*., **2003**,52 (5-6), 259-266.
[3] A. C. Dutta; Dictionary of Economic and Medicinal Plants, Doss Publications, Jorhat, India, **2006**.
[4] N. Mishra, K. K. Behal, *Der Pharmacia Sinica*, **2011**, 2(1), 31-35.

[5] Anonymous, *Tea Research Association, Jorhat, Assam, India*, **1999**, 59-80.
[6] Anonymous, *Tea Research Association, Jorhat, Assam, India*, **2000**, 70-86.
[7] A. Md. Hossain, *In: Forest, farm and community tree research papers (special issue)*, (Winrock International Institute for Agricultural Development, Morrilton AR, 1997), 125-127.
[8] J. K. Kanwar, S. Kumar, *Adv. Appl. Sci*. **2011**, 2(2), 357-366.
[9] M. Phukan, G. C. Mitra, *Two Bud*, **1983**, 30 (1/2), 54-58.
[10] V. Rajeshwari, K. Paliwal, *In Vitro Cell. Dev. Biol.-Plant*, **2006**, 42, 399-404.
[11] M. Phukan, *Two Bud*, **1992**, 39(1), 47-48.
[12] J. Mathur, S. Mukunthakumar, *Plant Cell Tiss. Org*., **1992**, 28, 119-121.
[13] R. K. Sinha, R. Mallick, *Plant Cell Tiss. Org*., **1993**, 32, 259-261.
[14] C. Pradhan, S. Kar, S. Pattnaik, P. K. Chand, *Plant Cell Rep.*, **1998**, 18, 122-126.
[15] A. S. Al-Wasel-, *J Arid Environ.*, **2000**, 46 (4), 425-431.
[16] M . Anis, M. K. Hussain, A. Shahzad, *Curr. Sci*., **2005**, 88(6), 861-863. 861-863.
[17] G. R. Rout, S. K. Senapati, S. Aparajeta, *Hort. Sci*., **2008**, 35, 22-27.
[18] R. K. Sinha, K. Majumdar, S. Sinha, *In Vitro Cell. Dev. Biol.-Plant*, **2000**, 36, 370-373.
[19] T. Murashige, F. Skoog, *Physiol. Plant.*, **1962**, 15, 473-497.
[20] B. K. Konwar, B. J. Bordoloi, R. K. Dutta, S. C. Das, *Two Bud*, **1999**, 46(2), 26-32.
[21] D. B. Duncan, *Biometrics*, **1955**, 11, 1−42.
[22] S. Singh, B. S. Tanwar, M. Khan, *Adv. Appl. Sci. Res*., **2011**, 2 (3), 47-52.
[23] K. N. Chaudhari, S. Ghosh, S. Jha, 2004, *Plant Cell Rep*., **2004**, 22, 731-740.
[24] N. Ahmad, S. A. Wali, M. Anis, *J. Hortic Sci Biotech*, **2008**, 83(3), 313-317.
[25] Ç. Işikalan, S. Namli, F. Akbas, B.E. Ak, *Aust. J. Crop Sci*., **2011**,5 (1), 61-65.
[26] M. Faheem, S. Singh, B. S. Tanwar, M. Khan, A. Shahzad, *Adv Appl Sci Res*., **2011**, 2 (1), 203-213.
[27] J. L. Vandermoortele, J.P. Billard, J. Boucaud, Th. Gaspar, *Plant Cell Tiss. Org*., **1996**, 44(1), 25-30.
[28] S. Shirani, M. Sariah, W. Zakaria, M. Maziah, *Am. J. of Agric. Biol. Sci*., **2010**, 5 (2), 128-134.
[29] M. Kumar, S. Singh, S. Singh, *Asian J. Plant Sci. Res*.. **2011**, 1(1), 31-40.
[30] A. R. Koroch, Jr. H.R. Juliani, H.R. Juliani, V.S. Trippi, *Plant Cell Tiss. Org. Cult*.,**1997**,48, 213-217.
[31] A. A. Jahan, M. Anis, *Acta Physiol. Plant*., **2009**, 31, 133-138.
[32] A. N. Shinde, N. Malpathak, D. P. Fulzele, *Records Nat. Prod.*, **2009**, 3 (1), 38-45.