Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Annals of Biological Research, 2011, 2 (5) :275-282
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0976-1233
CODEN (USA): ABRNBW

A protocol for in *vitro* shoot regeneration from apical buds of *Albizzia lebbeck* (L.) Benth.

Ananya Borthakur [*1], Suresh Chandra Das[1], Mohan Chandra 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

An in vitro approach became necessary in order to meet the increasing demand and conservation of the natural population of the economically important leguminous tree, Albizzia lebbeck. For this purpose, a study was undertaken to determine the amenability of apical buds from in vitro seedlings of A. lebbeck for direct shoot regeneration. The explants from 7-day-old seedlings were plated on Murashige and Skoog (MS) basal medium supplemented with 6-benzylaminopurine (BAP) and kinetin solely or in combinations of both at different concentrations. The best response (68.33%) with an average of 3-7 shoots per explant and shoot length of 4.47 ± 0.49 cms was observed on MS basal medium supplemented with 1 mg/L 6-benzylaminopurine. Kinetin was not found to be effective in induction of multiple shoot proliferation. In vitro 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 medium. Highest rooting (55%) of the microshoots was achieved on half strength MS media supplemented with 1 mg/L indole-3-acetic acid (IAA) with an average of 2 shoots per shoot and shoot length of 3.87 ± 1.19 cms. Soilrite was found to be 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

The woody legume, *Albizzia lebbeck* (L.) Benth., a native of Australia, Bangladesh, India, Indonesia, Malayasia, Myanmar, Nepal, Pakistan and Thailand is a dominant species in monsoon forests. *A. lebbeck*, a good source of fodder and green manure is also used for fuel production, furniture making, erosion control and as a shade tree in tea, coffee and cardamom plantations (http://www.worldagroforestrycentre.org). The tree is reported to possess antiprotozoal, hypoglycaemic, anticancer and analgesic properties [1]. Decoction prepared from the leaves and

barks of this tree are protective against bronchial asthma and other allergic disorders [1]. The methanolic extract of the pods was investigated for antifertility activity [2]. The plant extract was also evaluated for memory enhancement and learning in mice [3].

Indiscriminate and illegal logging, microclimatic changes, low regenerative potential have resulted in severe depletion of this species [4]. Conventional method of vegetative propagation for large scale multiplication of selected materials and establishment of clonal banks is also not brisk enough to meet the demands on time [5]. Propagation of this species through seeds is faced with the problem of long seed dormancy [4]. Moreover, the population derived from seeds are not homogenous with regard to canopy characteristics and disease and pest resistance. Tissue culture is a viable alternative for mass propagation of species with uniform characteristics [5]. Moreover, biotechnological approaches can also be employed for plant improvement through genetic transformation of the species [6]. The *in vitro* systems are an excellent source of genetically homogenous cells and tissues and are able to regenerate shoots which can be efficiently propagated either by organogenesis or by somatic embryogenesis. The regeneration of plants from cells or tissues is the first step for introduction of genetic variation by various transformation techniques for genetic improvement of species. Moreover, propagation of *A. lebbeck* through *in vitro* regeneration methods would ease the pressure to cut down trees from natural forests.

Shoot regeneration of *A. lebbeck* has been reported from hypocotyl, leaf, [7;8;9], root [5;7;8], cotyledons [7;8;10;11], stem [9; 12], petiole [12], rachis [8] and nodal segments [11]. Shoot tips or apical buds are usually preferred for *in vitro* propagation because of their strong growth potential and ability to produce virus-free plants [13]. So far, there is only one report on regeneration of shoots from apical buds of *A. lebbeck* [14] which yielded low number of shoots and therefore, could not be used for large scale multiplication.

The objective of the present study was to develop an efficient, rapid and reproducible protocol for *in vitro* propagation of *A. lebbeck* using apical buds from *in vitro* grown seedlings for mass multiplication and commercialization of selected genotypes.

MATERIALS AND METHODS

Source of plant material and explants preparation

Dried and mature seeds were collected from the tea gardens of Barbheta Division of Tea Research Association. The seeds were washed thoroughly for 5 min in running tap water and few drops of a commercial detergent 'Nocidet' (National Organic Chemical Industries Limited, Mumbai, Maharashtra, India) and then rinsed three times with distilled water. After surface sterilization with 0.1% (w/v) solution of mercuric chloride for 10 min, the seeds were subsequently washed three times with sterile distilled water. The seed coats were then slightly injured with a scalpel and the seeds germinated under aseptic conditions. For induction of multiple shoots, the apical buds (4-5mm) from 1 week- old *in vitro* grown seedlings were excised and cultured horizontally on shoot proliferation medium.

Basal nutrient medium for germination of seeds and optimal proliferation of shoots

The basal nutrient medium used in the present study consisted of MS medium [15]. Germination of the seeds was carried out on plant growth regulator-free MS basal medium containing half strengths of the MS macro and micro salts. The vitamins, sucrose and myoinositol were as per the original compositions of MS basal media. The pH of the medium was adjusted to 5.6 with 1N

NaOH or 1N HCl. The medium was finally solidified with 0.8% agar (Himedia, Mumbai, India) before autoclaving at 121°C for 20 min.

Effect of cytokinins and their concentrations on in vitro multiple shoot proliferation
For multiple shoot proliferation, MS medium supplemented with various concentrations of either BAP (0.25, 0.5, 0.75, 1.0, 1.25 mg/L) or kinetin at the same concentrations singly or in combinations of both. MS medium without the incorporation of any growth regulator was used as the control. The pH of the medium was adjusted to 5.6 and the medium was solidified with agar. The media were then sterilized by autoclaving for 20 min at 121ºC. Twenty explants were used for each treatment and every treatment was repeated three times. Observations for shoot proliferation were evaluated 4 weeks after the beginning of the experiment and the percentage of explants responding to shoot proliferation, 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. Subculturing of the cultures to fresh medium was carried out at 4 weeks interval.

Effect of auxins and their concentrations on in vitro rooting of the microshoots
Actively growing shoots with 4-5 leaves were used for *in vitro* root induction of the microshoots. The rooting medium consisted of half strength MS medium with and without the incorporation of auxins like IAA or IBA at various concentrations of 1.25, 1 and 0.75 mg/L. Sterilization of media and culture conditions were carried out as described previously for shoot proliferation experiments. Twenty microshoots were used for each rooting treatment. After 4 weeks of culture, the percentage of rooted microshoots, number of roots per microshoot and length of the roots were recorded.

Hardening and pot establishment of the plantlets
Healthy plantlets with well developed shoot and root systems were transferred to a hormone free minimal media of pH 5.2 incorporated with sucrose 7.5 g/L and solidified with 10 g/L agar [16]. The flasks with rooted plantlets were then brought outside the culture chamber and kept under room temperature (30°C) for 7 days. The plantlets were then taken out 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. The potted plantlets were first kept in dark conditions under room temperature. After 10 days, the plantlets were exposed to diffused light conditions. The plantlets were irrigated regularly 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 to test the reproducibility of the experiment. In shoot regeneration experiments, data on percentage of explants producing multiple shoots, number of shoots per explant, and length of the shoots were recorded after 4 weeks. Similarly, rooting experiments were also statistically evaluated after 4 week interval and data were collected on percentage of rooted microshoots, the number of roots per shoot and length of the roots. Hardening of plantlets was evaluated after 4 weeks of transfer to earthen pots

and percentage of survival of plantlets was recorded for different potting mixtures. All the data were subjected to one-way analysis of variance (ANOVA) with 5% significance level to determine the influence of the various treatments. Mean comparisons were carried out by Duncan's Multiple Range Test [17]. The Least significant difference test (LSD) was used to study differences between the different treatments.

RESULTS AND DISCUSSION

In vitro germination of seeds and effect of cytokinins on shoot regeneration

The seeds of *A.lebbeck* exhibited a germination of 70% after 3 days of inoculation on half strength MS media. After one week of culture, the seedlings achieved a height of 8-9 cms and produced 2 to 3 leaves (Fig. 1a). The apical buds from the one week old seedlings were excised and cultured vertically on MS media fortified with different concentrations of BAP and kinetin for multiple shoot induction. After 2 weeks of culture, green coloured shoot bud primordia were seen to develop from the basal region of the apical bud without the induction of an intervening callus phase.

Kinetin was found to be unsuitable for multiple shoot proliferation whether incorporated alone or in combination with BAP. On the other hand, BAP proved to be highly effective for induction of multiple shoots. Table 1 represents the response of apical bud explants in MS medium supplemented with BAP and kinetin at different concentrations and combinations.

Table 1. Effect of cytokinins on shoot multiplication from apical buds of *A. lebbeck* after 4 weeks 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	-	50.00 ± 5.0b	2.0 ± 1.0b	2.76 ± 1.63b
1.00	-	68.33 ± 2.9a	5 .0± 2.0a	4.47 ± 0.49a
0.75	-	41.67 ± 2.9d	2.0 ± 1.0b	2.85 ± 0.10b
0.50	-	41.67 ± 2.9d	1.0 ± 0.0b	1.90 ± 0.46b
0.25	-	23.33 ± 2.9e	1.0 ± 0.0b	1.80 ± 0.30b
1.00	1.00	28.33 ± 2.9e	1.0 ± 0.0b	1.83 ± 0.06b
1.00	0.75	40.00 ± 5.0d	1.0 ± 0.0b	1.87 ± 0.32b
1.00	0.50	43.33 ± 2.9cd	1.0 ± 0.0b	1.95 ± 0.39b
1.00	0.25	48.33 ± 2.9bc	2.0 ± 0.0b	2.10 ± 0.53b

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

Observations recorded after 4 weeks showed that MS medium without plant growth regulators (control) failed to induce multiple shoots though survival of all shoots was observed. MS medium fortified with BAP 1 mg l/L showed a significantly highest response (P<0.05) in which 68.33% of the explants produced 3-7 shoots/explant with length of the shoots ranging from 3.98 to 4.96 cms (Fig 1b). The apical buds from the *in vitro* derived microshoots when isolated and

subcultured MS medium supplemented BAP 1 mg l/L proliferated in the same manner like the mother apical bud and produced an average of 5 shoots per apical bud within another 4 weeks. Thus it was possible regenerate an average of 25 shoots from one mother apical bud within a period of 8 weeks. Earlier observations reveal that a combination of cytokinins and auxins in the basal medium enhanced shoot formation and plantlet regeneration from different explants of *A.lebbeck*. For example, it was reported to have obtained direct shoot regeneration from *in vitro* derived cotyledons, nodal segments and *in vivo* nodal segments of *A. lebbeck* on MS medium added with both BA and NAA [11]. A highest of 7.3 shoots were produced from *in vitro* nodal segments on MS medium supplemented with BA 2.5 mg/L and NAA 0.2 mg/L after 6 weeks of culture. Again, an average of 16.0±1.87 shoots was obtained on MS medium fortified with BA 7.5 µM and NAA 0.5 µM from root segments after 8 weeks of incubation [5]. On the contrary, in the present study, 25 shoots were derived from one apical bud within 8 weeks in MS medium containing only BAP 1 mg /L.

The use of BAP in the culture medium has been found to enhance multiple shoot proliferation from apical buds of other leguminous trees [18; 19]. A decrease in rate of shoot proliferation was observed at higher concentrations of BAP in the current study. An inhibitory effect on shoot multiplication at increased concentrations of BAP has also been reported by other workers [6;20;21] which are in agreement with our findings. A decline in the rate of shoot regeneration was also recorded at lowered concentrations of BAP. No induction of multiple shoots was observed in MS medium added with only kinetin, though the explants remained fresh upto 4 weeks. Kinetin did not produce any promoting effect on enhancement of shoot proliferation even when combined at different concentrations with BAP 1 mg/L. The shoots also exhibited stunted growth in kinetin supplemented medium. Kinetin was also found to be inefficient for shoot bud proliferation in *Petroselinum crispum* [22] and banana [23] which agree with our findings.

Table 2. Effect of half strength MS media supplemented with different concentrations of auxins on root induction from microshoots of *A. lebbeck* after 4 weeks of culture

Auxins (mg/L)		Percentage of rooting (%) (mean ± SE)	No. of roots per explants (mean ± SE)	Length of roots (cm) (mean ± SE)
IAA	IBA			
-	-	1.67 ± 2.9d	1 ± 0.0b	3.46 ± 1.1a
-	1.25	45.00 ± 5.0b	2 ± 0.0a	3.61 ± 0.9a
-	1.0	45.00 ± 5.0b	2 ± 0.0a	3.59 ± 0.9a
-	0.75	28.33 ± 7.6c	1 ± 0.0b	2.10 ± 0.3a
1.25	-	50.00 ± 0.0ab	2 ± 0.0a	3.75 ± 1.1a
1.0	-	55.00 ± 5.0a	2 ± 1.0a	3.87 ± 1.2a
0.75	-	45.00 ± 5.0b	2 ± 0.0a	3.53 ± 1.0a

Values presented are the mean ± SE of three replicated experiments with 20 explants per treatment. The values within a column followed by different letters are significantly different at $P<0.05$.

Rooting of the in vitro regenerated shoots

The healthy and actively growing regenerated microshoots of 6-7 cms in length, with 2-4 leaves, when isolated and transferred to plant growth regulator free half strength MS medium as well as in the medium supplemented with IBA or IAA showed root induction (Figure 1c). The induction of *in vitro* rooting was observed from the base of the microshoots after 4 weeks of incubation.

The best rooting response of 55% after 4 weeks of culture could be achieved in half strength MS medium incorporated with IAA 1 mg l/L (P<0.05) with an average of 2 roots per shoot and the length of the roots ranged from 2.67 to 5.07 cms (Table 2).

Table 3. Hardening of *in vitro* derived plantlets of *A. lebbeck* after 4 weeks of transfer to potting mixtures

Potting mixture	Response (%)
Soil	10.00 ± 5.0c
Sand:soil (1:1)	11.67 ± 2.9c
Sand:soil (2:1)	20.00 ± 5.0b
Soilrite	43.33 ± 2.9a

Values presented are the mean ± SE of three replicated experiments with 20 plants per treatment. The values within a column followed by different letters are significantly different at P<0.05.

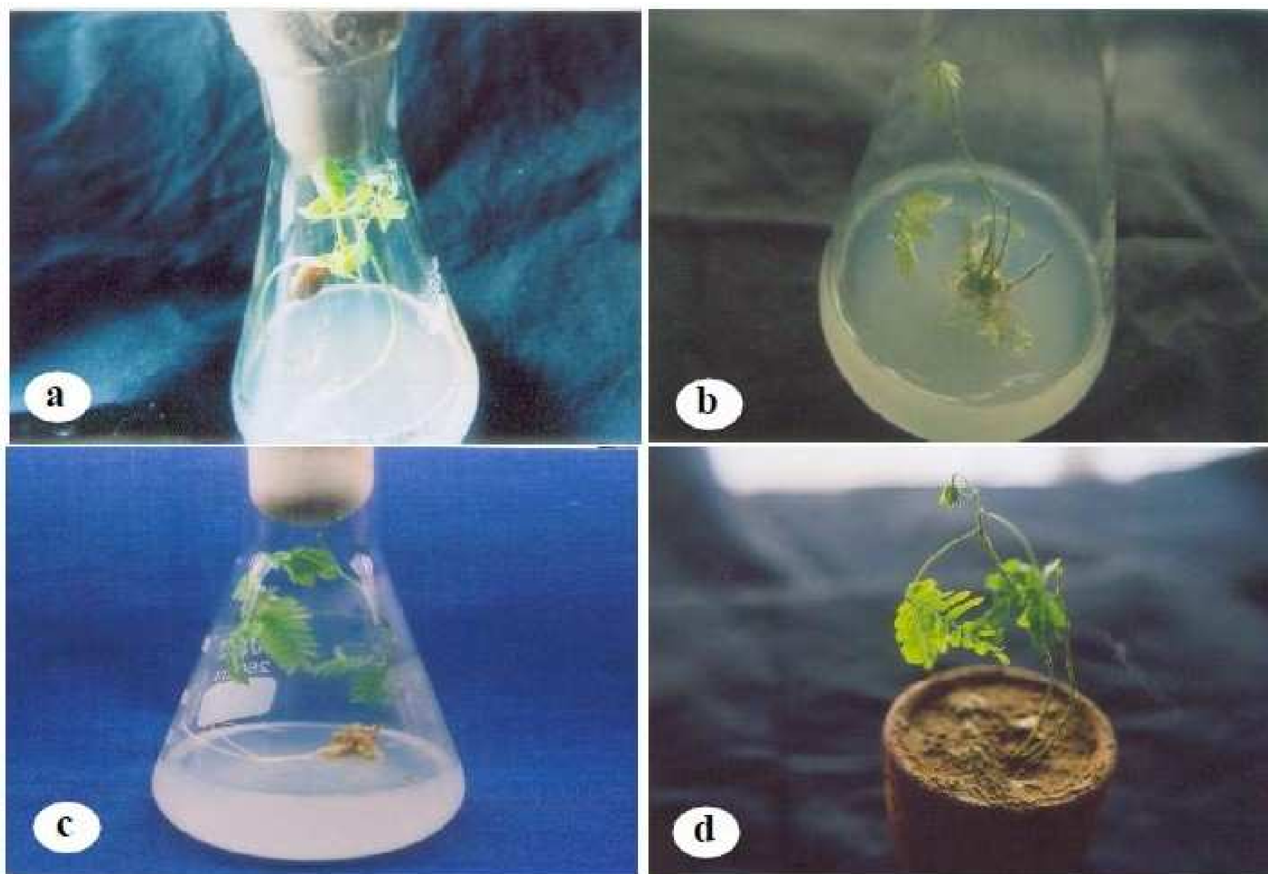

Plate 1 *In vitro* regeneration of shoots from apical buds of *A. lebbeck*

(a) *In vitro* grown seedling

(b) *In vitro* regenerated shoots from apical buds after 4 weeks of culture

(c) *In vitro* rooting of the microshoots

(d) Plant established in pot

At the same concentration IBA showed a decreased percentage of rooting. *In vitro* rooting is generally promoted by the presence of an auxin in the medium. Better rooting of this species was achieved in MS (half) medium incorporated with 2.0 µM IBA [5]. But in the current study, IAA was proved to be superior than IBA for *in vitro* rooting. IAA was also found to be better than other auxins in *Hedeoma multiflorum* [24] and *Cardiospermum halicacabum* [25]. Addition of IAA at increased concentration significantly lowered the percentage of rooting. Our results are in similarity with the findings obtained in *Psoralea corylifolia* where higher concentration of IAA was found to reduce the percentage of rooting and as well as elongation of roots [26].

Hardening of the rooted plantlets
The hardening success of the plantlets was observed to be highest in soilrite where 43.33% of the plants survived after 4 weeks of transfer as ($P<0.05$) (Figure 1d). Survival percentage of plantlets was found to be minimum in soil alone (Table 3). The plantlets exhibited normal growth when compared to 4 week old *in vivo* grown seedlings.

CONCLUSION

The protocol thus developed for *in vitro* propagation from apical buds of *A. lebbeck* was simple, highly efficient, easy and reproducible. It would be beneficial in developing technologies for the mass 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 manipulationg different concentrations of cytokinins for rapid and efficient shoot regeneration. In shoot proliferation experiments, BAP was found highly influential in induction of multiple shoot proliferation. Kinetin was not efficient for promoting shoot multiplication. In the rooting stage, IAA proved to be the better than IBA for *in vitro* rooting of the shoots. Rooted plantlets were best survived and acclimatized in soilrite. However, there is enough scope in improvement of the technique for better multiplication of shoots and acclimatization of the *in vitro* derived plantlets.

Acknowledgements
The authors express their gratitude to the Director, Tea Research Association, Tocklai Experimental Station, Jorhat, for his permission to carry out the work in the Tissue Culture Laboratory of Plant Improvement Division. They are also grateful to the scientists of the Tissue Culture team of the Division for their help and suggestions at different stages of the work.

REFERENCES

[1] A Saha; Ahmed M. *Pak J Pharm Sci*, **2009**, *22 (1)*, 74-77.
[2] RS Gupta; R Chaudhary; RK Yadav; SK Verma; Dobhal MP. *J Ethnopharmacol*, **2005**, *96(1-2)*, 31–36.
[3] SD Chintawar; RS Somani; VS Kasture; Kasture SB. *J Ethnopharmacol*, **2002**, *81(3)*, 299–305.
[4] RS Troup.The silviculture of Indian trees, Vol. 2, International Book Distributors, Dehradun, **1986** ; 467–468.
[5] S Perveen; A Varshney; M Anis; Aref IM. *J of Forestry Research*, **2011**, *22(1)*, 47-52.
[6] KN Chaudhari; S Ghosh; Jha S. *Plant Cell Rep*, **2004,** *22*, 731-740.
[7] PK Gharyal; Maheshwari SC. *Plant Cell Tiss Org Cult*, **1983**, *2*, 49-53.
[8] TM Varghese; Kaur A. *Curr Sci*, **1988,** *57(18)*,1010-1012.
[9] PVL Rao; De DN. *Plant Sci*, **1987**, *51 (2-3)*, 263-267.
[10] MA Reza; R Islam; ANK Mamun; Joarder OI. *Plant Tiss Org Cult*, **1996**, *6 (2)*, 67-71.
[11] ANK Mamun; MN Matin; MA Bari MA; NA Siddique; RS Sultana; MH Rahman; Musa ASM. *Pak J Biol Sci*, **2004**, *7(7)*, 1099-1103.
[12] PK Gharyal; Maheshwari SC. *Plant Cell Rep*, **1990**, *8*, 550-553.
[13] HS Chawla. Introduction to plant biotechnology, second edition, Oxford and IBH Publishing Company Private Limited, New Delhi, **2002**; pp. 39-56.
[14] AT Roy. In: V Dhawan, PM Ganapathy, Khurana DK. (Eds.) *Tissue Culture of forest tree species - Recent researches in India*, IDRC-TIFNET, Canada, **1993**; pp 9-17.
[15] T Murashige; Skoog F. *Physiol Plantarum*, **1962**, *15*, 473-497.
[16] BK Konwar; BJ Bordoloi; RK Dutta; Das SC. *Two Bud*, **1999**, *46(2)*, 26-32.

[17] DB Duncan. *Biometrics*, **1955**, *11*, 1–42.
[18] AS Al-Wasel-. *J Arid Environ*, **2000**, *46 (4)*, 425-431.
[19] GR Rout; SK Senapati; Aparajeta S. *Hort Sci,* **2008**, *35*, 22-27.
[20] N Ahmad; SA Wali; Anis M. *J Hort Sci Biotech*, **2008**, *83(3)*, 313-317.
[21] Ç Işikalan; S Namli; F Akbas; Ak BE. *Aust J Crop Sci,* **2011**, *5 (1)*, 61-65.
[22] JLVandermoortele; JP Billard; J Boucaud; Gaspar Th. *Plant Cell Tiss Org Cult*, **1996**, *44(1)*, 25-30.
[23] S Shirani; M Sariah; W Zakaria; Maziah M. *Am J Agri Biol Sci*, **2010**, *5 (2)*, 128-134.
[24] AR Koroch; HR Juliani Jr.; HR Juliani; Trippi VS. (1997) *Plant Cell Tiss Org Cult*, **2010,** *48*, 213-217.
[25] AA Jahan; Anis M. *Acta Physiol Plant*, **2009**, *31*, 133-138.
[26] AN Shinde; N Malpathak; Devanand N. *Rec Nat Prod*, **2009**, *3 (1)*, 38-45.