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In vitro organogenesis and plantlet regeneration of pigeonpea [*Cajanus cajan* (L.) Millsp.]

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ABSTRACT

Adventitious shoot regeneration was achieved from epicotyl and internode explants of *in vitro* grown seedlings of *Cajanus cajan*. Maximum number of multiple shoot formation was obtained by culturing the above explants on MS medium containing 1.0 mg/l BA and 0.5 mg/l NAA. The microcuttings were developed to complete plantlets after transferring them to the rooting media supplementation with auxins namely, IBA, NAA or IAA. Nevertheless, 0.1-0.2 mg/l IBA and NAA was found to be most suitable for root induction. The microplants were successfully established in the soil under natural environment with about 75% survival.
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KEYWORDS

In vitro culture;
Callus;
Cytokinin;
Auxin;
Proliferation.

INTRODUCTION

Cajanus cajan (L.) Millsp. belonging to the family Fabaceae and it is the only species of genus *Cajanus*. It has some common names such as pigeonpea, congobean, redgram, etc. It is grown widely in the entire Indian subcontinent, in the east and central African countries. It is also grown in some of the Central American countries. In recent years the crop has been cultivated commercially in Australia. In Bangladesh, it is grown all over the country, mostly in Kushtia, Rajshahi, Commilla, Jessore, Faridpur, Tangail, Noakhali, Rangpur and Rangamati[5].

Pigeonpea is a well known pulse yielding plants in our country. It is a multipurpose plant and all parts are used in some form or the other. The importance of protein enriched seeds (dhal) occupies an important position in tropical and sub-tropical regions. The plant also provides needed green vegetables, calcium and phosphorus in human diets, and fodder for cattle as well as fuel in rural society. Pigeonpea maintains and restores soil fertility by fixing atmospheric nitrogen[14].

Although there are reports on tissue culture and plant regeneration of pigeonpea from different countries[1,8], but little attempt has been made on tissue culture of pigeonpea varieties grown in Bangladesh. Therefore, present investigation was taken to develop the tissue culture techniques for inducing callus, proliferating shoots and regenerating complete plants form seedling explant of pigenonpea, as a prerequisite for taking any initiative to the improvement this important pulse crop using *in vitro* techniques.

MATERIALS AND METHODS

The immature seeds of *Cajanus cajan* were used

as experimental materials. For raising aseptic seedlings green pods were collected from the mature plants and washed thoroughly under running tap water for ten minutes to reduce the level of surface micro-organisms. Surface sterilization of the material was done following treatment of pods with 1% Savlon for 10 min with constant shaking, second washing with running tap water to make the material free from Savlon-foam and finally washing carefully 2-3 times with distilled water. The pods were then taken under running laminar airflow cabinet and transferred to 250 ml sterilized conical flask. After rinsing in 80% ethanol they were immersed in 0.1 % $HgCl_2$ for different duration of time. After washing with autoclaved distilled water and socking in sterilized filter paper the immature seeds were taken out from pods and finally implanted on the surface of semisolid MS medium[16]. The aseptic seedlings thus grew, attained a height of 8-10 cm after 8-10 days of incubation.

The explants consisting of epicotyl and internode were prepared from the aseptically grown two-week-old seedlings. They were then cultured on agar-gelled MS medium supplemented with 0.5-2.0 mg/l BA along with 0.1-0.5 mg/l of either NAA, IBA, IAA or 2,4-D. Microcuttings prepared from *in vitro* grown shoots were rooted in ½MS and full strength of MS media fortified with 0.1-1.0 mg/l of either NAA, IBA or IAA. All media were fortified with 3% sucrose, gelled with 0.8% agar (Hi-media, India), adjusted to pH 5.7 ± 0.1 and steam sterilized for 20 min at 121°C under 1.2 kg/cm^2 pressure. The cultures were grown at 26 ± 1°C under 14-hr photoperiod with light intensity of about 2500 lux.

RESULTS

Epicotyl and internode explants were cultured on MS medium supplemented with different concentrations and combinations of BA, NAA, IBA, IAA and 2,4-D for inducing shoot regeneration. The data on intensity of callus growth, percentage of shoot formation, number of total shoots per culture, number of usable shoots per culture and average length of shoots per culture form different treatments were recorded after 10 weeks of culture initiation. Results obtained on morphogenic responses of the cultured explants are presented in TABLE 1. BA alone and BA-IAA or BA-2,4-D combination in the medium failed to produce any adventitious shoot whereas it produce considerable amount of non-morphogenic callus only at higher concentrations. Out of the nine BA-NAA combinations, the medium containing 1.0 mg/l BA with 0.5 mg/l NAA, 0.5 mg/l BA with 0.2 mg/l NAA and 1.0 mg/l BA with 0.2 mg/l NAA produced calli with shoots 90%, 80% and 76% from epicotyl explants, respectively (Figure 1A). Frequency of shoot proliferation was maximum at 1.0 mg/ BA with 0.5 mg/l NAA and the number of shoots was 10.5 ± 0.31 per culture (Figure 1C & 1E). Among the combinations of BA-IBA maximum frequency of shoot bud formation was observed in 75% cultures at 1.0 mg/l BA with 0.1 mg/l IBA where the maximum 7.5 ± 0.46 shoots per cultures was recorded at the same combination.

TABLE 1 : Effects of different concentrations and combinations of cytokinin and auxin on induction of callus and regeneration of adventitious shoots from epicotyl and internode explants.

	Epicotyl			Internode		
Growth regulators (mg/l)	% of shoot formation	*Intensity of callus growth	No. of total shoot per culture	% of Shoot formation	*Intensity of callus growth	No.of total shoot per culture
BA						
0.5	-	+	-	-	+	-
1.0	-	+	-	-	+	-
2.0	-	++	-	-	+	-
BA+NAA						
0.5 + 0.1	55.0	+	6.5 ± 0.24b	55.0	+	4.5 ± 0.55c
0.5 + 0.2	80.0	++	8.0 ± 0.53b	78.0	++	7.5 ± 0.37b
0.5 + 0.5	10.0	+++	3.2 ± 0.44b	10.0	+++	2.5 ± 0.32c
1.0 + 0.1	70.0	+	5.6 ± 0.32b	68.0	+	4.0 ± 0.37c
1.0 + 0.2	76.0	++	7.2 ± 0.61b	72.0	++	5.5 ± 0.67c
1.0 + 0.5	90.0	+++	10.5 ± 0.31a	90.0	+++	10.0 ± 0.65a
2.0 + 0.1	50.0	++	4.5 ± 0.62b	50.0	++	4.2 ± 0.42c
2.0 + 0.2	25.0	+++	2.5 ± 0.47b	20.0	+++	2.5 ± 0.22c
2.0 + 0.5	-	-	-	-	+++	-
BA+IBA						
0.5 + 0.1	55.0	+	5.5 ± 0.46b	45.0	+	4.0 ± 0.52c
0.5 + 0.2	35.0	+	4.5 ± 0.51b	30.0	+	3.0 ± 0.12
0.5 + 0.5	-	++	--	-	+++	-
1.0 + 0.1	75.0	+	7.5 ± 0.46b	65.0	+	5.5 ± 0.42c
1.0 + 0.2	64.0	+	6.0 ± 0.17b	55.0	++	4.6 ± 0.52c
1.0 + 0.5	40.0	++	4.4 ± 0.32b	40.0	++	3.5 ± 0.52c
2.0 + 0.1	40.0	++	4.0 ± 0.12b	35.0	++	2.8 ± 0.32c
2.0 + 0.2	18.0	+++	2.5 ± 0.22b	10.0	+++	1.6 ± 0.22c
2.0 + 0.5	-	+++	-	-	+++	-

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Growth regulators (mg/l)	Epicotyl % of shoot formation	Epicotyl *Intensity of callus growth	Epicotyl No. of total shoot per culture	Internode % of Shoot formation	Internode *Intensity of callus growth	Internode No.of total shoot per culture
BA+IAA						
0.5 + 0.1	-	+	-	-	+	-
0.5 + 0.2	-	+	-	-	+	-
0.5 + 0.5	-	++	-	-	++	-
1.0 + 0.1	-	+	-	-	+	-
1.0 + 0.2	-	+	-	-	+	-
1.0 + 0.5	-	++	-	-	++	-
2.0 + 0.1	-	+	-	-	++	-
2.0 + 0.2	-	++	-	-	++	-
2.0 + 0.5	-	+++	-	-	+++	-
BA+2,4-D						
0.5 + 0.1	-	+	-	-	+	-
0.5 + 0.2	-	++	-	-	++	-
0.5 + 0.5	-	+++	-	-	+++	-
1.0 + 0.1	-	++	-	-	+	-
1.0 + 0.2	-	++	-	-	+	-
1.0 + 0.5	-	+++	-	-	++	-
2.0 + 0.1	-	++	-	-	++	-
2.0 + 0.2	-	+++	-	-	+++	-
2.0 + 0.5	-	+++	-	-	+++	-

***Intensity of callusing: (+) slight callusing ; (++) considerable callusing; (+++) intensive callusing; and (-) no callusing; Values represent means ± standard error of 15 explants per treatment. Means followed by the same letters are not significantly different by Duncan's multiple Range Test at 0.05% probability level.**

On the other hand, in case of internode explants among various combinations of BA-NAA, the cultured explants showed the maximum percentage (90%) of adventitious shoot proliferation with 1.0 mg/l BA + 0.5 mg/l NAA (Figure 1B). Maximum number of 10.0 ± 0.65 shoots per culture was observed on the same medium (Figure 1D & 1F). Among different combinations of BA-IBA, maximum frequency of shoot formation was 65%; and the highest number of shoots per cultured explant was 5.5 ± 0.42. The combinations of 0.5 mg/l BA with 0.5 mg/l of NAA or IBA, and 2.0 mg/l BA with 0.5 mg/l of NAA or IBA failed to produce any adventitious shoots however, these combinations produced large amounts of non-morphogenic callus. In this experiment it was found that 1.0 mg/l BA as cytokinin with 0.5 mg/l NAA as auxin was the best media combinations for proliferation of the adventitious shoots from both the epicotyl and internodes explants.

TABLE 2 : Effects of different concentrations of various auxins in ½MS and MS media on adventitious root formation from *C. cajan* microcuttings.

Media and Types of auxins	Conc.of auxins mg/l	% of cutting rooted	No of roots per rooted cutting	Average length of roots (cm)	Days to root formation	Callus Formation at the cutting base
	0.1	100.0	5.5 ± 0.12a	3.0 ± 0.52a	7-10	-
½MS+IBA	0.2	100.0	3.3 ± 0.46b	2.6 ± 0.32a	8-12	+
	0.5	80.0	2.0 ± 0.22b	2.2 ± 0.55b	10.12	++
	1.0	-	-	-	-	+++
	0.1	100.0	5.0 ± 0.25a	2.8 ± 0.25a	7-10	+
½MS+NAA	0.2	90.0	2.5 ± 0.32b	2.2 ± 0.33b	9-12	++
	0.5	65.0	1.0 ± 0.48b	1.5 ± 0.32c	10-15	+++
	1.0	-	-	-	-	+++
	0.1	60.0	2.5 ± 0.23b	1.8 ± 0.42b	10-12	-
½MS+IAA	0.2	45.0	1.8 ± 0.42b	1.3 ± 0.36c	10-15	-
	0.5	20.0	1.3 ± 0.32	1.0 ± 0.55c	12-15	++
	1.0	-	-	-	-	+++
	0.1	90.0	5.0 ± 0.20a	3.0 ± 0.10a	8-10	-
MS+IBA	0.2	85.0	3.0 ± 0.42b	2.5 ± 0.25a	10-12	+
	0.5	68.0	2.0 ± 0.15b	2.0 ± 0.45b	10-15	++
	1.0	-	-	-	-	+++
	0.1	85.0	4.7 ± 0.33a	2.5 ± 0.75a	10-12	+
MS+NAA	0.2	75.0	2.3 ± 0.56b	2.2 ± 0.14b	10-15	++
	0.5	52.0	1.0 ± 0.12b	1.4 ± 0.16c	12-18	+++
	1.0	-	-	-	-	+++
	0.1	55.0	2.2 ± 0.36b	1.5 ± 0.75c	10-12	-
MS + IAA	0.2	40.0	1.5 ± 0.25b	1.2 ± 0.26c	12-15	-
	0.5	20.0	1.0 ± 0.32b	1.0 ± 0.12c	12-18	+
	1.0	-	-	-	-	+++

(-) indicate no response; (+) slight callusing; (++) considerable callusing and (+++) profuse callusing; Values represent means ± standard error of 20 explants per treatment. Means followed by the same letters are not significantly different by Duncan's multiple Range Test at 0.05% probability level.

In vitro propagation is meaningless without successful establishment of the proliferated shoots in the soil. For this reason induction of roots to the *in vitro* proliferated shoots is essential for successful establishment of the microshoots into the soil. Microcuttings (3-4 cm) prepared from the *in vitro* proliferated shoots were rooted on ½MS and MS media supplemented with different concentrations (0.1-1.0 mg/l) of a single auxin, namely IBA, NAA and IAA. Both the ½MS and MS media were found to support root formation in pigeonpea shoots cultured *in vitro* (TABLE 2). The maximum percentage of root formation (100%) was found in ½MS with 0.1-0.2 mg/l IBA, and in ½MS with 0.1 mg/l NAA (Figure 1G). In MS me-

dium the maximum 90% root formation was observed with 0.1 mg/l IBA. The highest number of 5.5 ± 0.12 roots per shoots was observed in ½MS with 0.1 mg/l IBA, and in MS it was 5.0 ± 0.02 with 0.1 mg/l IBA. The maximum length of the longest root per shoot was 3.0 ± 0.52 cm found in ½MS with 0.1 mg/l IBA and 3.0 ± 0.10 cm in MS with 0.1 mg/l IBA. When microcutting were cultured either on ½MS or MS medium supplemented with 1.0 mg/l IBA, NAA or IAA, they could not produced any root. Besides, malformation and slow growth of roots were also observed at the high concentration of NAA and IAA supplemented media (both in ½MS and MS). Callus formed slightly at the base of the microcuttings more or less in both the ½MS and MS media with almost all auxin treatments, however, it could not hamper the emergence of roots.

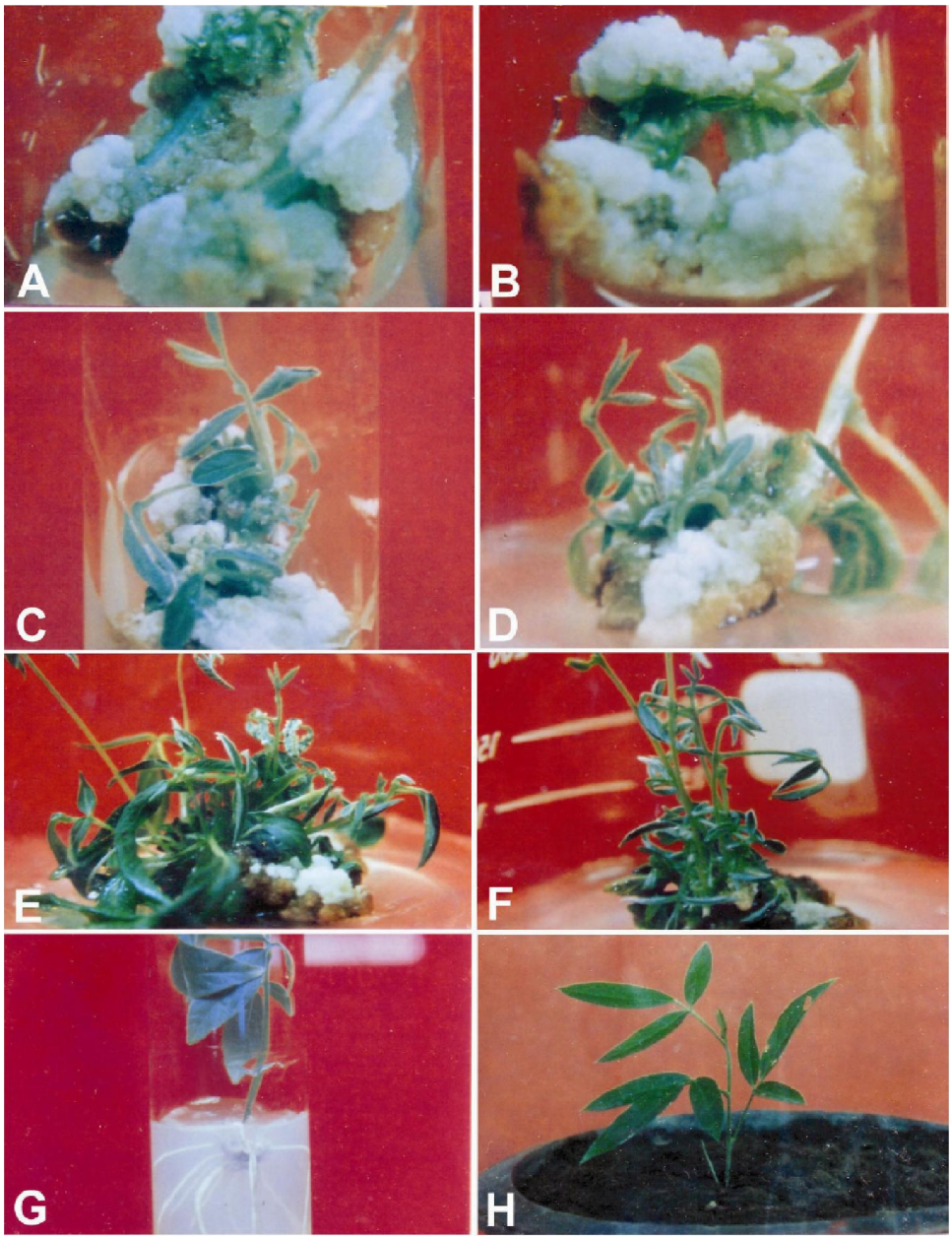

Figure 1 : A-H Regeneration of plantlets *in vitro* from epicotyl and internode explants of *Cajanus cajan*. A-B Proliferation of callus and development of adventitious shoots at the cut margins of an epicotyl (Figure A) and internode (Figure B) explants after three weeks of culture. C-F Elongation and multiplication of adventitious shoots on epicotyl and internode-derived callus after six weeks (Figure C & D) and ten weeks (Figure E & F) of culture initiation, respectively. G Rooting of the in *vitro* proliferated shoots. H Regenerated plantlet on soil after eight weeks of transfer under *ex vitro* condition.

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DISCUSSION

The results of the present investigation demonstrate that epicotyl and internode explants of *C. cajan* were amenable to *in vitro* culture. With proper manipulation of cytokinin and auxin combinations and concentrations, it was possible to induce callus and subsequently plantlet regeneration from the two types of explants. Epicotyl explants were cultured on MS medium supplemented with different concentrations and combinations of cytokinin and auxin for inducing callus as well as shoot regeneration. Among the different combinations the media containing 1.0 mg/l BA with 0.5 mg/l NAA produced calli with shoots from the cut margin of 90% epicotyls explants, the responses were better than that on other combinations. These observations support the classical cytokinin - auxin balance hypothesis of Skoog and Miller[7]. On the other hand Zeng *et al.*[10] showed that shoots were regenerated from callus cultures of epicotyl of *Citrus reticulata* on MS medium with 2.0-4.0 mg/l BA. Cheruavathur et al.[11] also noted that epicotyls explants of *Caesalpina bonduc* produce callus on MS medium supplemented with 4.0 mg/l BA and 1.0 mg/l NAA followed by 3.0 mg/l BAP and 1.0 mg/l IAA induced shoots. Results of the above investigations are in agreement with the present study where a cytokinin-auxin combination was found to be most suitable for shoot regeneration from the cultured explant. Adventitious shoots were produced successfully also from the internode explant of *C. cajan*. These explants were cultured on MS medium supplemented with different concentration and combinations of cytokinin and auxin. It was found that BA alone or in combination with IAA or 2,4-D failed to produce any shoot bud. But they produced different amounts of callus. Internode explants have been previously shown to be the most productive explant source for several species, including *Verbena officinalis*[2], *Euphorbia tirucalli*[9], and *Adenophora triphylla*[7]. This effect might be partly attributed to the intense vascular nature of the stem tissue since, in some cases, parenchyma cells surrounding the vascular bundle or cambial cells could be actively involved in the initiation of primordia development, which then leads to an organogenic process[6].

Our data indicated that cultured explants certainly required the presence of auxin in addition to cytokinin in the culture medium in order to achieve shoot organogenesis, as no shoot development could be obtained when BA was used alone or in combination of IAA. In this study it was revealed that 1.0 mg/l BA with 0.5 mg/l NAA was most suitable for shoot induction from internode explant of pigeonpea which was followed by 1.0 mg/l BA with 0.1 mg/l IBA. Similar observation was found in *Adenophora triphylla* for internode culture[4]. Uchida *et al.*[9] reported that adventitious buds were efficiently induced on LS medium supplemented with 0.02 mg/l thidiazuron. On the other hand Cuenca *et al.*[3] mentioned that Woody Plant medium supplemented with 4.5 μM TDZ and 2.9 μM IAA showed best performance for shoot induction from the internode explant of *Fagus sylvatica*.

Percentage of root induction and number of roots per shoot were obviously influenced by the concentrations, types of auxin and basal medium. Among the IBA or NAA concentrations tested, rooting response of microcutting in IBA containing medium was better than that in NAA containing medium, while least rooting response was found with all IAA concentrations tested. The findings are in agreement with those observed in *Ricinus communis*[18], *Arachis hypogaea*[15], *Caesalpinia bonduc*[11] and other plant species as well. Among the two types of media, ½MS medium showed better rooting than MS medium. Roy *et al.*[13], Jaiswal and Amin[17] and Niraula and Rajbhandary[12] reported that roots obtained in ½MS with IBA and NAA either individually or in different combinations were satisfactorily better than that in MS medium from the proliferated shoots of *Mitragyna parvifolia, Psidium guajava* and *Poncirus trifoliata,* respectively. Cheruvathur *et al.*[11] also observed maximum frequency of culture that produced roots was 100% when the shoots of *Caesalpinia bonduc* were cultured on ½MS medium with 6.0 mg/l IBA. The regenerated plantlets of *C. cajan* were successfully established under *ex vitro* condition (Figure 1H). The regeneration protocol developed through the present study may be used for conducting experiments on *in vitro* manipualtion of *Cajanus cajan.*

REFERENCES

[1] A.S.Kumar, T.P.Reddy, G.M.Reddy; Multiple shoots

from cultured explants of pigeon pea and *Atylosia* species. SABRAO J., **16**, 101-105 **(1984)**.

[2] A.Uçar Türker, B.Yücesan, E.Gürel; Adventitious shoot regeneration from stem internode explants of *Verbena officinalis* L., a medicinal plant. Turk J.Biol., **34**, 297-304 **(2010)**.

[3] B.Cuenca, A.Ballester, A.M.Vieitez; *In vitro* adventitious bud regeneration from internode segments of beech. Plant Cell, Tissue and Organ Cult., **60**, 213–220 **(2000)**.

[4] C.Chung-Chuan, C.Suh-Jau, P.Abhay, Sagare, T.Hsin-Sheng; Adventitious shoot regeneration from stem internode explants of *Adenophora triphylla* (Thunb.) A. DC. (Campanulaceae) – an important medicinal herb. Bot.Bull.Acad.Sin., **42**, 1-7 **(2001)**.

[5] C.L.L.Gowda, A.K.Kaul; Pulses in Bangladesh. Bangladesh Agricultural Research Institute and FAO, Dhaka, **(1982)**.

[6] E.Gurel, M.J.Wren; *In vitro* development from leaf explants of sugar beet (*Beta vulgaris* L.): Rhizogenesis and the effect of sequential exposure to auxin and cytokinin. Ann.Bot., **75**, 31-38 **(1995)**.

[7] F.Skoog, C.O.Miller; Chemical regulation of growth and organ formation in plant tissue cultures *in vitro*. Symp.Soc.Exp.Biol., **11**, 118-131 **(1957)**.

[8] H.K.S.Rao, S.Narayanaswamy; Effect of gamma irradiation on cell proliferation and regeneration in explanted tissues of pigeonpea (*Cajanus cajan* L. Millsp. Rad.Bot., **15**, 301-305 **(1975)**.

[9] H.Uchida, O.Nakayachi, M.Otani, M.Kajikawa, Y.Kohzu, T.Y.Katsuyuki, H.Fukuzawa, T.Shimada, K.Ohyama; Plant regeneration from internode explants of *Euphorbia tirucalli*. Plant Biotechnol., **21(5)**, 397–399 **(2004)**.

[10] L.Zeng, H.Xu, Y.Zeng, A.Luan, H.Wang; High efficiency in vitro plant regeneration from epicotyls explants of Ponkan Mandarin (*Citrus reticulata* Blanco). In Vitro Cell.Dev.Biol.—Plant **45**, 559–564 **(2009)**.

[11] M.K.Cheruvathur, J.Britto, T.D.Thomas; Callus induction and shoot regeneration from epicotyls explants of ethnomedicinally important *Caesalpinia bonduc* (L.) Roxb. Iranian J.Biotechnol, **8(4)**, 263-269 **(2010)**.

[12] R.Niraula, S.B.Rajbhandary; *In vitro* propagation of trifoliate organ (*Poncirus trifoliata*). Nat.Conf.of Sci.Technol., 141-144 **(1988)**.

[13] S.K.Roy, L.Rahman, P.C.Datta; Rapid clonal propagation of forest tree *Mitragyna parvifolia* by tissue culture. Plant Cell, Tissue and Organ Cult., **12**, 75-78 **(1987)**.

[14] S.K.Sinha, B.Lal, K.R.Koundal, R.Khannachopra; Nitrogen fixation does not limit yield in pulses. Curr.Sci., **52**, 916-918 **(1983)**.

[15] S.Palanivel, S.Parvathi, N.Jayabalan; Callus induction and plantlet regeneration from mature cotyledonaury segments of groundnut *(Arachis hypogaea* L.). J.Plant Biol., **45(1)**, 22-27 **(2002)**.

[16] T.Murashige, F.Skoog; A revised medium for rapid growth and bioassays with tobaco cultures. Physiol.Plant., **15**, 473-497 **(1962)**.

[17] V.S.Jaiswal, M.N.Amin; *In vitro* propagation of Guava from shoot cultures of mature trees. J.Pant Physiol., **130**, 7-12 **(1987)**.

[18] Y.-J.Ahn, L.Vang, T.A.McKeon, G.Q.Chen; High-frequency plant regeneration through adventitious shoot formation in castor (*Ricinus communis* L.). In Vitro Cell.Dev.Biol.-Plant, **43**, 9–15 **(2007)**.