The Bioscan 7(1) : 123-127, 2012
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

CALLUS INDUCTION AND *IN VITRO* MULTIPLICATION OF *BOERHAAVIA DIFFUSA* - MILESTONE MEDICINAL PLANT OF JHARKHAND

PINKI RAJ SAHU* AND A. S. KHALKHO[1]
Department of Biotechnology, Marwari College, Ranchi - 834 001, Jharkhand
[1]Department of Botany, Marwari College, Ranchi - 834 001, Jharkhand
E-mail: pinki_biotech@yahoo.co.in

KEY WORDS
Callogenesis
Genetic Improvement
Micropropagation
Punarnava
Tissue culture

Received on :
27.11.2011

Accepted on :
13.02.2012

***Corresponding author**

ABSTRACT
Boerhaavia diffusa commonly known as Punarnava is an important plant of India having wide range of pharmacological, clinical, antibacterial and antiviral properties.The commercial bulk of *Boerhavia diffusa* represent a heterogeneous population. Consequently, it quit often result in poor quality roots and biomass. The modern cultivation of plant with impressive uniformity and high productivity for end product is still grossly lacking in Boerhavia diffusa. The need, therefore is to genetically improve the available commercial bulk of Boerhavia diffusa in order to meet the natural requirement of the valuable products. In this context, an attempt was taken to improve the yield and quality of Boerhavia diffusa, by callogenesis from leaf and nodal explant. Different combination of Auxin and Cytokinin with or without addatives (citric acid, ascorbic acid and coconut water) were tested. Among 20 different hormonal combination of MS media, media supplemented with BAP -3mg/L + NAA- 0.5mg/L +citric acid - 0.5mg/L + 2, 4-D-0.1 mg/L shows high efficacy to induce callus and then organogenesis (shoot bud differentiation). Callus induction was observed within 12 days and the callus was subultured once in 2 week in sub culturing media. The multiple shoot regeneration was observed with max. no. of 8-10 in media supplemented with BAP -0.5mg/L +NAA -1.0mg/L + coconut water - 5mL/L. The regenerated shoots having size of 5-8 mm were excised and transferred to liquid medium supplemented with low concentration of IAA, NAA for rooting.

INTRODUCTION

Boerhaavia diffusa commonly known as punarnava is an important herbaceous medicinal plant. The whole plant of *B. diffusa* is a very useful source of the drug punarnava, which is documented in India Pharmacopoeia as a diuretic (Chopra, 1969). It has a long history of uses by indigenous and tribal people and in Ayurvedic or natural herbal medicines (Dhar *et al*., 1968). Mass scale collection of this plant from natural habitats is leading to a depletion of this plant species. *B. diffusa* is propagated by seeds, but the seed viability is poor and has very low germination percentage. Micropropagation method is specifically applicable to species in which clonal propagation is needed (Gamborg and Phillips, 1995). Tissue culture in propagation and its importance in conservation of genetic resources and clonal improvement have been described in different books (Barz *et al.,* 1977; Datta and Datta, 1985, Kukreja *et al.*, 1989). A large number of publications on the chemistry, pharmacology and several other aspects have been made, but here have been a few reports on *in vitro* regeneration of *Boerhaavia diffusa(Bhansali et al.,* 1978, Shrivastava and Padhya, 1995; Nagarajan *et al.,* 2005). Bhansali *et al.*, 1978, reported induction of adventious shoots using stem explants of *Boerhaavia diffussa*. Shrivastava and Padhya (1995) reported that increase of IAA in MS medium reduced the no of roots generation from the leaf segment and alkaloid content.But no significant reporting on genetic improvement and callogenesis was found till now.

The cardinal feature of modern cultivation of the plant with impressive uniformity and high productivity for end product is thus grossly lacking in *B. diffuse (*Awasthi *et al.,* 1989). The need, therefore, is to genetically improve the available commercial bulk of *B. diffusa* in order to meet the natural requirement of the valuable products. Improved varieties with enhanced drug yields hold great promise. Callogenesis, somaclonal variation and Mutation breeding may have an important role in this direction and may improve the yield and quality. Callogenesis, somaclonal variation are recognized as a source to generate useful genetic variability for crop improvement (Larkin and Scowcroft, 1981; Bright, 1985; Evans, 1989; Brar and Jain, 1998). The generation of somaclonal variation has been applied in crop improvement with the intention of including and exploiting useful and economically valuable characters that may not be readily available within other sources of germplasm (Juned *et al*., 1991). Somaclonal variation and callogenesis is cheaper than any other method of genetic manipulation. It has been most successful in crops with limited genetic systems and/or narrow genetic bases,where it can provide a rapid source of variability for crop improvement (Angela Karp, 1995). Another source of generating variations in plants is by mutagenesis (physical and chemical). Radiation induced mutations and chemical mutagenesis have been also extensively used for the improvement of crop plants (Shah *et al*., 2008). Mutagenesis/ callogenesis techniques allow induction of variation, selection and multiplication of the desired genotypes in a much shorter

duration and smaller space than conventional methods (Humera *et al.,* 2010). Recently, Shukla (2002) has reported a substantial amount of genetic variability in *B. diffusa*. Of the seventy-one genotypes tested, only a few were elite lines and were found to have desirable material for commercial use. Therefore, commercial manufacture of active constituents from these improved elite lines would be useful and profitable. Keeping all these in mind, an attempt has been made for genetic improvement of this dynamic plants by the approach of Callogenesis, in different combination of auxin and cytokinin.

MATERIALS AND METHODS

Explants source and preparation

Actively growing young leaves and nodal segment of *B.diffusa* with 6 to 8 nodes were collectd from the field grown plants maintained in the botanical and medicinal garden of Birsa Agriculture University of Ranchi, for the present study. For callus initiation, internode segments and leaves of young vegetative stem with a size of 1.5-2.0 cm were excised from the ex-vivo plant and washed under running tap water for 30 min. and then with liquid detergent + 10% SDS followed by rinsed with sterile double distilled water. The explants were then disinfected using 0.1%(w/v) $HgCl_2$ (Hi-media) for 2 min. there after the segments were washed 5-7 times with sterile distilled water. Nodal and leaf explants were trimmed using a sterile surgical blade and kept in a solution of ascorbic acid (0.1%)for 2 min and blotted on sterile filter paper before implanting on the culture media. The process was aseptically done under LAF.

Culture media preparation and explants implantation

Murashige and Skoog's (MS) medium supplemented with sucrose (3%) and agar (0.8%) were prepared with 20, 12 and 12 different concentrations of auxin (NAA, 2, 4-D) and cytokinin(BAP, KIN) with or without addatives (citric acid, coconut water) for callus induction, shooting, and rooting respectively. The pH of media was adjusted to 5.8 before gelling with 0.8% agar (Hi-media, India) &was subjected for

Table 1: Effect of different combination of auxin and cytokinin in MS medium for callus induction of *Boerhaavia diffusa*

Sl.no	HormonalRegime(mg/mL)	Source of explant	No. of explant	Response %	Callogenesis	Time required for response(days)	Colour
M1	NAA(0.5) + 2,4-D(3.5) + BAP(0.5) +	Internode	3	40	-	15-	Green
	+ Additives Citric acid (0.5)	Leaf	-	-	-	-	-
M 2	NAA(0.5) + 2,4D(2.5) + BAP(1.5) +	Internode	3	40	-	15-	-
	Additives Citric acid (0.5)	Leaf	-	-	-	-	-
M 3	NAA(0.5) + 2,4D(1.5) + BAP(1.5) +	**Internode**	**3**	**100**	**+ + + + +**	**08**	**Green & compact**
	Additives Citric acid (0.5)	Leaf	5	60	**+ +**	**15**	**brownish & friable**
M 4	NAA(0.5)24D(1.5) + BAP(3) +	**Internode**	**3**	**80**	**+ + + +**	**08**	**Green & friable**
	Additives Citric acid (0.5)	Leaf	5	40	-	-	-
M 5	NAA(3.5) + 2,4-D(.25) + BAP(0.5) +	Internode	3	50	-	15	-
	Additives Citric acid (0.5)	Leaf	-	-	-	-	-
M6	NAA(2.5) + 2,4-D(0.25) + BAP(1.5) +	Internode	3	50	+ +	12	Brown &compact
	Additives Citric acid (0.5)	Leaf	-	-	-	-	-
M7	NAA(1.5)24-D(0.25) + BAP(3.0) +	**Internode**	**3**	**80**	**+ + + +**	**10**	**Green & friable**
	Additives Citric acid (0.5)	Leaf	5	40	-	15	-
M8	NAA(0.5) + 2,4-D(0.25) + BAP(3.5) +	Internode	3	40	-	18	-
	Additives Citric acid (0.5)	Leaf	-	-	-	-	-
M 9	NAA(0.5) + 2,4-D(3.5) +	Internode	3	40	-	18	-
	Kinetin(0.5) + Coccont water(5)	Leaf	-	-	-	-	-
M 10	NAA(0.5) + 2,4-D(2.5) +	Internode	**3**	**60**	**+ + +**	**11**	**Green & compact**
	Kinetin(1.5) + + Coccont water(5)	Leaf	-	-	-	-	-
M 11	NAA(0.5) + 2,4-D(0.5) + BAP(1.5) +	Internode	3	40	-	20	-
	Kinetin (3.0) + Coccont water(5)	Leaf	-	-	-	-	-
M 12	NAA(0.5) + 2,4-D(0.5) +	**Internode**	**3**	**75**	**+ + + +**	**10**	**Green & friable**
	Kinetin(3.5) + Coccont water(5)	Leaf	5	50	**+ +**	**15**	**Brown & friable**
M 13	NAA(3.5) + 2,4-D(0.25) +	Internode	3	40	-	20	-
	Kinetin(0.5) + + Coccont water(5)	Leaf	-	-	-	-	-
M 14	NAA(2.5) + 2,4-D(0.25) +	Internode	3	40	-	20	-
	Kinetin(1.5) + + Coccont water(5)	Leaf	-	-	-	-	-
M 15	NAA(1.5) + 2,4-D(0.25) +	Internode	3	50	+ +	12	Brown &compact
	Kinetin(3.0) + + Coccont water(5)	Leaf	-	-	-	-	-
M 16	NAA(0.5) + 2,4-D(0.25) +	**Internode**	**3**	**75**	**+ + + +**	**10**	**Green & friable**
	Kinetin(3.5) + Coccont water(5)	Leaf	5	50	-	15	-
M 17	NAA(0.5) + 2,4-D(3.5) + BAP(0.5) +	Internode	3	50	-	18	-
	Kinetin(3.5) + + Coccont water(5)	Leaf	-	-	-	-	-
M 18	NAA(0.5) + 2,4-D(2.5) + BAP(1.5) +	**Internode**	**3**	**60**	**+ + +**	**11**	**Green & friable**
	Kinetin(3.0) + + Coccont water(5)	Leaf	-	-	-	-	-
M 19	NAA(0.5) + 2,4-D(1.5) + BAP(3.0) +	Internode	3	50	+ +	12	Brown &compact
	Kinetin(1.5) + Additives Citric acid (0.5)	Leaf	-	-	-	-	-
M 20	NAA(0.5) + 2,4-D(0.5) + BAP(3.5) +	Internode	3	50	-	18	-
	Kinetin(0.5) + Additives Citric acid (0.5)	Leaf	-	-	-	-	-

Table 2: Effect of growth regulators in MS medium on shoot bud differentiation from callus culture of nodal segment of *B. Diffusa*. Mean value (±) were collected from triplicate set up. Data were taken after 6 weeks

S.no.	Harmonal regime	% of callus response for shoot induction	Time period for response(days)	Average no of shoot explant	Average length of shoot(mm)
S1	BAP(3.0) + Additives Citric acid (2.5)	60	12	4.00± 0.30	6.10± 1.70
S 2	BAP(2.0) + Additives Citric acid (2.5)	40	15	3.00± 0.20	3.25± 2.20
S 3	BAP(1.0) + Additves Citric acid (2.5)	70	10	5.00± 0.10	10.62± 1.72
S 4	BAP(0.5) + Additives Citric acid (2.5)	70	10	6.00± 0.20	16.22 ±2.10
S 5	NAA(1.0) + BAP(0.5) + Kinetin(0.5) + coconut water(5.0)	100	7	12.12± 0.33	19.25± 2.20
S 6	NAA(1.0) + BAP(1.0) + coconut water(5)	75	8	10.42± 0.62	12.06 ±1.02
S 7	NAA(1.0) + BAP(2.0) + coconut water(5.0)	50	15	2.25± 0.32	2.32± 0.23
S 8	NAA(0.5) + BAP(3.0) + coconut water(5)	60	12	4.10 ±0.23	10.12± 0.33
S 9	NAA(1.5) + Kinetin(0.5) + Additives Citric acid (2.5)	75	8	10.36± 0,58	14.25 ±2.60
S 10	NAA(2.0) + Kinetin(0.5)) + coconut water(5.0)	80	7	11.06 ±0.21	18.50 ±1.70
S 11	NAA(2.5) + Kinetin(0.5)	40	15	2.25± 0.32	3.25± 0.32
S 12	NAA(3.0) + Kinetin(0.5) + coconut water(5.0)	50	15	1.32 ±0.23	2.25± 0.24

autoclaving at 121·c for 20 min. The media was then poured in test tubes (12x10cm) stored at room temprature. The prepared explants were implanted on the media and were maintained for data analysis. The experiment was set in triplicate.

Culture maintainance

Culture was maintained at 25 ± 2ºC with a photoperiodism of 16/8h under illumination of 4000 lux under aseptic condition of culture room and were observed for callus induction and organogenesis.

Subculturing of Callus for organogenesis

The callus developed in different hormonal regime were subjected to sub culturing media for re differentiated into shoot buds. Primary cultures with single and multiple shoots regenerated from the callus originated from nodal explants were carefully taken out from the culture vessels and once again cultured on MS medium fortified with hormones as given in Table 2 for further multiplication. Multiplication of shoots was carried out by repeated harvest of microshoots followed by sub-culturing on medium supplemented with BAP (0.5-3.0 mg/L), Kin (0.5-3.0 mg/L),NAA(1.0-3.0 mg/L) and with or without citric acid(2.5mg/L) and Coconut Water (5mL/L). For *in vitro* rooting, healthy microshoots regenerated from the above experiments were subjected to rooting on half-strength MS medium fortified with different concentrations of indole butyric acid (IBA) (0.5-2.0 mg/L),NAA(0.5 mg/L) and IAA(0.5-2.0mg/L). Microshoots measuring the height of 4-5 cm with 2-3 pairs of shoots were cultured on different concentrations of auxins and maintained under the cultural conditions as described earlier.

RESULTS AND DISCUSSION

Callus induction of nodal and leaf explant

Callus induction of nodal and leaf explant of *B.diffusa* were cultured on MS medium supplemented with 20 different combination of auxin and cytokinin with additives citric acid and coconut water. Nodal explant showed better response (100%) for callogenesis than leaf explant among most of the combination. The best callogenesis was observed within 8 days in C3 media supplemented with NAA(0.5mg/L) + 2-4-D(1.5mg/L) + BAP(3.0mg/L) and citric acid (0.5mg/L) Nodal explant also give good responses in media M4, M7, M10, M12, M16 and M18 ranges from 60% to 80% callogenesis, but it takes longer time (8-12 days) than M3 media while M1,M2,M5,M8,M9,M11,M13,M14 and M17 gave negative response on callus induction. The responded explant in the C3 media,developed callli faster than others and the callus was vigourous in growth. The noticeable characteristic of the responded callus was the variation of the texture and colour in the callus along with changes in the hormonal combinations. It varied from compact to loose and to loose with wet surface (Fig. 1). The similar finding was reported by Farzana et al., (2011). Work on studies with callus induction of *vitex negundo*. It has been found that NAA, 2, 4-D supplemented media with BAP and citric acid showed better callogenesis than the media supplemented with NAA, 2, 4-D, kinetin and coconut water.In leaf explant,callus induction was best only in M3 media (60%) and M4,M7,M12 and M16 also showed positive response ranges from (40%-50%).In non of the media composition 100% callogenesis was observed for leaf explant with brownish compact texture. Almost all the hormonal concentration in the experiment formed green friable /green compact callus but brownish compact callus was also observed in M6,M15 and M19(M6-2.5 mg/L NAA, 2, 4D0.25mg/L, 1.5mg/L BAP and 0.5mg/L citric acid M15-1.5mg/L NAA, 0.25mg2, 4-D, 3mg/L Kinetin and 5mg/L coconut water, M-19 0.5mg/L NAA, 1,5mg/L 2, 4-D, 3.0mg/mL BAP, 1.5mg/L Kinetin and 0.5mg/L Citric acid.

The efficacy of exogenous 2,4-D found in this experiment, was also been reported with other medicinal plants by various authors. Results described by Rani *et al*. (2003), Thomas and Maseena (2006), Hassan *et al*. (2009) were also in agreement with our result for using this synthetic plant growth regulator in the culture medium for callus induction of *Withania somnifera, Cardiospermum halicacabum* Linn and *Abrus precatorious*, respectively. Synergism of 2, 4-D and NAA found by the authors also agree with results obtained by Nikolaeve *et al*. (2009) with *Camellia chinensis*. However, effectiveness of auxin-cytokinin combination for callus induction found in this present study differ from the findings of Olivira *et al*. (2001), Kiong *et al*. (2005) and Baldi and Dixit (2008).

Our finding in the experiment regarding type of explant responses contradict the finding of Hassan *et al*. (2009),found the best responses only from young leaves with same texture

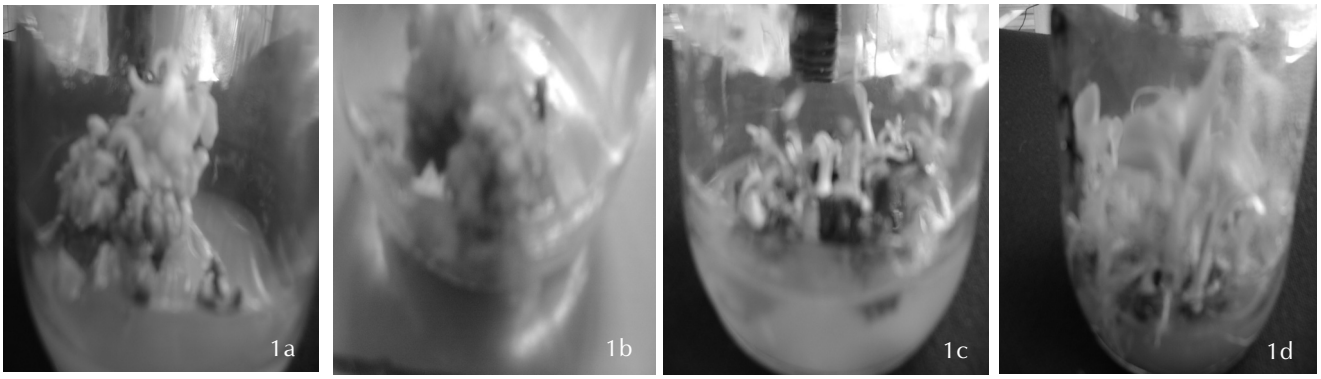

Figure 1: Callusing and shooting in different hormonal composition for *B.diffussa,nodal explants* (a) Callusing in M3 media (b) Callusing in M12 media; (c) multiple shooting in S4 media and (d) multiple shooting in S12 media

of callissses but in this experiment nodal segment showed better response than leaf explantwith varying texture of calli.

Shoot regeneration and multiplication

The *invitro* induced callus from nodal explants were subcultured on shooting media supplemented with 1.0-3.0mg/ L NAA + 0.5-3.0 mg/L BAP + 0.5 mg/L NAA in addition to 2.5 mg/L citric acid and 5 mg/L coconut water in 12 different combinations. Among 12 different combinations highest percent of shoot induction was observed in S5 (NAA-1.0mg/L +BAP-0.5mg/L + coconut water 50mg/L) showed 100% regeneration within 7 days with average number of shoot 8-12 having length of 19-20mm.Next to S5 media were S10>S9>S6.(S10media supplemented with 2mg/L NAA,0.5 mg/L Kinetin and 5mg/L coconut water, S9 supplemented with-1.5mg/L NAA,0.5mg/L lKinetin and 2.5mg/L citric acid and S6 supplemented with- 1mg/L NAA,1mg/mL BAP and 5mg/ mL coconut water responded within 8-10 days with average shoot number 6to 11 with average shoot length of 16-18mm. Other hormonal combination dosent show any significant findings.

In the experiment it has been reported that with increase in concentration of cytokinin, there is decrease in shoot number and shoot length. This finding contradict the finding of Mercy Stephen *et al.,*2010, worked on Vitex Negundo, reported low concentration of cytokinin(BA 0.1-0.5 mb/L) exhibited poor shoot length ranging from 14.2 with 1-2 pairs of leaves,while increase in concentration BA(1mg/L) induced longest shoot (28.1mm) with 3-4 pairs of leaves. The leaves produced at lower concentration of BAP are longer compared with the shoots obtained at higher concentration of BAP. The similar report was found by stephen *et al.,* 2010 and also by Kukreja *et al.,* 1990 in *Pogostemon cablin,* Sen and Sharma 1991 in *Withania somnifera,* Vicent *et al.* (1992) in *K. galango*.

On the medium containing0.5mg/L BAP with 1.0 mg/L NAA responded well and produced more shoots than the medium containing high concentration of NAA (1.5-3.0mg/L) with kinetin. It has been also reported that coconut water supplemnted media show better response than citric acid supplemented media. Similar report was observed by Roy *et al.* (1995), Reported that addition of 10% CM in the medium increase the number of shoots in *E. robustus*. Rahman *et al.* (1999) also observed similar effects *on embca officinalis* culture.

The synergetic effect of auxin (NAA) and cytokinin (BAP, Kinetin) was also observed in the experiment and was observed that the explant responded well and produced more shoots than the medium containing only cytokinin. Similar response was reported by Roy *et al.* (1995) in case of *Rauvolfia serpentia*.More or les similar response was also observed by Nagaranjan *et al.* (2005) in *B.Diffusa* and Ahmad *et al.* (2001) in *Holarrhena antidysenterica*.

ACKNOWLEDGEMENT

The authors are thankful to Centre for Biotechnology, Marwari College Ranchi for the research facility for carrying out this study .We are also thankful to Principal of Marwari college and UGC/CPE project grants to make the work accessible and smooth.

REFERENCES

Ahmed, G., Roy, P. K. and Mamun, A. N. K. 2001. High frequency shoots regeneration from nodal and shoot tip explants in *Holarrhena antidysenterica* L. *Indian J. Experimental Biology*. **39:** 1322-1324.

Angela Karp 1995. Somaclonal Variation as a tool for crop improvement, Department of agricultural sciences,University of Bristol,Institute of Arable Crop Research, Long Ashton Research Station,Bristol,BSI89AF;UK,Euphytica 85; 295-302, K Academic Publisher. Printed in Netherlands

Awasthi, L. P., Kluge, S. and Verma, H. N. 1989. Characteristics of antiviral agents induced by *Boerhaavia diffusa* glycoprotein in host plants. *Indian J. Virology*. **3:**156–169.

Baldi, A. and Dixit, V. K. 2008. Enhanced artemisinin production by cell cultures of Artemisia annua. *Current Trends in Biotechnology and Pharmacology*. **2:** 341-348.

Barz, W., Reinhard, E. and Zenk, M. H. 1977. Plant tissue culture and its biotechnological application. Springer Verlag, Berlin, New York, 27 - 43.

Bhansali, R. R., Kumar, A. and Arya, H. C. 1978. *In vitro* induction of adventitious shoots on stem explants of *Boerhaavia diffusa* L. *Current Science*. **47:** 551–552

Brar, D. S. and Jain, S. M. 1998. Somaclonal variation: Mechanism and application in crop improvement. Academic Publishers. London. pp.15-37.

Bright, S. W. J. 1985. On the causes and origins of somaclonal variation. *Oxford Survey of Plant Molecular and Cell Biology*. **2:** 199-234.

Chopra, G. L. 1969. Angiosperms. Systematics and Life Cycle. S. Nagin and Co., Jalandhar, Punjab, India. pp. 361-365.

Datta, P. C. and Datta, S. C. 1985. Applied biotechnology on medicinal, aromatic and timber plants. Calcutta University, Calcutta, India

Dhar, M. L., Dhar, M. M., Dhawan, B. N., Mehrotra, B. N. and Ray, C. 1968. Screening of Indian plants for biological activity: Part I. *Indian J. Experimental Biology*. **6:** 232–247.

Evans, D. A. 1989. Somaclonal variation— genetic basis and breeding application. *Trends in Genet*. **5:** 46-50.

Farzana, B. C., Safiul Azam, F. M., Maruf, H., Farhana, I. J., Anita, R. C., Syeda, S., Zubaida, K., Rahmatullah Karp, N. 2011. Studies of callus induction of Vitex Negundo:An aromatic medicinal plant. *American –Eurasium J. Sustainable Agriculture*. **S(1):** 6-14.

Gamborg, O. L. and Phillips, G. C. 1995. Laboratory facilities, operation and management. *In :* Gamborg OL and Phillips GC (Ed.) Fundamental methods of plant cell, tissue and organ culture. Springer, Berlin, New York, pp. 3-20.

Hassan, M. M., Azam, F.M. S., Chowdhury, M.H. and Rahmatullah, M. 2009. Callus Induction of *Abrus precatorius*: Screening of Phytohormones. *American-Eurasian J. Sustainable Agriculture*. **3(3):** 512-518.

Humera, A. and Javed, I. 2010. *Department of Botany, University of the Punjab, Lahore, Pakistan. In vitro* techniques and mutagenesis for the genetic improvement of potato cvs.desiree and diamant*2school of Biological Sciences, University of the Punjab, Lahore. Pakistan. Pak. J. Bot*. **42(3):** 1629-1637.

Juned, S. A., Jackson, M.T. and Ford-Lloyd, B. V. 1991. Genetic variation in potato cv. record: Evidence from *In vitro* regeneration ability. *Ann. Bot*. **67:** 199-203.

Kiong, A. L., Mahmood, M., Fodzillan, N. M. and Daud, S. K. 2005. Effects of precursor supplementation on the production of triterpenes by *Centella asiatica* callus culture. *Pakistan J. Biological Sci*. **8:** 1160-1169.

Kukreja, A. K., Mathur, A. K., Ahuja, P. S. and Thakur, R. S. 1989. Tissue culture and biotechnology of medicinal and aromatic plants. ICSIR, Lucknow.

Kukreja, A. K., Mathur, A. K., Zaim, M. 1990. Mass production of virus free patchouli plants [*Pogostemon cablin* (Blanco) Benth] by in vitro culture. *Trop Agric*. **67:** 101-104.

Larkin, P. J. and Scowcroft, W. R. 1981. Soamclonal variation -a novel source of variability from cell cultures for protoplast improvement. *Theor. Appl. Genet*. **60:** 197-214.

Nagarajan, S. M., Suresh, T., Rajasekaran, S., Kannan, T. M. S. and Kulothungan, S. 2005. *In vitro* micropropagation of *Boerhaavia diffusa* L. *Geobios-Jodhpur*. **32:** 169-172.

Nikolaeva, T. N., Zagoskina, N. V. and Zaprometov, M.N. 2009. Production of phenolic compounds in callus cultures of tea plant under the effect of 2, 4-D and NAA. Russ. *J. Plant Physiology*. **56:** 45-49.

Olivira, A. J. B., Koika, L., Reis, F. A. M. and Shepherd, S. L. 2001. Callus culture of *Aspidosperma ramiflorum* Muell.-Arg. Growth and alkaloid production. *Acta Scientia*. **23:** 609-612.

Rani, G., Virk, G. S. and Nagpal, A. 2003. Callus induction and plantlet regeneration in *Withania somnifera (L.)* Dunal. *In vitro* Cellular & Developmental Biology - Plant. **39(5):** 468-474

Rahman, M. M., Roy, P. K., Mannan, M. A. and Roy, S. K. 1999. Clonal propagation of *Emblica officinalis* through *in vitro* culture. *Plant Tissue Cult*. **9(1):** 17-23

Roy, S. K., Roy, P. K., Rahman, M. and Hossain, T. 1995. Clonal propagation of *Rauvolfia serpentina* through *in vitro* culture. *Acta Hort*. **390:** 141-146.

Sen, J. and Sharma, A. K. 1991. Micropropagation of *Withania somnifera* from germinating seeds and shoot tips. *Plant Cell Tissue Organ Cult*. **26:** 71-73.

Shah, T. M., Mirza, J. I., Haq, M.A. and Ata, B.M. 2008. Induced genetic variability in chickpea (*Cicer arietinum* L.) II. Comparative mutagenic effectiveness and efficiency of physical and chemical mutagens. *Pak. J. Bot*. **40(2):** 605-613

Shrivastava, N. and Padhya, M. A. 1995. Punarnavine profile in the regenerated roots of *Boerhaavia diffusa* L. from leaf segments. *Current Science*. **68:** 653–656.

Stephen, M., Nagaranjan, S. and Ganesh, D. 2010. Phlorogyllucinol and silver nitrate enhances axillary shoot proliferation in nodal explants of Vitex Negundo. *An aromatic medicinal plant-Iranian Journal of Biotechnology*. **8(2):** 82-89.

Thomas, T. D. and Maseena, E. A. 2006. Callus induction and plant regeneration in *Cardiospermum halicacabum* Linn. An important medicinal plant. *Scientia Horticulturae*. **108(3):** 332-336.

Vincent, K. A., Mathew, K. M. and Hariharan, M. 1992. Micropropagation of *Kaempferia galanga L.* -a medicinal plant. *Plant Cell Tissue Organ Cult*. **28:** 229-230.