IN VITRO STUDIES ON ORGANOGENIC POTENTIAL OF SOME IMPORTANT COTTON VARIETIES

Aneela Yasmin*, Shabana Lochi, Noorunisa Memon, Shazia Yasin, Shahneela Khaskhali, Rani Abro and Shahid Hussain Abro

Sindh Agriculture University, Tandojam, Pakistan

*Corresponding Author: Dr. Aneela Yasmin, Department of Biotechnology, Faculty of Crop Production, Sindh Agriculture University Tandojam, Sindh, Pakistan.

Email: aneelayasmin@yahoo.com

ABSTRACT: *Present study was aimed to optimize a protocol for in vitro based propagation of cotton through callus and shoot tip culture. In this regard four cotton varieties including G. arboreum (diploid wild cotton) and three tetraploid varieties of G. hirsutum i.e. Marvi, Cris-121 and Cris-9, were selected. 5-7 days old seedlings of selected varieties were excised and cultured on MS media containing different concentrations of 2,4-D. The callus growth was quite profound on 1.0 mgL^{-1} of 2,4-D as compared to 0.5 mgL^{-1}. The induced callus was transferred to 2,4-D (0.25 mgL^{-1}) to induce somatic embryogenesis (SE) and BAP (0.5 mgL^{-1}) to induce shoots. Meanwhile shoot tips of diploid cotton G. arboreum, were used to initiate in vitro culture on MS medium supplemented with various concentrations of BAP (0.0, 0.5 and 1.0 mgL^{-1}) and IBA (0.0, 0.5 and 1.0 mgL^{-1}). BAP at the concentration of 1.0 mgL^{-1} initiated shoots after 41.67 days, it also revealed the highest numbers of shootlets (4.07), numbers of leaves (3.23) and length of shootlets (3.61 cm). When these shoots were transferred to IBA at the concentration of 1.0 mgL^{-1}, it demonstrated the highest number of roots (8.40) with good length (3.22 cm) per shootlet. The complete healthy plantlets were successfully transferred to pots after 3-weeks of acclimatization under high humidity.*

Keywords: *G. hirsutum, G. arboreum,* Callus induction, shoot tip culture

1. INTRODUCTION

Cotton crop (Gossypium hirsutum L.) is an economically important cash crop of Pakistan. Although, the plant breeders and researchers have improved its yield and fiber quality using conventional breeding methods, conventional breeding is not enough to improve cotton genome due to lack of variation available in commercial germplasm & lengthy process of breeding cycle. In this scenario, plant biotechnology can facilitate conventional breeding by using techniques such as ovule culture [1], shoot apex culture and genetic transformation [2], somatic embryogenesis [3], protoplast culture [4] etc. Nevertheless, genetic engineering of cotton again has a major setback of unavailability of well-established tissue culture protocols. Callus culture followed by proper regeneration into plantlets is an important source of adding variation in present germplasm [5]. Therefore development of a reproducible tissue culturing protocol will help to harness the proper advantages of plant biotechnology especially genetic engineering.

However, the application of tissue culture technology will depend on the availability of an effective regeneration system through callus and/or shoot tip culture. The production of callus and its subsequent regeneration through somatic embryogenesis can facilitate the production of transgenics [6]. Regeneration of plants through somatic embryogenesis is a prime process because a single cell can give rise to a somatic embryo, thus chimeric transformants are rare in this process. Therefore, somatic embryogenesis is a method of choice as compared to direct organogenesis.

Additionally, shoot tip culture can also be used to generate transgenics [7]. When there is no somatic embryogenesis in some cotton variety due to its recalcitrant nature then organ cultures are a method of choice to apply genetic engineering techniques.

The first report of cotton somatic embryogenesis demonstrated the regeneration of cotton plantlets from somatic embryos of Coker variety 310 using 2 years old callus [8]. Since then, Coker varieties were found to respond somatic embryogenesis and regeneration. Thus desirable traits are initially introduced into Coker through genetic engineering & later cross backed to established commercial cotton varieties. This adds many undesirable traits also into commercial varieties and to get rid of these undesired traits again need many years of back crossing. Therefore, there is a need to screen different commercial cotton varieties in routine to find out non-recalcitrant cotton varieties for tissue culture that can be transformed directly without using Coker varieties.

Keeping in view the importance of cotton tissue culture and its usage in Plant Biotechnology, the present study is aimed to screen the callogenesis and regeneration potential of some selected cotton varieties for the development of an optimal in vitro protocol through callus or shoot tip culture.

2. MATERIAL AND METHODS

Desi cotton (*G. arboreum*) and cultivated cotton varieties e.g. Marvi, Cris-121 and Cris-9 were used as plant material. Cotton seeds of selected varieties were de-linted in concentrated H_2SO_4 and soaked overnight in sterile distilled water. Next day seeds were cultured in vitro on ½ MS media. 5-7 days old seedlings were sterilized in 15 % of bleach comprising one to three drops of Tween-20 for 20 minutes followed by a dip in 70% ethanol and rinsing this material three times in sterile distilled water. Further manipulation of the explants was occurred in a laminar air flow cabinet with autoclaved sterile surgical tools. The excised shoot tips or leaves discs (1x1 cm2), stem discs (1-2 cm) and root discs (1-2 cm) of cotton seedlings were cultured on MS basal medium (Murashige & Skoog, 1962) supplemented with various concentrations of BAP i.e. 0 mgL^{-1}, 0.5 mgL^{-1}, 1 mgL^{-1} or of 2,4-D (0 mgL^{-1}, 0.5 mgL^{-1} & 1 mgL^{-1}). The explants were incubated at 22+3°C, under a 16-hour photoperiod and in luminous intensity of 2000 lux.

Data was collected for the callus growth response, its morphology and regeneration potential for somatic embryogenesis or direct shoots or roots induction. In shoot tip culture data was recorded for the days taken to initiate new shoots, number of shoots per explant, number of leaves per explant, length of shoots, number of roots per explant and length of roots on different concentrations of BAP or IBA. The collected data was evaluated through analysis of variance and L.S.D (Least Significant Difference) test as integrated in Statistix 8.1.

3. RESULTS AND DISCUSSION

In vitro propagation is one of the major steps leading to successful exploitation of plant biotechnology or genetic engineering. Therefore, present study was aimed to optimize a protocol for in vitro based propagation of cotton through callus and shoot tip culture.

Callogenesis

Callus induction, its morphology, growth response and regenerative potential were studied in one diploid wild cotton G. arboreum and three tetraploid varieties of G. hirsutum namely Marvi, Cris-121 and Cris-9. Different explants were used to initiate callus culture such as discs of seedling stem, cotyledonary leaves and root. Among them stem and leaf discs showed callus induction within 35-45 days after inoculation whereas root discs never responded in vitro and became dead within a month. All four cotton varieties induced callus at 0.5 and 1.0 mgL^{-1} concentrations of 2,4-D (table 1). The callus growth was quite profound on 1.0 mgL^{-1} as compared to 0.5 mgL^{-1}. The morphology of callus was recorded by observing its color and texture. *G. arboreum* and Marvi produced hard reddish brown and hard reddish green callus respectively, whereas Cris varieties (121 & 09) showed granular whitish yellow and loose sticky yellowish green callus respectively (figure 1). The induced callus was transferred to a lower concentration of 2,4-D (0.25 mgL^{-1}) to induce somatic embryogenesis (SE) and BAP (0.5 mgL^{-1}) to induce direct organogenesis that is, shoots or roots. During the period of 16-weeks these calli did not show any regeneration in the form of somatic embryogenesis (SE) or direct shooting or rooting. Most of these calli became necrotic and dead during 4th month of culturing. These cultures were maintained on MS basal media containing 0.25 mgL^{-1} of 2,4-D or 0.5 $mgL^{-1}$of BAP with regular sub-culturing on every 15th day. Based on the observation, it was found that the callogenesis in selected cotton varieties (*G. arboretum*, Marvi, Cris-121 and Cris-9) is possible using 0.5 and 1.0 mgL^{-1} of 2,4-D in MS basal media. However, the induced calli did not reveal any kind of regeneration on the tested regime of growth regulators in MS medium i.e. 0.25 mgL^{-1} of 2,4-D and 0.5 $mgL^{-1}$of BAP.

The production of callus and its subsequent regeneration through somatic embryogenesis can facilitate the production of transgenics [6]. Callus induction and somatic embryogenesis is a method of choice as compared to direct organogenesis because a single cell give rise to a somatic embryo, thus chimeric transformants are rare. According to previous studies of cotton tissue culture, callus culture can be initiated from different explants such as hypocotyls, radicals, tissues of embryo, shoot apex, cotyledonary leaves etc. [9]. In this study different explants were excised from 5-7 old seedling such as tissues of cotyledonary leaves, stems, roots etc. and were cultured on MS media supplemented with different concentrations of 2,4-D (0.5 and 1.0 mgL^{-1}).

Although there are many reports on cotton (*G. hirsutum* L. or wild genotypes) somatic embryogenesis [3,10,11] these reports demonstrated that the in vitro somatic embryogenesis depends on genotype and age of the callus and majority of embryos resulting from somatic embryogenesis are abnormal showing lack of shoot elongation or difficulty in forming complete plantlets. In agreement to these studies callus induction was quite easy on 2,4-D. All varieties produced good quantities of calli within a month or in six weeks at both concentrations of 2,4-D (0.5 and 1.0 mgL^{-1}). *G. arboreum* revealed hard reddish brown callus without any regeneration after 16 weeks the same response was observed in all *G. hirsutum* varieties. The embryogenic callus of *G. hirsutum* could be granular or friable and cream, yellow or green in colour [10,12]. In agreement to this *G. hirsutum* varieties revealed granular, yellowish green calli in present study, but in contrast these selected cotton varieties did not show any induction of somatic embryos, direct shooting or rooting on proliferated calli. Such contrasting results could be due to genotypical effect and age of calli. In present study the callus stimulated for somatic embryogenesis was only one month old. According to [8] the first report of cotton somatic embryogenesis demonstrated the regeneration of cotton plantlets from somatic embryos of Coker variety 310 using 2 years old callus. In *G. arboreum* the callus growth and proliferation was very fast but that callus became brown on subculturing. Similar observations were recorded by [10] in cotton genotype *G. africanum*. They also claimed that the identification of embryogenic callus is quite difficult in tissue culture studies of cotton.

Although in present study embryogenic calli were not formed on the tested hormones concentration, there are many tactics reported that can improve the induction of embrogenic callus. These include different combinations of growth hormones, application of suspension cultures, addition of different organics (amino acids, glucose, maltose) or inorganic factors such as KNO3 or MgCl2 [10,11]. Combination of these tactics can improve somatic embryogenesis in cotton. The cotton is well known for its recalcitrant nature to regeneration through callus and somatic embryogenesis. The reason for this is some unknown genetic factors that vary from one genotype to other [13]. With accordance to this none of the studied genotypes responded to 2,4-D and did not induce somatic embryogenesis in present study. Therefore, there is a need for such a protocol that does not require plantlet regeneration through somatic embryogenesis and the best option to this is direct transformation of commercial germplasm through shoot tip/ apices culture.

Shoot tip culture of *G. arboreum*

Initially, [7] demonstrated direct transformation of cotton using shoot tip culture. Later, meristem based many cultures were used for *Agrobacterium* mediated transformations in cotton [14], rice [15], banana [16], corn [17], pea [18] etc. Before transformation an optimized protocol for shoot tip

culture is a must. Keeping in view these facts shoot tip. Table 1: Cotton callogenesis on MS media supplemented with different concentrations of 2, 4-D.

Table 1

Cotton species	Conc. of 2,4-D mgL^{-1}	Growth response	Callus Morphology		Regeneration potential
			Color	Texture	
G. arboreum	0.0	-	-	-	No SE, shooting or rooting
	0.5	+	Brown	Hard	
	1.0	++	Reddish brown	Hard	
Marvi	0.0	-	-	-	No SE, shooting or rooting
	0.5	+	Reddish green	Hard	
	1.0	++	Reddish green	Hard	
Cris-121	0.0	-	-	-	No SE, shooting or rooting
	0.5	+	Yellow	Granular	
	1.0	++	Whitish yellow	Granular, soft	
Cris-9	0.0	-	-	-	No SE, shooting or rooting
	0.5	+	Green	Loose	
	1.0	++	Yellowish green	Soft & sticky	

Table 2: Cumulative effect of various concentrations of BAP on shooting related parameters

BAP Concentrations	Days taken to shoot	Number of shootlets	Number of Leaves/ shoot	Length of shootlets
0.0 mgL^{-1}	0.00b	0.00c	0.00c	0.00c
0.5 mgL^{-1}	41.20a	1.87b	2.18b	2.58b
1.0 mgL^{-1}	42.13a	4.07a	3.23a	3.61a

Table 3: Cumulative effect of various concentrations of IBA on rooting related parameters

IBA Concentrations	**Number of roots per shootlet**	**Length of roots**
0.0 mgL^{-1}	0.00b	0.00c
0.5 mgL^{-1}	7.73a	2.30b
1.0 mgL^{-1}	8.40a	3.22a

culture of *G. arboreum* was studied as the second part of present investigation and this organ culture resulted the production of healthy plantlets within 6-months.

In this regard, shoot tips of diploid cotton *G. arboreum*, were cultured in vitro and data was recorded for days taken to shoot, number of shootlets per explants, number of leaves per shoot, length of shootslets, number of roots per shootlet and length of roots (table 2 & 3). The basal media utilized for this purpose was MS medium supplemented with various concentrations of BAP (0.0, 0.5 and 1.0 mgL^{-1}) and IBA (0.0, 0.5 and 1.0 mgL^{-1}). BAP was used to induce shooting whereas IBA as root inducer.

The analysis of variance for days taken to initiate new shoots under different concentrations of stimulator BAP suggests that the different concentrations of BAP are significantly effecting the duration to induce shoots at p= 0.05. When BAP was not added to media there was no shoot initiation even after 12 weeks whereas at concentration 0.5 and 1.0 mgL^{-1} shoot initiation took place after 42.13 and 41.67 days respectively. Although on average 0.5 mgL^{-1} BAP concentration took minimum days to initiate the shoots (41.2 days), the two duration means (41.2 and 42.13 days) were not significantly different from each other showing that the any tested concentration of BAP will take about the same time to initiate shooting. Negative control that was the media without BAP showed no shoot initiation that indicates that the growth regulators are a must to initiate shooting.

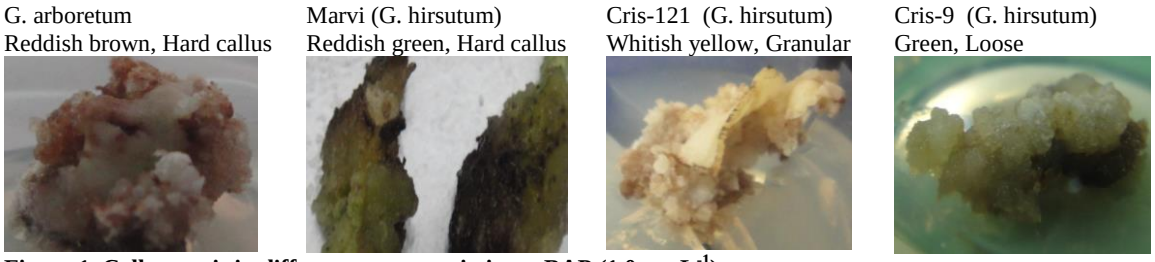

Figure 1. Callogenesis in different cotton varieties at BAP (1.0 mg L^{-1})

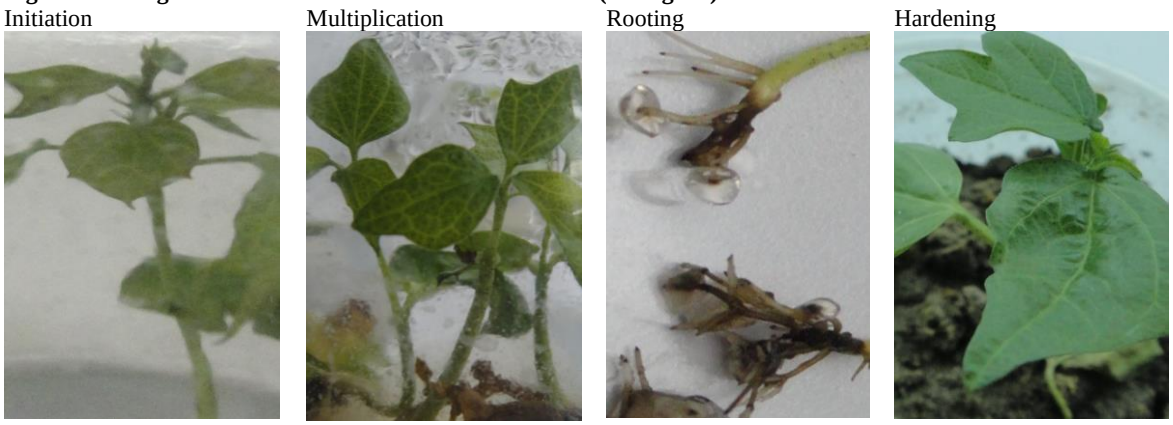

Figure 2. Shoot tip culture of *G. arboreum*.

After shoot initiation, data for number of shootlets per explants, numbers of leaves per shoot and length of shoot was collected after 8-weeks with two subcultures, each after 3-weeks in the same media and treatment. Based on ANOVA and LSD three different mean populations were found showing significant differences among the hormone concentrations and their effect on the induction of shootlets, leaves or shoot length. When no hormone was added to the media there was no growth but as the concentration of BAP was increased in media from 0 to 0.5 and 1.0 mgL^{-1} the numbers of shoots were also increased from 0 to 1.87 and 4.07 respectively. The highest numbers of leaves (3.23) and shoot length (3.61 cm) were also recorded on 1.0 mgL^{-1} concentration. As the highest no of shootlets, number of leaves and shoot length was recorded at 1.0 mgL^{-1} of BAP therefore, the optimal concentration of BAP that effected shootlet formation in *G. arboreum* is 1.0 mgL^{-1}.

The mean number of roots formed on different concentrations of IBA were analyzed through ANOVA and LSD (p= 0.05) and results showed that there were only two mean populations. One mean population represent negative control which did not show any kind of rooting whereas the remaining two means of root numbers obtained on MS media containing 0.5 mgL^{-1} and 1.0 mgL^{-1} concentrations of IBA represent only one population. Although it is obvious from the table that 1.0 mgL^{-1} of IBA demonstrated the highest number of roots (8.40) followed by 0.5 mgL^{-1} of IBA (7.73), these differences were non-significant. Therefore, to obtain the highest root number per shootlet, 1.0 mgL^{-1} of IBA can be added to MS basal media as this concentration of IBA can be considered the most successful quantity of IBA among the two other concentrations because it produced the longest roots (3.22 cm) per shootlet. When the shoots became 3-5 inches long having 3-4 leaves and a well-developed root system then these were transferred to pots with sterile soil (mixture of clay and sand). Pots were kept in covered boxes to maintain high humidity. During 24-hours the plantlets were uncovered for a limited time to harden during three weeks of acclimatization. All plants showed new growth and turned into healthy plants when shifted to net house (figure 2).

Abdellatef and Khalafalla [2] demonstrated adventitious shoots and complete plantlet formation in *G. hirsutum* L. cv Barac [67] B. They used B5 medium with benzyladenine (BA) or Kinetin (KIN) in combination with α naphthalene acetic acid (NAA). However, KIN at the concentration of 2.0 mgL^{-1} revealed the best shoot induction whereas the best rooting was obtained on ½ strength B5 medium containing 0.1 mgL^{-1} NAA with sucrose (2.0%). Instead of this, we used MS basal media supplemented with BAP (1.0 mgL^{-1}) for shooting and IBA (1.0 mgL^{-1}) for rooting. It is worthwhile to describe here the difference between the genotypes of both studies; we used a wild diploid genotype of cotton *G. arboretum* whereas Abdellatif and Khalafalla (2007) used *G. hirsutum*. Thus the differences found in both studies could be attributed to the difference in the concentration of growth hormones and the genotypes.

In accordance to Ozyigit et al. [19] we also found that the shoot induction depends on the presence of cytokinin. Cytokinin actually, reprograms the shoot tips for multiple shoot induction. The highest number of shoots, leaves per shoot and length of shoots were observed on BAP at 1.0 mgL^{-1} (table 2 & 3). This was the optimum concentration of BAP and further increase in its concentration did not increase the shoot growth in any way, however, fewer shoots were observed. Hemphil et al. [20] also studied the effect of

single hormone BA on shoot formation in *G. hirsutum* L. cv. Stoneville 7A and observed the highest number of multiple shoots and shoot elongation on BA at the concentration of 0.3 mgL^{-1}. There are many reports on shoot tip culture of cotton demonstrating the use of different cytokinins as BAP, KIN, BA alone or in combination to get multiple shooting [19,21,22] This difference in usage of hormone is actually due to the differences in studied cotton genotypes. Different genotypes induce shooting on different hormones. Thus, obtaining a genotype independent protocol for shoot tip culture of cotton is very difficult. Similar observations are reported by Bazargani et al. [23].

In agreement to present study, Bushra et al. [24] also used MS basal media [25] to culture shoot apices of twenty two (22) cotton varieties and got promising growth. Interestingly, they did not use any growth regulators in media. Although cotton shoot apices revealed good growth response in MS media without hormones as demonstrated by Bushra et al. (2004), we did not find shoot and root formation without growth hormones. In another report *G. arboreum* produced multiple shoots on MS basal media containing BAP (2.0 mgl^{-1}) and KIN (1.0 mgl^{-1}) with the frequency of 8.5 to 17.2 shoot number/ explant [26]. In contrast we used BAP alone and the highest numbers of shootlets observed were 4.07 at the 1.0 mgl^{-1} of BAP in MS basal media. According to Bushra et al. [24] such differences are may be due to different seed lots and genotypes.

There are many reports demonstrating no root formation on in vitro regenerated shoots [9,24,27]. In present study regenerated shoots induced roots on IBA (1.0 mgl^{-1}) and after complete plantlet formation on MS media containing growth hormones, the plantlets were successfully transferred to pots. Similar to these findings, Ozyigit et al. [19] also used MS basal media supplemented with 1.0 mgL^{-1} IBA and observed rooting in in vitro generated shoots. Similarly, Mushke et al. [28] also regenerated well rooted plantlets on MS media containing growth hormones and acclimatized these plantlets in greenhouse with 98% survival rate.

CONCLUSION

In this study the shoot tip culture of G. arboreum was optimized according to which shoot tips of 5-7 days old seedling could be cultured on MS media containing BAP (1.0 mgL^{-1}) to get the highest number of shoots with good length and number of leaves. Regenerated shoots can be rooted on IBA (1.0 mgL^{-1}). The well-established complete plantlets can be transferred to pots after 3-weeks of weaning. This protocol can be utilized to generate transgenics using gene gun or Agrobacterium mediated transformation systems.

REFERENCES

[1] Lee J.J., A.W. Woodward and Z.J. Chen. 2007. Gene expression changes and early events in cotton fiber development. Ann. Bot., 100(7): 1391-1401.

[2] Abdellatef E. and M. M. Khalafalla. 2007. Adventitious shoot and plantlet formation in medium staple cotton cultivar (Gossypium hirsutum L. cv Barac [67] B). International Journal of Agriculture & Biology, 9(6): 913-916.

[3] Rao A. Q., S. Hussain, M.S. shahzad, S. Bokhari. A. Yassir, M. H. Raza, A.rakha, A. A. Majeed, A. Shahid, Z. Saleem, T. Husnain and S. Riazuddin. 2006. Somatic embryogenesis in wild relatives of cotton (Gossypium Spp.). J. Zhejiang. Univ. SCI. B., 7(4): 291-298.

[4] Peeters M., K. Willems and R. Swennen. 1994. Protoplasm to plant regeneration in cotton (Gossypium hirsutum L. cv. Coker 312) using feeder layers. Plant Cell Rep., 13: 208-211.

[5] Barzali P., A.J. Mofidabadi, M. R. Zangi and R. Bozorgi. 2011. The using of gibberellic acid hormone on cotton mature embryo resulted by crossing between wild and commercial species on artificial medium. African Journal of Biotechnology, 10(46), 9322-9327

[6] Pushpa R. and T.S. Raveendran. 2010. Optimization of shoot apex based cotton regeneration system. Electronic Journal of Plant Breeding, 1(4): 1235-1240.

[7] Gould J.H. and M. Magallanes-Cedeno. 1998. Adaptation of Shoot apex agrobacterium inoculation & culture to cotton transformation. Plant Mol. Biol. Rept., 16 (3) 284-289

[8] Ghaemi M., A. Majd, F. Fallahian and K. GhasemiBezdi. 2011. Comparisonof call us induction and somatic Spp.) with Coker 312 and histology of somatic embryogenesis. Afr. J. Biotechnol., 10(15): 2915-2922.

[9] Abdellatef, E and M. M. Khalafallah. 2008. Influence of growth regulators on callus induction from hypocotyls of medium staple cotton (Gossypium hirsutum L.) Cultivar Barac B -67. J. Soil. Nature, 2: 17-22.

[10] Sun, Y., X. Zhang, C. Huang, X.Guo and Y.Nie. 2006. Somatic embryogenesis and plant regeneration from different wild diploid cotton (Gossypium) species Plant Cell Rep., 25: 289-296.

[11] Kumria R, V.G. Sunni Chan, D.K. Das, S.K. Gupta, V.S. Reddy, R.K. Bhatnagar and S. Leekavathi. 2003. High-frequency somatic embryo production and maturation into normal plants in cotton (Gossypium hirsutum L.) through metabolic stress. Plant Cell Rep., 21: 635-639

[12] Sakhanokho H. M., Z. F. Allan, K. Rajasekaran, S. Saha and G. C. Sharma. (2001). Induction of highly embryogenic calli and plant regeneration in Upland (Gossypium hirsutum L.) and Pima (Gossypium barbadense L.) Cottons. Crop. Sci., 41: 1235-1240.

[13] Trolinder N. L. and J. R. Goudin. 1987. Somatic embryogenesis and plant regeneration in cotton (Gossypium hirsutum L.). Plant Cell Rep., 6: 231-234.

[14] Wilkins T. A., K. Rajasekaran and D. M. Anderson. 2000. Cotton biotechnology. Crit. Rev. Plant Sci., 19: 511–550

[15] Park S.H., S.R.M. Perison and R.H. Smith. 1996. T- DNA integration into genomic DNA of rice following Agrobacterium inoculation of isolated shoot apices. Plant Mol. Biol., 32: 1135–1148

[16] May G., R. Afza, H. Mason, A. Wiecko, F. Novak, and C. Arntzen. 1995. Generation of transgenic banana (Musa acuminata) plants via Agrobacterium-mediated transformation. Biotechnology, 13: 486-492

[17] Gould J., M. Devey, O. Hasegawa, E.C. Ulian and G. Peterson. 1991a. Transformation of zea mays L., using Agrobacterium tumefaciens and the shoot apex. Plant Physiol., 95:426–434

[18] Hussey G., R.D. Johnson., S.Warren. 1998. Transformation of meristematic cells in the shoot apex of cultured pea shoots by Agrobacterium tumefaciens and A. rhizogenes. Protoplasma, 148: 101-105.

[19] Ozyigit Ibrahim I. 2008. Phenolic changes during in vitro organogenesis of cotton (Gossypium hirsutum L.) shoot tips. African Journal of Biotechnology, 7(8): 1145-1150.

[20] Hemiphill J.K., C.G.A. Maier, K.D. Chapman. 1998. Rapid in-vitro plant regeneration of cotton (Gossypium hirsutum L). Plant Cell Rep., 17: 237-278

[21] Pathi K, Tuteja N. 2013. High-frequency regeneration via multiple shoot induction of an elite recalcitrant cotton (Gossypium hirsutum L. cv. Narashima) by using embryo apex. Plant Signal Behav, 3(8): 22763-22795; PMID: 23221745.

[22] Laud D., S.B. Nandeshwar, M. Sandhya and P. Kulkarni. 2010. Effect of growth regulators and explant types on regeneration of diploid cotton G. arboretum. Asiatic Journal of Biotecnology Resources, 03: 214-219.

[23] Bazargani M. M., E.S.T. Badraldin and O. Mansoor. 2011. Multiple shoots regeneration of cotton (Gossypium Hirsutum L.) via shoot apex culture system. African Journal of Biotechnology, 10(11): 2005-2011.

[24] Bushra R., Tayyab H, Riazuddin S (2004). In-vitro shoots tip culture of cotton (Gossypium hirsutum). Pakistan J. Biotechnol. Sci., 36(4): 817-823.

[25] Murashige T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures: Physiol Plant.; 15:473-497.

[26] Chinchane B.N., L.A. Deshpande, S.B. Nandeshwar and V.N. Chinchane. 2005. In vitro plant regeneration through multiple shoot induction in diploid cotton Gossypium arboreum L. (cv. PA 255 and PA 402). J. Cotton Res. Dev., 19 (1): 1-6

[27] Aslam M., M. Ashfaq, T. Saeed, Sarni Ul Allah and Y. Zafar. 2010. In vitro morphogenic response of cotton (Gossypium hirsutum L.) from apical meristem culture. American-Eurasian J. Agric. & Environ. Sci., 7 (1): 07-11, ISSN 1818-6769

[28] Mushke R., S. Tasleem, K. P. Pavan. 2012. High frequency regeneration and multiple shoot induction in indian cotton (Gossypium hirsutum L.) cultivar. Research Journal of Agricultural Sciences, 3(5): 1109-1112.