Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 6 [2] January 2017: 83-90

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.533
Universal Impact Factor 0.9804

ORIGINAL ARTICLE **OPEN ACCESS**

Standardization of preculture Treatments to enhance Transformation efficiency in *Gossipium hirsutum* (L.)

Dipika Ashokrao Padole[1], Gahukar S.J.[2], Amrapali A. Akhare[3]
[1]Junior Research Assistant, Biotechnology Centre, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, India, dipikapadole@gmail.com, 09372138220
[2]Associate Professor, Biotechnology Centre, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, India, sj_gahukar@yahoo.com, 09921004345
[3]Assistant Professor, Biotechnology Centre, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, India, atulakhare@yahoo.com, 07588961132

ABSTRACT
Cotton is an important cash crop worldwide. Due to lack of effective regeneration protocol, cotton transformation efficiency remained poor. In the present study, attempt was made to develop efficient Agrobacterium mediated cotton transformation. The various explants such as shoot tip, cotyledonary node and mature embryo of cultivar PKV-Rajat were precultured with different treatments such as dehydration, desiccation and chilling. Mannitol and sorbitol at 0.1M concentration, desiccation for 15min using silica beads and chilling for 30 min found superior amongst the different levels tried. The various explants tried viz. shoot tip, cotyledonary node and mature embryo, mature embryo performed better followed by cotyledonary node and shoot tip in all the treatments.
Keywords: *Preculture treatments, dehydration, desiccation, chilling injury, Agrobacterium, transformation*

Received 20.10.2016 Revised 15.12.2016 Accepted 02.01.2017

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) has economic value worldwide as it is important fiber and oil crop. Early bud formation, lint strength, and high yield are the major properties of *Gossypium hirsutum* [1].Genetically modified cotton is cultivated on more than 24 million hectares worldwide and makes a significant contribution to the income of 16.5 million poor farmers in developing countries [2, 3]. The global adaptation rates of genetically modified cotton have increased 70% compared with conventional cotton [2].

Recent trends towards development of traits like fiber-quality, resistance against insect pests, herbicides, insecticides, and pathogens has been increased using genetic transformation techniques [4-6]. Different cultivars of cotton have been bred by hybridization as most regeneration protocols are genotype specific, and researchers need to develop genotype-independent protocols for cotton. Cotton is very difficult plant to regenerate (recalcitrant) under in vitro conditions .Although many shoot-regeneration and genetic transformation protocols have been developed in cotton culture, there generation and transformation frequency is very low and highly variable among cultivars [4-7].

Agrobacterium mediated genetic transformation technique is comparatively less expensive, very effective and remains the most successful gene transfer methods in plants. Because of its advantages of low copy number, defined and preferential integration of transgene into transcriptional active regions in the chromosome and produced fertile transformed plants [8-10]. but the transformation efficiency is very low in cotton[7]. Keeping this view, the present investigation was undertaken to enhance the transformation efficiency in cotton through the standardization of different preculture treatments.

MATERIALS AND METHODS

Selection of Plant Material: PKV-Rajat (hirsutum hybrid) released by Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra recommended for Vidarbha region. As the hybrid shows moderate tolerance to bacterial blight, jassids and bollworm it is widely cultivated in this region. But due to

continuous use of synthetic pesticides, insects are acquiring resistance against the pesticides used which can be overtaken only with the use of either biopesticides or transgenic.

Seed Delinting- Delinting was done with sulphuric acid @ 100ml/kg seed for 10 min and neutralized using lime. Further seeds were washed and dried.

Surface sterilization- Seeds were washed under running water and sterilized with fungicide one percent (w/v) bavistin and few drops of tween-20 for 10 minutes which were further treated with 1 percent (w/v) streptocycline for 10 minutes followed by rewashing with sterile distilled water for 2-3 times and were transferred to LAF unit in order to treat with 0.1 percent (w/v) mercuric chloride ($HgCl_2$) for 7 minutes followed by rinsing with sterile distilled water for 3-4 times.

In vitro germination of seeds

Surface sterilized seeds were aseptically inoculated on half strength MS media and kept in dark for 3 days at 27±2^{0}C for germination. After 3 days the germinated seeds were transferred in light until the seedling becomes ready for explants isolation.

Preparation of Explants

Shoot tip and cotyledonary node were excised from 7-10 days old seedlings grown on ½MS, whereas, mature embryos were obtained from overnight soaked seeds carefully. The explants were then precultured for 72 hrs on MS supplemented with 0.1mg/lit.

Media preparation

Tissue culture media with growth regulators

Different media were used either plain MS or MS in combination with growth regulators were depending on purpose of the experiment as tabulated in table 1.

Table 1: Different culture media used for transformation

Stage	**Media composition**
Seed Germination medium	½ MS
Preculture medium	MS + 0.1 kinetin
Shoot Induction medium	MS+0.1kinetin+cefotaxime @ 200mg
Selection medium	MS+0.1kinetin+kanamycin @ 100 mg

The regeneration protocol as previously standardized was used for the present experiments[11]. For culturing and maintenance of *Agrobacterium*, yeast extract mannitol medium either solidified or broth was used as per standard protocol, pH adjusted to 7.0 before autoclaving. When it cooled down to 45-50^{0}C after autoclaving, filter sterilized antibiotics were added to it.

***Agrobacterium* strain maintenance**

The *Agrobacterium* strain EHA-105 containing *cry*1F and *npt*-II as selectable gene was maintained either on solid medium, YEMA with kanamycin 50 mg/lit and 10 mg/lit rifampicin or glycerol stock. A single colony was streaked on YEMA from glycerol stock and allowed to grow for 48 hrs at 25 ^{0}C. These plates were then stored at 4 ^{0}C for further use.

Preparation of *Agrobacterium* for colonization

A single colony of bacterial growth of 48 hours was taken from mother plate and inoculated in 20 ml of YEM broth (without agar) containing 50 mg/lit of kanamycin and 10 mg/lit rifampicin, followed by incubation for 48 hours at 25 ^{0}C in orbital shaker with 120 rpm.

Preculture treatment

Any treatment given to explants prior to infection which makes explants sturdy and may increase the explants survival, regeneration and transformation frequency. During this study, explants were precultured using various treatments tabulated in table 2 prior to colonization with Agrobacterium inoculum. A separate experiment was conducted (data not presented here), to evaluate the effect of vacuum infiltration in combination with and without injury on explant survival on selection media, showed that the survival was higher when injured explant vacuum infiltrated for 15 min.

Dehydration: The principle behind dehydration is that osmoticum generated due to mannitol and sorbitol enhance the competency of plant cells for T-DNA uptake, and facilitate plant cell recovery after infection. The isolated explants were further inoculated on preculture medium for 72 hrs supplemented with mannitol and sorbitol at the rate of 0.1M, 0.2M, and 0.3M, to study transformation efficiency.

Desiccation: Desiccator containing silica beads completely dried and sterilized at 60^{0}C for 2-3hrs. in hot air oven was used. The explants precultured for 72 hrs in light were kept in the petridish and allowed to desiccate for 0 min, 15 min, and 30 min. During desiccation, moisture from cells absorbed by the silica beads so that the cells become dehydrated and becomes prone to absorb moisture from outside. When we

infect it with *Agrobacterium* culture, the cells absorbs it more efficiently, thus may enhance the T-DNA uptake and integration in to plant cell genome.

Table 2: Details of preculture treatments given to explants along with treatment combination

Method	Concentration/Time	Total No. of treatments
Dehydration using Mannitol		
Dehydration	Mannitol Levels (04) 0.0M, 0.1M, 0.2M, 0.3M	04
Dehydration using Sorbitol		
Dehydration	Sorbitol Levels (04) 0.0M, 0.1M, 0.2M, 0.3M	04
Desiccation Using Silica Beads		
Desiccation	Duration of desiccation (03) 0min, 15min, 30min	03
Chilling Injury at 4^0C		
Chilling Injury	Duration of chilling injury (04) 0min, 30min, 60min, 24hrs	04

Chilling Injury: The explants were chilled at 4^0C for 0 min, 30 min, 60 min and 24 hrs and infected with *Agrobacterium* culture, the cells absorbs it more efficiently, thus may enhance the infection efficiency.
For all the above treatments, the observations were recorded after 4 weeks, 8 weeks and 12 weeks and on rooting media and on selection medium.

Screening of putative transformants

Primary screening was done on shoot induction medium containing kanamycin and observations were recorded as percent survival of the putative transgenic plants after 4 weeks, 8 weeks and 12 weeks after inoculation on selection medium. If survived, rooted putative transformants were further screened using *npt*-II gene specific primers.

Statistical analysis

Ten culture bottles each with ten explants were used per replication for each treatment. Every treatment was replicated thrice. Thus, observations were recorded for 300 explants per treatment.
The observations were recorded for the number of shoot survived and regenerated on selection media after 4 weeks, 8 weeks 12 weeks and number of shoots transferred to rooting medium. Values of observation recorded in percentages and transformed to arcsine or square root values depending upon its range for statistical analysis. The data of present investigation was analyzed and ANOVA was carried out by using factorial completely randomized design (FCRD). The mean standard error and critical difference were calculated as per procedure given by Panse and Sukhatme in 1958 [12]**.** F-test at 5 percent level of significance was used to test the significance.

RESULT AND DISCUSSION

Dehydration using mannitol and sorbitol

A comparison based on mean percent survival of all the explants precultured on media containing mannitol and sorbitol showed, mature embryo performed better followed by cotyledonary node and shoot tip on selection media. When explants dehydrated with preculture media containing mannitol and sorbitol at varying concentrations such as 0.1 M, 0.2 M and 0.3 M and preculture media without mannitol served as control, the highest mean percent survival was shown when dehydrated with mannitol and sorbitol at 0.1 M concentration for 72 hrs (Table 3a and table 3b).

Table 3a: Effect of dehydration using mannitol on transformation efficiency

Explants	Variables	percent survival on selection medium			Mean
		4 weeks	8 weeks	12 weeks	
Shoot Tip	Control	0.67	0.00	0.00	0.22
	0.1 M	0.67	0.33	0.00	0.33
	0.2 M	0.33	0.00	0.00	0.11
	0.3 M	0.00	0.00	0.00	0.00
Mean		**0.42**	**0.08**	**0.00**	
Cotyledonary Node	Control	0.67	0.00	0.00	0.22
	0.1 M	0.67	0.33	0.00	0.33
	0.2 M	0.33	0.00	0.00	0.11
	0.3 M	0.00	0.00	0.00	0.00

Mean		**0.42**	**0.08**	**0.00**	
Mature Embryo	Control	0.67	0.33	0.00	0.33
	0.1 M	1.00	0.67	0.00	0.56
	0.2 M	0.67	0.33	0.00	0.33
	0.3 M	0.33	0.00	0.00	0.11
Mean		**0.67**	**0.33**	**0.00**	
Grand Mean		**0.50**	**0.16**	**0.00**	
SE (m) ± (A)		0.21	0.15	-	
CD (5 percent) (A)		0.60	0.44	-	
SE (m) ± (B)		0.18	0.13	-	
CD (5 percent) (B)		0.52	0.38	-	
SE (m) ± (AxB)		0.36	0.26	-	
CD (5 percent) (AxB)		1.04	0.77	-	

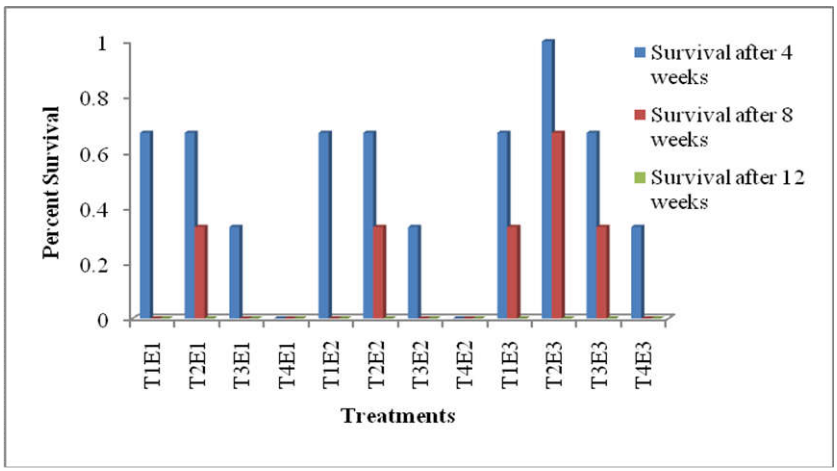

Fig 1: Graphical representation of explant survival after dehydration with mannitol

Table 3b: Effect of dehydration using sorbitol on transformation efficiency

Explants	**Treatments**	**percent survival on selection medium**			**Mean**
		4 weeks	**8 weeks**	**12 weeks**	
Shoot Tip	Control	0.33	0.00	0.00	0.11
	0.1 M	0.67	0.33	0.00	0.33
	0.2 M	0.33	0.00	0.00	0.11
	0.3 M	0.00	0.00	0.00	0.00
Mean		**0.33**	**0.08**	**0.00**	
Cotyledonary Node	Control	0.33	0.00	0.00	0.11
	0.1 M	0.67	0.33	0.00	0.33
	0.2 M	0.33	0.00	0.00	0.11
	0.3 M	0.33	0.00	0.00	0.11
Mean		**0.42**	**0.08**	**0.00**	
Mature Embryo	Control	0.33	0.33	0.00	0.22
	0.1 M	1.00	0.67	0.00	0.56
	0.2 M	0.67	0.33	0.00	0.33
	0.3 M	0.33	0.00	0.00	0.11
Mean		**0.58**	**0.33**	**0.00**	
Grand Mean		**0.44**	**0.16**	**0.00**	
SE (m) ± (A)		0.22	0.14	-	
CD (5 percent) (A)		0.64	0.42	-	
SE (m) ± (B)		0.19	0.12	-	
CD (5 percent) (B)		0.55	0.36	-	
SE (m) ± (AxB)		0.38	0.25	-	
CD (5 percent) (AxB)		1.11	0.72	-	

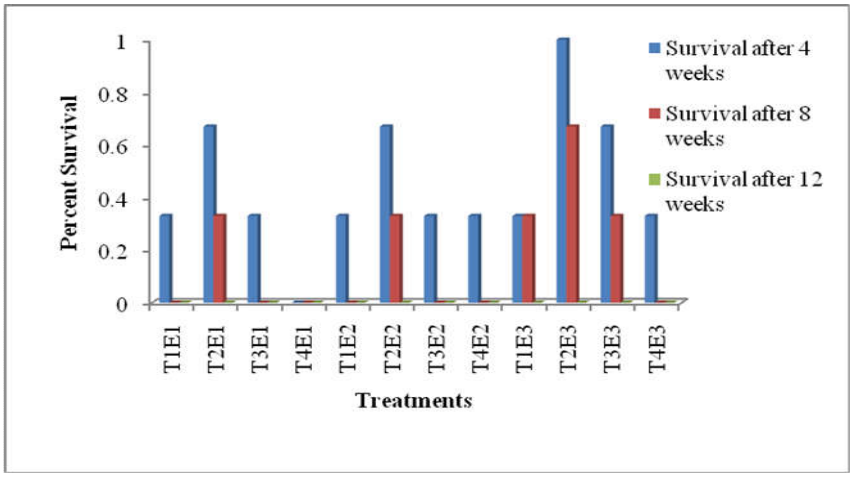

Fig 2: Graphical representation of explant survival after dehydration with sorbitol

Fig.1 and fig. 2 clearly indicates that the survival reduced from 0.1 M to 0.3 M mannitol and sorbitol in case of all the explants but as compared to other treatments, the survival was highest when mature embryo dehydrated with 0.1M mannitol for 72 hrs.

Ye and co-workers in 1990 also reported a similar results and found 20-fold increase in transformation efficiency in chloroplasts using sorbitol and mannitol in the bombardment and incubation medium [13].

Desiccation

Desiccation of the explants leads to dehydration and creates an empty space between intercellular spaces with an expectation of increasing the bacterial entry in the intercellular spaces of meristematic tissue and to enhance more genetic transformation. A comparison based on mean percent survival of all the explants desiccated showed that mature embryo performed better followed by cotyledonary node and shoot tip on selection media. When precultured explants desiccated prior to colonization at varying durations, the highest mean percent survival was shown when desiccated for 15 min.

Table 4: Effect of desiccation on transformation efficiency

Explants	Treatments	Percent survival on selection medium			Mean
		4 weeks	8 weeks	12 weeks	
Shoot tip	Control	0.33	0.00	0.00	0.11
	15 min.	0.67	0.33	0.00	0.33
	30 min.	0.00	0.00	0.00	0.00
Mean		**0.33**	**0.11**	**0.00**	
Cotyledonary node	Control	0.33	0.00	0.00	0.11
	15 min.	0.67	0.33	0.00	0.33
	30 min.	0.33	0.00	0.00	0.11
Mean		**0.44**	**0.11**	**0.00**	
Mature Embryo	Control	0.67	0.33	0.00	0.33
	15 min.	1.00	0.67	0.00	0.57
	30 min.	0.67	0.33	0.00	0.33
Mean		**0.78**	**0.44**	**0.00**	
Grand Mean		**0.52**	**0.22**	**0.00**	
SE (m) ± (A)		0.19	0.17	-	
CD (5 percent) (A)		0.56	0.52	-	
SE (m) ± (B)		0.19	0.17	-	
CD (5 percent) (B)		0.56	0.52	-	
SE (m) ± (A x B)		0.33	0.30	-	
CD (5 percent) (A x B)		0.97	0.89	-	

Table 4 and fig. 3 shows that the survival was higher when mature embryo desiccated for 15 min, followed by cotyledonary node and shoot tip and survival reduced when the duration of dessication increased to 30 min.

Sakhanokho and co-workers in 2001 tried desiccation treatment to improve the conversion efficiency of somatic embryos to plants in diploid cotton, *G. hirsutum* accession, A2-9 (PI-529712). Improved SE numbers and their subsequent conversion into plantlets were achieved with a Murashige and Skoog (MS)/sucrose-based medium M2 [0.04M sucrose, 0.3 mM α-naphthaleneacetic acid (NAA)]. A 3 days

desiccation period resulted in improved plant regeneration. When immature *G. arboreum* somatic embryos induced on M1 (0.2 M glucose, 2.6 mM NAA, and 0.2 mM kinetin) medium underwent a 3 days desiccation treatment, 49 percent of these immature somatic embryos were converted to plantlets after a 4 week period on M2 medium. These improved results will help to pave the way for future genetic transformation [14].

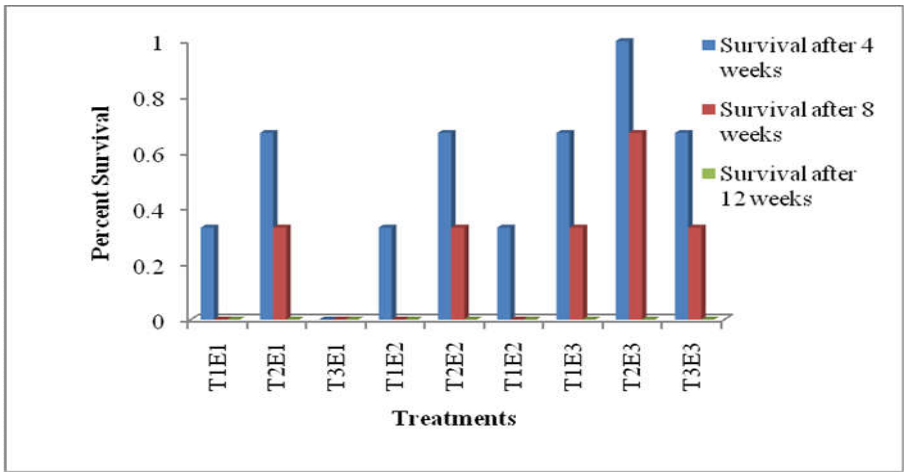

Fig 3: Graphical representation of survival after desiccation on selection media

Chilling injury at 4^{0}C

Chilling injury is a term used to describe the physiological damage that occurs in many plants and plant commodities as a result of exposure to a chilling temperature. Therefore, there are chances for *Agrobacterium* entry through damaged tissue or plant cell. The mean percent survival of the explants desiccated showed that mature embryo performed better than cotyledonary node and shoot tip on selection media. When precultured explants chilled for various durations, the highest mean percent survival shown was when chilled for 30 min.

Table 5: Effect of chilling injury on transformation efficiency

Explants	**Duration of chilling injury**	**percent survival on selection medium**			**Mean**
		4 weeks	**8 weeks**	**12 weeks**	
Shoot Tip	Control	0.33	0.00	0.00	0.11
	30 min.	0.67	0.33	0.00	0.33
	60 min.	0.33	0.00	0.00	0.11
	24 hrs.	0.00	0.00	0.00	0.00
Mean		**0.33**	**0.08**	**0.00**	
Cotyledonary Node	Control	0.33	0.00	0.00	0.11
	30 min.	0.67	0.33	0.00	0.33
	60 min.	0.33	0.00	0.00	0.11
	24 hrs.	0.00	0.00	0.00	0.00
Mean		**0.33**	**0.08**	**0.00**	
Mature Embryo	Control	0.33	0.33	0.00	0.22
	30 min.	1.00	0.67	0.00	0.56
	60 min.	0.67	0.33	0.00	0.33
	24 hrs.	0.00	0.00	0.00	0.00
Mean		**0.50**	**0.33**	**0.00**	
Grand Mean		**0.39**	**0.17**	**0.00**	
SE (m) ± (A)		0.19	0.16	-	
CD (5 percent) (A)		0.55	0.45	-	
SE (m) ± (B)		0.16	0.13	-	
CD (5 percent) (B)		0.48	0.39	-	
SE (m) ± (AxB)		0.33	0.27	-	
CD (5 percent) (AxB)		0.96	0.79	-	

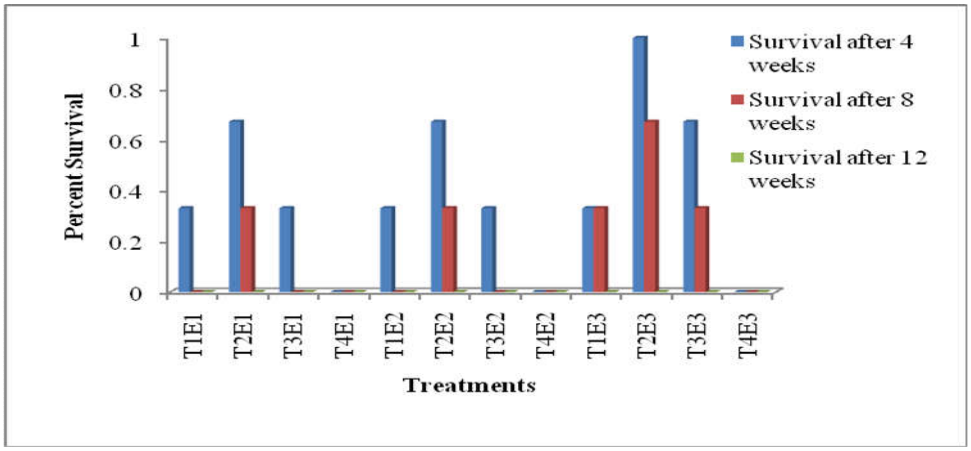

Fig 4: Graphical representation of survival after chilling at 4^0C on selection media

From table 5 and fig. 4 it is clear that mature embryo performed better than other two explants when chilled for 30 min showed higher survival than rest of the durations.

Various durations for chilling injury for efficient transformation were tried and observed that thirty minutes of chilling injury showed higher per cent of plant regeneration and survivability than chilling for 1 and 2 days [15]. Shoot apices with SAM showed higher regeneration when they were chilled for 10 minutes with and without vertical cut at SAM in both the genotypes by Sangannavar in 2008. Higher the time of chilling, lower the regeneration and plant survivability. One putative transgenic plant was obtained in DLSa-17 when chilled for 10 min [16].

CONCLUSION

All the above preculture treatments attempted were principally based on moisture loss from the explants and then colonize it with *Agrobacterium* culture which makes explant prone to absorb moisture from surrounding environment. The results showed that when mannitol and sorbitol at 0.1M concentration, desiccation for 15min and chilling injury for 30 min was given, the explant survival on selection media was highest as compared to control which shows that the preculture treatments optimized at said level, can be used in order to enhance transformation efficiency.

REFERENCES

1. Mehetre S, Thul A, More A & Dahat D, (2006). Interspecific hybridization between diploid and tetraploid cultivated Gossypium spp. and its role in cotton improvement—A review, J Cotton Res Dev, 20; 149-159.
2. James C (2014). Global status of commercialized biotech/GM crops: ISAAA Brief No. 49 ISAAA, Ithaca, NY, USA.
3. Bakhsh A, Emine A, Özcan SF, Hussain T, Aasim M, Khawar KM, Özcan S (2015). An insight into cotton genetic engineering (*Gossypium hirsutum* L.): current endeavors and prospects. Acta Physiol Plant 37: 171-179.
4. Mishra R, Wang HY, Yadav N, Wilkins T (2003). Development of a highly regenerable elite Acala cotton (*Gossypium hirsutum* cv. Maxxa)—a step towards genotype-independent regeneration. Plant Cell Tiss Organ Cult 73: 21-35.
5. Ahmed HA, Hajyzadeh M, Barpete S, Özcan S (2014). In vitro plant regeneration of Iraqi cotton (*Gossypium hirsutum* L.) cultivars through embryonic axis. J Biotechnol Res Cent (special edition) 8: 90-94.
6. Barampuram S, Allen G, Krasnyanski S (2014). Effect of various sterilization procedures on the in vitro germination of cotton seeds. Plant Cell Tiss Organ Cult 118: 179-185.
7. Satyavathi VV, Prasad V, Gita LB, Sita LG (2002). High efficiency transformation protocol for three Indian cotton varieties via *Agrobacterium tumefaciens.* Plant Sci 162: 215-223.
8. Hiei, Y., Komari, T., Ishida, Y., and Saito, H. (2000). Development of *Agrobacterium* mediated transformation method for monocotyledonous plants. Breed. Res. 2: 205–213.
9. Kumar, R., Singh, B., Yadav, A., Goswami, A., and Sharma, A., (2011). The use of *Agrobacterium*-mediated transformation methodology in tomato (*Lycopersicon esculentum*): current trends and developments. Ann.Hortic. 4: 1–9.
10. Ling, H.Q., Kriseleit, D., and Ganal, M. (1998). Effect of ticarcillin/potassium clavulanate on callus growth and shoot regeneration in *Agrobacterium*-mediated transformation of tomato (*Lycopersicon esculentum* Mill.). Plant Cell Rep. 17: 843–847.
11. Ugale P. R. (2010). Development of shoot regeneration protocol in cotton (*Gossypium hirsutum* L.). M.Sc. thesis, Dr. Panjabrao Deshmukh Krishi Vidyapeth, Akola, M.S.
12. Panse, V. G. and Sukhatme, P. V., 1958, Statistical Methods for Agricultural Workers, ICAR Publication, New Delhi, India.
13. Ye G. N., D. Stone, S. Z. Pang, W. Creely, K. Gonzalez and M. Hinchee. (1999). *Arabidopsis* ovule is the target for *Agrobacterium in planta* vacuum infiltration transformation. The Plant J. 19:249-257.

14. Sakhanokho, H.F., Zip, F.A., Rajasekaran, K., Saha, S., Sharma, G.C., (2001). Induction of highly embryogenic calli and plant regeneration in upland (*Gossypium hirsutum* L.) and Pima (Gossypium barbadense L.) cottons. *Crop Science*, 41:1235-1240.
15. Trolinder, N.L., Koonce, L.K. and Dever, J.K., (2006), Methods for producing transgenic cotton plants using chilled apical shoot tips. www.freepatentsonline.com/7122722.html.
16. Sangannavar P. (2008). Genetic transformation studies in cotton (*Gossypium arboreum* and *G. barbadense*). M.Sc. Thesis. Univ. Agric. Sci., Dharwad.

CITATION OF THIS ARTICLE

D A Padole, Gahukar S.J., Amrapali A. Akhare. Standardization of preculture Treatments to enhance Transformation efficiency in *Gossipium hirsutum* (L.). Bull. Env. Pharmacol. Life Sci., Vol 6[2] January 2017: 83-90