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A rapid and simple method for *in-vitro* plant regeneration from petiolar region of diploid *G. arboreum* cotton cultivar *(cv. Ambika)*.

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Abstract: *In the current study, four diploid cotton cultivars viz. Ambika, NSGA-A4, NSGA-A9 and NSGA-B5 (G. arboreum L.) have been used to review the effects of multiple shoot formation in MS (Murashige and Skoog) basal media subsidize with distinctive concentrations of BAP (6-benzylaminopurine) and 2,4-D growth regulator for in vitro generation of shoot bud origin, multiple shoot induction and regeneration using petiolar excised explants from 5-6 day-old seedlings.*

The mean sprouting response in four cultivars Ambika, NSGA-A4, NSGA-A9 and NSGA-B5 (25.15% to 94.24 %) was considered on MS medium. The most effective treatment for the formation of adventitious shoots (72.55%) in Ambika and NSGA-B5 was MS + 1.00 mg/l BA and 0.02mg/lit 2,4-D with addition of Adenine sulphate, whereas MS + 0.75mg/l BA and MS +0.012 mg/lit kinetin was top of the line the generation of adventitious shoots (42.58%). The maximum quantity of shoots/explant (16 to 18, respectively) were obtained when cultured on MS supplemented with 1.00 mg/l BA + 0.02mg/lit 2,4-D supplemented with adenine sulphate 5 mg/lit as an additive. The elongation of multiple shoots was obtained in media supplemented with 0.1mg/lit of kinetin and 0.075 mg/lit gibberellic acid (GA3).

Highest root development (71.00%) used to be acquired when shoots had been cultured on ½ MS medium supplemented with 0.25 mg/l NAA in Ambika, NSGA-A4, NSGA-A9 and NSGA-B5 cultivars. Plantlets raised in vitro were then shifted to thermaocol cups containing JP pots (Nucitera, India) under greenhouse condition till they grew into healthy vegetative condition and mature. The consequences of this study will facilitate the utility in transgenic diploid cotton development for improvement of elite breeding germplasm.

Key words: *Gossypium arboreum, petiolar region, in vitro culture, BA 6-Benzylaminopurine· 2,4-D(2,4 Dichlorophenoxy acetic acid, MS (Murashige and Skoog)*

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I. Introduction

The cotton *Gossypium* species is the exceptional source of textile having elevated profitable crop among the world fibre industry. This *Gossypium* genus has been found in approximate 50 species of them four (*G. hirsutum L., G. barbadense L., G. arborum L and G. herbaceum*) are cultivated to produced spinnable fibre. Diploid (*G. arboreum*) genotypes are grown in various regions of rain-feed area in India covering about 25-30% cotton production[26]. This cotton is highly tolerant to drought environmental condition and suitable for rain-fed cultivation where low rainfall and poor soil present henceforth attempt wider adaptability than tetraploids cotton. The noticeable industry demand has been increasing day by day considering high economic value in cloth manufacturing industries for progressive development of cotton through traditional plant breeding, but which required more time up to 8-10 years to commercialize new cotton varieties [3].

Conventional crop improvement has certain constraints for creating variation such as biotic as well as abiotic stress. In vitro plant tissue culture adds an advantage for genotypically improvement of somaclonal and induced variation, somatic hybridization, double haploid line development in favor of insertion of certain foreign gene through genetic engineering [11]. The tissue culture based somatic embryogenesis approach that is regeneration from preexisting meristem explants was reported by different biologist [4,8,23]. A stable, reliable regeneration protocol is important for plant regeneration through organogenesis which is an essential step in improvement of crop. Despite the application of in vitro technologies primarily depending on reproducible regeneration method such as somatic embryogenesis which is durable in *Arboreum* cotton [25]. Regeneration through somatic embryogenesis can be substituted by using pre-existing meristematic explants source such as apical meristem [8,17], Shoot tip [5], cotyledonary node [24], Embryonic explants [26] and petiole based regeneration in pigeon pea [21&9], *Jatropha curcas* L. [15]. Henceforth, comprehensive research work has been done to standardized competent regeneration system using meristematic tissue, petiole as explants source in diploid cotton. Regeneration of cotton through apical shoot tip has been reported 5-6 shoots [29]. Therefore, present research findings are aimed to standardize *G. arboreum* cotton regeneration through multiple shoot organogenesis using petiole as explants source with higher rate of shoot multiplication. Efficient regeneration systems were used to

develop genetically modified cotton plants[17,13].

The fundamental goal of this research study was to learn the effect of cytokinin and auxin hormone combination for callus development and multiple shoot formation in *G. arboreum* cotton genotypes. Therefore, present investigation is aimed to established a rapid and highly efficient *in-vitro* regeneration for Nirmal Seeds *G. arboreum* cotton cultivar Ambika, NSGA-A4, NSGA-A9 and NSGA-B5. The shoot organogenesis by petiolar region explants were obtained after seven weeks. This protocol can be used in future research work on genetic transformation experiment of different *Arboreum* genotypes.

II. Materials and Methods

Seed germination

Seeds of *Gossypium arboreum* delinted cotton Ambika, NSGA-A4, NSGA-A9 and NSGA-B5 have been collected from gene bank of Nirmal Seeds (P) Ltd. Pachora, Dist. Jalgaon. The uniform healthy seeds were undertaken for germination and continuously washed thrice with sterile distilled water (SDW). Seeds were then floor surface sterilized in 30% hydrogen peroxide (H_2O_2) for 60 min on orbital shaker followed by treatment with 0.1% mercuric chloride ($HgCl_2$) for 20 minutes. The seeds were then overnight soaked in 1% bavistin solution on orbital shaker at 120 rpm followed by 3 SDW washes in early morning. Again seeds were treated with the 0.1% mercuric chloride for three minutes followed by the aid of three SDW washes and stored in water until they used for germination. The seed coat has been then removed using scalpel and forceps, seed embryo was inoculated in tissue culture glass bottle containing 30 ml of half MS media [16] for sprouting seeds at 26 ± 2°C temperature under dark circumstances in growth room condition.

Multiple shoots development

In vitro grown 5-7 days old seedlings of Ambika, NSGA-A4, NSGA-A9 and NSGA-B5 genotypes were used to isolate petiole explants from apical region of seedlings. The petiole explants were separated from the groove region by taking sharp cut with the surgical blade in a LAF cabinet [21] and inoculated on MS basal media containing 6-Benzylaminopurine (BAP 0.25 to 2.0 mg/lit) in combination with (2,4-D 0.01-0.05 mg/lit) Table-1 for callusing mass development. All the tissue culture media were incorporated with 3% sucrose and 0.7% agar as gelling agent, the pH of media was set at 5.8 before autoclaving. The inoculated cultures were incubated for six weeks in growth room under favorable condition at 24±2°C temperature and photoperiod of 16-h light and 8-h dark using cool white fluorescent light. The shoot responses were observed using sprouted shoots induction in percentages after completion incubation period.

Table-1 List of Media used for multiple shoot formation

Sr. No.	Media name	BAP (mg/lit)	2,4-D(mg/lit)	Kinetin(mg/lit)
1	MSAS-1	0.25	0.01	-
2	MSAS-2	0.5	0.01	-
3	MSAS-3	0.75	0.01	-
4	**MSAS-4**	**1.00**	**0.02**	-
5	MSAS-5	1.25	0.03	-
6	MSAS-6	1.50	0.04	-
7	MSAS-7	1.75	0.05	-
8	MSAS-8	2.00	-	0.10
9	MSAS-9	0.5	-	0.05
10	MSAS-10	0.75	-	0.10
11	MSAS-11	1.00	-	0.15
12	MSAS-12	1.25	-	0.20
13	MSAS-13	1.50	-	0.25

Shoot elongation and root development:

Shoot bud with developed callus sprouted mass on MSAS-4 media have been cut from the basal region for keeping apart single shoots along with mass of callusing body and shifting to MSAE-3 basal elongation media supplemented with 0.1mg/lit of kinetin and 0.075mg/lit gibberellic acid (GA-3) (Table-2) along with 3% sucrose and 0.7% agar powder. The cultures were incubated for 4 weeks under favorable growth room condition such as 16-h light and 8-h dark with 24±2°C temperature.

Table-2: List of media used for shoot elongation

Sr.No	Media name	Kinetin (mg/lit)	GA3 (mg/lit)	Adenine sulphate (mg/lit)
1	MSAE-1	0.025	0.025	2.5
2	MSAE-2	0.050	0.050	2.5
3	**MSAE-3**	**0.100**	**0.075**	**2.5**
4	MSAE-4	0.025	0.100	2.5
5	MSAE-5	0.050	0.125	2.5
6	MSAE-6	0.100	0.150	2.5

The number of petiole explants used for each individual treatment and genotypes (Ambika, NSGA-A4, NSGA-A9 and NSGA-B5) were 100-110. All the experiments were repeated three times, data recorded and evaluated according to randomized block designed (RBD) using statistical analysis .

III. Results and Discussion

Germination of seeds with maximum rate for isolating petiole explant retrieved using healthy seeds of *G. arboreum* and were delinted by using sulfuric acid which resulted in removal of surface microflora clunged to seed coat [6]. Effective seed treatment was done using hydrogen peroxide, mercuric chloride (0.1%) along with bavistin enhanced slacken seed coat and increased rate of healthy germination (80-92%) with reduced contamination up to (0.8%) in all four cotton genotypes, similar observations were obtained[19].

The seed germination of *G. arboreum* (Fig.1a) was observed after 3-4 days of incubation in dark condition. The competent germination on MS half basal media has been reported[22]. After seven days, seedlings completely developed and expanded cotyledonary leaf and roots (Fig.1b & 1c). Petiole explants (0.4-0.6 cm) inoculated on different MS media combination grown very well with on BAP and 2,4-D hormone ratio (Table-1) exhibiting shoot with callus induction. Petiole explants inoculated on media to grow within 9-10 days on variable media. The results of basal media effect on sprouting and callus development, different plant growth regulator on multiple shoot development with callus formation at basal region in Nirmal *G. arboreum* cotton genotypes Ambika, NSGA-A4, NSGA-A9 and NSGA-B5 are mentioned as below.

Fig.1a, 1b&1c: Seed inoculation, germination and petiole explant development

Effects of growth medium on shoot sprouting response:

Petiole explants of *Arboreum* cotton (Ambika, NSGA-A4, NSGA-A9 and NSGA-B5) genotypes, isolated from 6-7 days old in vitro grown germinated seedlings in aseptic condition were cultured on MS media enriched with different concentration of BAP, 2,4-D and kinetin hormone for multiple shoot development (Table-4). Earlier, petiole explants isolated from 6-8 days old seedling have been used in pigeon pea for multiple shoot with callus development, the research work reported by Gawali, et al. (2010), who stated that maximum number of shoots was obtained 2-6 per explants.

Arboreum cotton shows different response on variable age of germinating seedling and were used for shoot development [17,22]. The effect of different media formulation for shoot sprouting results using petiole explants was studied for four *arboreum* cultivar (Ambika, NSGA-A4, NSGA-A9 and NSGA-B5) mentioned in Table-3. Higher rate of shoot sprouting was observed in MS basal medium incorporated with B5 vitamins however absence of vitamin growth response was seen very slow. The MS basal media with addition of various additive for in vitro cotton regeneration from various meristem has been reported[8].

Table-3: Effect of basal media on shoot sprouting and morphogenesis

Sr.No.	Media type	Sprouting response (%)	Multiple shoot induction (%)	Morphogenetic response
1	MS plane	25.15	23.50	1.2 shoot, dwarf
2	MS+MS vit	70.01	48.05	4-5 shoot,tall
3	MS + B5 vit	76.22	62.02	10-12 shoot, tall
4	MS+B5+$AdSO_4$	94.24	72.55	14-15 shoots, medium

In MS media composition addition of B5 vitamin shown efficient sprouting response for all the four cultivar (Table-3). However, multiple shoot germination was higher in vitamin reached medium for Ambika and NSGA-B5 genotype than vitamin free medium (Fig. 2). NSGA-A4 and NSGA-A9 shown 48.05% and 62.02% in vitamin reached medium, multiple shoot formation, and same genotypes were exhibit lower 25.06% and 31.07% of shoot in culture medium without vitamin and adenine sulphate.

Fig.2: Regeneration of in vitro *Arboreum* cotton using petiole explants. (2a) Petiole explants on preculture media, (2b) Explants shoot bud with callus at base, (2c) Explants sprouted shoots, (2d&e) Multiple shoot development in 1-2 month, (2f) Petiole explants with callus induction and multiple shoot formation, (2g) Individual shoot separated from callus, (2h) Shoots with root development, (2i) Primary hardening.

Effect of BAP and 2,4-D on multiple shoots induction:

Various concentration of BAP, 2,4-D and BAP + 2,4-D were used in MS basal media to produce multiple shoot in *arboreum* cotton genotypes Ambika, NSGA-A4, NSGA-A9 and NSGA-B5. For Ambika and NSGA-B5, higher multiple shoot formation (72.55%) was observed in MS + 1.0 mg/lit BAP in combination with 0.02mg/lit 2,4-D followed by 42.58% in MS+ 0.75 mg/lit BAP and 0.012,4-D. The role of 2,4-D was cell division at higher rate for induction of callus alongside multiple shoot formation (Fig. 2f). The percentage of multiple shoot germination found decreasing level (5.36-48.8%) when concentration of BAP (1.5-2.0 mg/lit) and 2,4-D(0.03-0.05mg/lit) alone or in combination (Table-3). Increased concentration of hormone changes morphology of the shoots and leaves, slower growth of shoots and became dehydrated and ultimately leads to death. Pathi and Tuteza (2013) stated that regeneration of plants on BAP (2.0 mg/lit) and 2 mg/lit kinetin supplemented media shows multiple shoot formation in embryo apex were 16 per explants. However, in our current research experiment, with BAP and 2,4-D shows multiple shoot formation 4-18 per explants.

Multiple shoot induction per explants using petiole explants varies from different concentration of BAP, 2,4-D and BAP+2,4-D in MS media and shown 1.0 to 18.00 on the basis of various cultivar and media composition (Table-4). The maximum number of shoots 18.00 was found in NSGA-B5 on MS media in

combination of BAP+2,4-D in addition with Adenine sulphate followed by 6.00 seen when alone BAP 1.0 mg/lit + kinetin 0.05 mg/lit was used. Proliferation of shoots was observed, when explants subculture on same combination of fresh medium and incubated for another 4 weeks. Khatoon et al. (2014) studied the multiple shoot induction in *G. arboreum* on MS medium containing 1.0 mg/lit BA and 1.0 mg/lit Kinetin. Similarly, Yasin & Yasmin (2018) reported 6.3 shoots per explants using shoot tip in MS media containing 2.0 mg/lit BAP and 0.5 mg/lit Kinetin.

Table-4: Effect of phytohormone on multiple shoot induction in cotton

Sr. No	Media name	Phytohormone conc. (mg/lit)			Multiple shoot induction (%)				Shoot/explants			
		BAP	2,4-D	Kinetin	Ambika	NSGA-A4	NSGA-A9	NSGA-B5	Ambika	NSGA-A4	NSGA-A9	NSGA-B5
1	MSAS-1	0.25	0.01	-	32.1	36.3	35.6	38.8	1	3	2	4
2	MSAS-2	0.5	0.01	-	11.3	12.2	19.1	33.2	1	1	2	3
3	MSAS-3	0.75	0.01	-	37.1	66.1	55.2	70.1	5	11	9	12
4	**MSAS-4**	**1**	**0.02**	**-**	**88.01**	**80.1**	**75.01**	**92.14**	**16**	**14**	**13**	**18**
5	MSAS-5	1.25	0.03	-	54.2	49.1	53.4	54.5	9	6	8	10
6	MSAS-6	1.5	0.04	-	48.8	35.8	38.2	5.36	6	3	5	9
7	MSAS-7	1.75	0.05	-	11.1	11.3	28.9	40.5	1	1	3	5
8	MSAS-8	2	-	0.1	21.5	12.4	13	23.4	2	1	1	2
9	MSAS-9	1.0	-	0.05	33.1	24.1	12.4	13.1	3	2	1	6
10	MSAS-10	0.5	-	0.1	10.1	11	11.4	12.4	1	1	1	1
11	MSAS-11	0.5	-	0.15	9	9.8	8.1	11.3	1	1	1	1
12	MSAS-12	0.5	-	0.2	11.6	10.2	11.4	25.3	1	1	1	2
13	MSAS-13	0.5	-	0.25	11.5	11	19.5	11.4	1	1	2	1

Effect of PGR on morphogenetic response on plants

Isolated petiole explants of *Arboreum* cotton from 6-7 days old seedling was cultured on MS basal media incorporated with BAP (0.25 mg/lit to 2.5 mg/lit), 2,4-D (0.01 mg/lit to 0.05 mg/lit) and a combination of both BAP and 2,4-D exhibited reproducible shoot development with varying rate of response (10.1 to 90.2) on the basis of growth hormones used (Table-5). The main goal of adding BAP in media was to induce multiple shoot along with callusing development by the addition of 2,4-D growth regulator, similar study was earlier conducted by Kumar and Tuli (2004), and reported BAP was the ultimate effectual for multiple shoot development. Likewise, several researchers used 2,4-D + kinetin[27,10,12] and stated 2-4-D is effective for higher cell division and callusing formation during multiple shoot development. These results are in resemblance with the research conducted by Kumar and Tuli (2004) who reported the impact of various cytokinins and auxins for mass multiplication and cotton regeneration of cotton, also stated that BAP and 2,4-D concentration above 1.5 mg/lit and 0.05 mg/lit showed limited feedback for shoot development. Similar discovery recorded by Xu et al., (2013) and Aydin et al., (2013) with shoot multiplication of cotton for plant regeneration, concluded higher concentration of BA (2.0-5.0) and 2,4D (0.1) alone induced only 1 or 2 shoots per explants after 30 days of culture incubation. The elongation of regenerated shoot was obtained on MS medium supplemented with concentration of 0.1 mg/lit kinetin and 0.075mg/l GA3 (Fig. 3).

Table-5: Effect of different phytohormone on sprouting and morphogenetic response in cotton

Sr. No	Media name	Phytohormone conc(mg/lit)			Explant response(%)				Nature of response				Callus formation (%)	Regeneration (%)
		BAP	2,4-D	Kinetin	Ambika	NSGA-A4	NSGA-A9	NSGA-B5	Ambika	NSGA-A4	NSGA-A9	NSGA-B5		
1	MSAS-1	0.25	0.01	-	30.2	34.1	33.5	36.2	S	DF	D	S	12.01	35.1
2	MSAS-2	0.5	0.01	-	10.1	11.6	17.8	32.4	DF	S	S	C	50.6	40.1
3	MSAS-3	0.75	0.01	-	36.2	64.2	54.2	68.1	S	C	S	C	88.01	73.1
4	**MSAS-4**	**1.00**	**0.02**	**-**	**85.2**	**78.1**	**73.1**	**90.2**	**C,ES**	**C,ES**	**C**	**C,ES**	**95.01**	**92.6**
5	MSAS-5	1.25	0.03	-	53.1	48.6	52.1	52.3	ES	ES	C	C	89.5	80.5
6	MSAS-6	1.5	0.04	-	46.2	33.5	36.8	4.25	S	C	C	C	66.4	40.5
7	MSAS-7	1.75	0.05	-	10	10.9	27.9	38.5	DS	S	DS	DS	50.2	38.4
8	MSAS-8	2	-	0.1	19.8	11.3	12.5	21.7	ES	ES	ES	ES	22.3	42.5
9	MSAS-9	0.5	-	0.05	32.5	21.6	11.4	12.5	S	S	DS	S	33.4	48.1
10	MSAS-10	0.5	-	0.1	10.1	10.8	9.6	11.1	DS	ES	DS	ES	20.1	38.9
11	MSAS-11	0.5	-	0.15	10.5	41.2	9.5	11	S	S	S	S	25.4	29.8
12	MSAS-12	0.5	-	0.2	10.5	10.1	9.4	22.5	S	S	S	S	36.4	32.4

13	MSAS-13	0.5	-	0.25	11.7	10.6	18.5	10.6	S	DF	DF	S	38.4	40.9

*Note: S-shoot, C-Callus, ES-Elongated shoot, DF-dwarf shoot

The concentration of 2,4-D used in this study produced callus surrounding to petiole explants. 2,4-D at 0.01 to 0.05 mg/lit responded in plentiful callus bodies (89.5-95.01%) which resulted in dwarfing of shoot growth (Fig-2f). Similar research work had been reported by Finner et al. (1988) that explants at higher concentration of 2,4-D shoot with callus growth stunted.

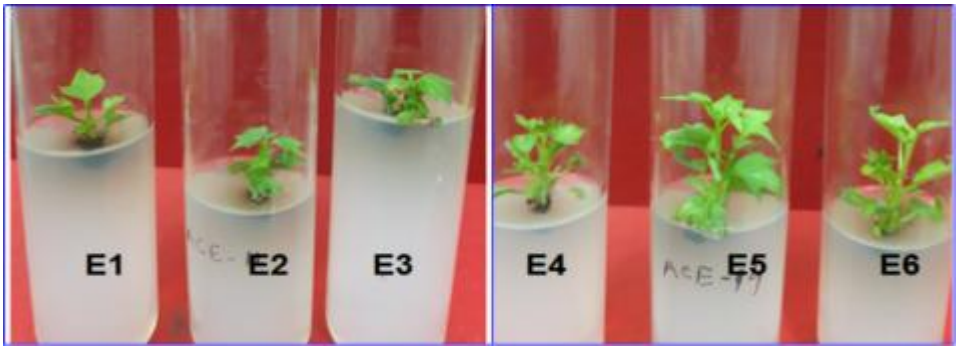

Fig. 3: Shoot elongation in *Arboreum* cotton using GA3

***In vitro* root development**

Petiole explants with mass of growing callus and multiple sprouted shoots were separated from dorsal region after 4-5 weeks of incubation and were subcultured to MS half strength medium enriched with NAA under incubation for root development (Table-6). The experiment was replicated three times and root induction were observed after 3rd and 4th week of shoot incubation resulting all the genotypes Ambika, NGAC-A4, NGAC-A9 and NGAC-B5 genotype shown different responses (Fig 2h). The response of genotypes towards root percentage formation was not indicative among the all media tested however symbolic variations remain among the different rooting media (Table-6). Highest root development observed in MS media (MSAR-2) with 0.25mg/lit of NAA (71%) however MS half basal hormone free media and IBA enriched media proven least root formation (12.0-41.0%). Previously Abdellatef and Khalafalla (2007) reported higher percentage of root formation (87%) in hirsutum cotton L. cv Barac(67)B in MS half media enriched with 0.1 mg/lit of NAA. However, Obembe et al., (2011) reported efficient root development in *Arboreum* cotton on hormone free MS half media.

Rooted plants were later shifted to small thermaocol cups with JP pots (Nucitera, India) followed by transferred in soil:sand:coco-peat in plastic polythene bag after 25-30 days of incubation in polyhouse condition. The plants were finally transferred to 10 kg size black gunny bag filled with soil:sand and monitored in greenhouse up to maturity (Fig 2i).

Table-6: Effect of different media composition on root induction in cotton

Sr. No.	Media name	NAA (mg/lit)	IBA(mg/lit)	Charcoal l (%)	Ave. root length(cm)	Rood induction (%)
1	MSAR-1	0.10	00	0.1	2.1	60
2	MSAR-2	0.25	00	0.1	2.2	71
3	MSAR-3	0.30	00	0.1	4.6	50
4	MSAR-4	0.40	00	0.1	3.0	40
5	MSAR-5	0.50	00	0.1	2.1	35
6	MSAR-6	00	0.1	0.1	3.3	41
7	MSAR-7	00	0.2	0.1	4.2	20
8	MSAR-8	00	0.3	0.1	3.5	15
9	MSAR-9	00	0.4	0.1	2.5	13
10	MSAR-10	00	00	0.1	1.6	12

This research work was conducted for the intention of petiole explants based regeneration in *Arboreum* cotton of cultivar Ambika, NSGA-A4, NSGA-A9 and NSGA-B5 varieties. Considering *Arboreum* cotton is recalcitrant in nature and has demonstrated to manipulate in tissue culture[23]. This work can be referenced for development of elite cotton breeding germplasm reduplication through multiple shoot development.

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