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Research Article

Optimisation of *in vitro* propagation of *Gossypium arboreum* L.

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Abstract

A protocol for micropropagation of *Gossypium arboreum* was standardized. The shoot tip of one week old cotton seedlings were aseptically excised and cultured on Murashige and Skoog (1962) medium. The medium was supplemented with different concentrations (0 mgL^{-1}, 1 mgL^{-1}, 2 mgL^{-1}) of Benzyl-amino-purine (BAP) and (0 mgL^{-1}, 0.5 mgL^{-1}& 1 mgL^{-1}) Kinetin (KIN) for shoot proliferation. The study was carried out for twelve-weeks with three repeats. Among the tested concentrations 1.0 mgL^{-1}Benzyl-amino-purine (BAP) and 0.5 mgL^{-1}Kinetin (KIN) was found optimal and initiated shooting within 26 days. These hormones revealed 6.3 shoots per explant on average. Indol-butyric acid (IBA) at the concentration of 0.75 mgL^{-1} demonstrated 4.0 cm long 3.0 roots per plantlet on average. The *in vitro* generated healthy plantlets were acclimatized for a month under high humidity with 100% survival rate. The optimized protocol is quite simple, less laborious and less expensive to rejuvenate cotton plantlets. These plantlets can further be utilized for the *Inplanta* transformation experiments and/or molecular studies to overcome off season unavailability of plant material to explore genetic basis of diversity and organization of desirable traits in diploid germplasm.

Keywords: Benzyl-amino-purine; *Gossypium arboreum*; *In-vitro*; Kinetin

Introduction

Cotton is a major source of natural fiber and seed oil throughout the world. This fiber yielding crop is grown almost in sixty-five countries of world with an annual contribution of $500 billion, and more than 180 million people are somehow connected to this crop as it provide raw material to textile mills, ginning factories and oil expellers **[1]**. In Pakistan cotton shares 60% of our exports and fulfills about 85% of our local demand of oil **[2]**. Pakistan started cotton breeding program in 1914 by releasing American upland cotton by Department of Agriculture, Faisalabad followed by numerous breeding programs which were launched with major focus on yield and fiber improvement that had limited the genetic diversity of disease resistance in cultivated cotton varieties **[3]**. Right now commercial cotton suffers from many biotic and abiotic stresses that not only reduce its actual economic potential but also more than half of the total pesticide/ insecticide utilization across the world is due to this crop **[4]**. Therefore, the future of cotton breeding programs should depend on the understanding genetic diversity of the local cotton germplasm and development of disease resistant cultivars including *cotton leaf curl virus* (CLCuV) resistance cultivars with particular reference to Pakistan **[5, 6]**.

The *Gossypium* genus include 51 species, 46 species of them are diploid and remaining five species are tetraploid [7]. This diploid germplasm is a source of genetic diversity and contain favorable traits like drought and salt tolerance, fiber quality, male cytoplasmic sterility, high and low temperature tolerance and pest and disease resistance [8, 9]. Due to these valuable traits they carry, the diploid wild species are still cultivated in limited areas of India, China and Pakistan. Although the transfer of desirable genes from diploid germplasm to commercial tetraploid varieties could be achieved through interspecific hybridization due to the non-availability of genes conferring resistance within the crossable gene pool however, this approach is laborious and time consuming [10]. Furthermore, the evaluation of new varieties mostly takes 6 to 10 years through conventional breeding methods [11]. On the other hand, genetic engineering has the solution to these problems and it is an inevitable tool to transfer new genes into commercial cotton varieties, in fact 81 % of cotton grown throughout the world is genetically modified [4]. Despite these accomplishments, there are several constraints lagging cotton tissue culture and transformation behind. The callus based indirect *in vitro* proliferation of cotton crop considered as recalcitrant [12]. The plant regeneration and somatic embryogenesis has been obtained from hypocotyls, while the response of plant generation was extremely genotypic dependent [13-15]. The recalcitrance of the *Gossypium* species in callus and somatic embryo culture hamper the transfer of desirable traits to the cultivated tetraploid species through genetic engineering. The majority of somatic embryos derived from callus culture usually fail to mature and could not develop into normal plantlets, require lengthy culturing period and produce undesirable soma clones [8]. This problem is even more manifest in diploid cottons. In addition, indirect morphogenesis of cotton is limited to Coker cultivars that are non-indigenous and reveal low efficiency of somatic embryogenesis [16]. Thus, this necessitates evolution of easy, reliable and efficient protocols for cotton transformation for particularly in the Pakistani cultivars which are adapted well to local conditions.

Direct organogenesis through shoot tip culture technique is an alternate method to acquire plantlets and also resolve the troubles of obtaining plantlets through callus culture method [17, 18] (Gould, *et al.*, 1991., Saeed *et al.*, 1997). It is therefore a technique to increase the potential and ability of genetic engineering to transform commercially cultivated local cotton varieties. There are numerous reports on in vitro morphogenesis of tetraploid cotton through direct and indirect organogenesis techniques representing the efforts of the researcher of Punjab, Pakistan [17, 19- 22]. However, to our knowledge, present study is the first report on exploitation of local diploid germplasm of Sindh in tissue culture studies. This study is aimed to utilize wild local cotton germplasm namely *G. arboreum* L. to optimize a shoot tip culture based cotton regeneration system. The optimized protocol is quite simple, less laborious and less expensive to rejuvenate cotton plantlets. These plantlets can further be utilized for the *Inplanta* transformation experiments and/or molecular studies to overcome off season unavailability of plant material to explore genetic basis of diversity and organization of desirable traits in diploid germplasm.

Materials and methods

Plant material was collected in the form of seeds from Nuclear Institute of Agriculture, Tandojam. De-linted Seeds were soaked in sterile distilled water for 24 hours. On the following day seeds submerged in ½ MS liquid media or plain water until germinated. Seven days old seedlings were washed in 10

% bleach solution for 20 minutes. Bleach sterilized seeds were dipped in 70% ethanol and rinsed 3-4 times in sterilized water. Following protocol was adopted with minor modification [23]. Sterilized seedlings were excised under clean bench for the preparation of shoot tips to initiate sterile cultures. The excised shoot tips were without cotyledons and measuring about 0.5cm in length. These explants were cultured on MS basal medium [24] supplemented with various concentrations of Benzyl-amino-purine (0 mgL^{-1}, 1 mgL^{-1}, 2 mgL^{-1}) and/or of Kinetin (0 mgL^{-1}, 0.5 mgL^{-1}& 1 mgL^{-1}) for two months and sub-cultured every 15-days on same medium. The cultures were incubated at 22±3°C, under a 16-hour photoperiod and 2000 lux luminous intensity.

Shoots produced from the multiplication stage were cultured individually on the MS medium [24] containing various concentrations of IBA as 0, 1, 2mgL^{-1} in liquid MS media [24] and were incubated at 22±3°C, under a 16-hour photoperiod and 2000 lux luminous intensity for 1-month. Individual rooted plantlets were hardened in *in vivo* conditions using a sterilized mixture of soil and grinded coconut husk in 1:1 ratio for 2 weeks.

Data was collected for seed germination %, number of explants with shoots %, number of shoots/explant, days taken to shoot initiation and number roots per shoot. The data obtained for the above parameters was subjected to one-way, two-way ANOVA and DMRT in (Microsoft Excel) where it deemed fit.

Results and discussion

The first transgenic cotton plants were obtained in 1987 [25]. Since then despite a significant progress in the field of genetic engineering, only Coker cultivars have been successfully transformed [16]. Transformation of *Gossypium* species remains difficult for a number of reasons. One prerequisite for high efficiency transformation is an effective and robust tissue culture system. Response of cotton to regeneration media is genotype specific. Therefore it is necessary to optimize these media individually for each genotype that is to be studied. In this study, we have optimized media for in vitro regeneration of *Gossypium arboreum* L. For this purpose initially the seed germination was optimized. Obtaining highest germination and good plant stand is an important step to reduce losses and cost of the protocol. There are various treatments of seed hardening to overcome moisture stress during cotton germination such as soaking cotton seeds in water, potassium chloride, sodium or calcium salts, succinic acid, potassium dihydrogen phosphate etc. [26]. In present study simple distilled water and ½ strength MS media were used as seed priming substances to germinate cotton seeds with and without seed coats. The highest germination was observed when seed coats of overnight soaked seeds were removed and sown in ½ MS media. With this treatment seed germination became 90% that was otherwise as low as 46.7% when seeds with seed coats were soaked in water (Table 1). This data also showed the seed coat as a barrier for proper seed germination. Halo-priming using KCl, KNO_3 and Calcium salts have also been found quite effective in increasing cotton seed germination [27]. However, in this study half strength MS medium was used that also contain specific micronutrients, vitamins, myo-inositol and iron salts in addition to KCl, KNO_3 and Calcium salts [24]. Table 1 represents the importance of seed priming substance (1/2 MS) and seed coat removal that enhanced seed germination from 73.3% to 90.0% and from 76.7% 90% respectively.

Table 1. The effects of different treatments on percentage *in vitro* germination of *G. arboreum* seeds

	Distilled water	½ MS liquid medium
With seed coat	46.7c	73.3b
Without seed coat	76.7ab	90.0a

Note: Means represent three replications with ten seeds in each. DMRT is applied to find the difference between means at p= 0.05. Means followed by different letters are significantly different

Germinated seedlings were then allowed to grow for a week. Shoot tips of 7 days old seedlings were aseptically cultured on MS basal media with two concentrations of BAP i.e. 0, 1, 2 mgL^{-1} and two concentrations of KIN i.e. 0, 0.5, 1 mgL^{-1} to study their effect on shooting and growth of explants. Shoot tip and cotyledonary node were excised from 7-10 days old seedlings grown on ½MS also reported **[28]**. The effect of different treatment on the shoot initiation was significant. In control, MS media without growth regulators did not revealed any new growth during the course of this study, however inoculated shoot tips remained green and alive whole time. The highest number of shoots per explant (6.30) was recorded in treatment 5 (T5) that correspond to 1.00 mgL^{-1} BAP and 0.50 mgL^{-1}Kinetin (Table 2) followed by treatment 08 (T8: 1.00 mgL^{-1}Benzyl-amino-purine and 2.0 mgL^{-1}Kinetin) and treatment 02 (T2: 1.00 mgL^{-1}BAP and 0.0 mgL^{-1}Kinetin). Furthermore as the concentration of BAP was increased in media to 2.00 $mgL^{-1}$the number of shoots decreased to the level that the shoot numbers were comparable to treatments without BAP (Table 2). This observation reveals the importance of optimal concentration of BAP in media. BAP is a shoot inducing growth regulator known as cytokinin. Nonetheless, Kinetin and Benzyl-amino-purine in combination were found more effective as compared to alone in media. In present study when high concentration of Benzyl-amino-purine (2 mgL^{-1}) was used in media it induced callus at the base of shoots resulting in decreased shoots. Similar results are reported by **[29]** that when callus was induced at the base of shoots, multiple shoots were not stimulated from the cotton shoot apex. They studied *Gossypium herbaceum* and *Gossypium hirsutum*. They also reported that the Kinetin alone in media only supports the induction of single shoot. In present study we found multiple shoot in Kinetin alone (treatments 4 and 7) in *Gossypium arboreum*. The observed differences could be due to the genotypes used in both studies.

Table 2. The effect of different concentrations of Benzyl-amino-purine and Kinetin on number of shoots per explant of *Gossypium arboreum in vitro*

	Benzyl-amino-purine concentrations		
Kinetin concentrations	**0 mgL^{-1}**	**1.0 mgL^{-1}**	**2.0 mgL^{-1}**
0 mgL^{-1}	0.00d	3.00bc	1.20cd
0.5mgL^{-1}	1.60cd	6.30a	1.50cd
2.0 mgL^{-1}	1.30cd	4.00b	1.20cd

Note: Means represent two replications with five explants in each. DMRT is applied to find the difference between means at p= 0.05. Means followed by same letters are not significantly different

Different concentrations of BAP and Kinetin also effected the time required for shoot induction significantly. Within 26 days after shoot tip culture new shoot growth was observed inT5 followed by 30 days in T8. Treatments 3, 4, 6, 7 and 9 took highest time

to show shoot proliferation. Shoot tips took maximum time when cultured on 2.0 mgL^{-1} of Benzyl-amino-purinealone (46 days; Table 3). As described earlier, in most of the cultures, callus was induced at 2.0 mgL^{-1} of Benzyl-amino-purine that inhibited the further growth of shoots. These calli proliferated very well but did not show any regeneration or somatic embryogenesis. Among the nine tested combinations, two media regimes (T5 and T8) were found promising to initiate shooting within shortest time (Table 2, 3 & 4). However, T8 is actually MS media containing 1.00 mgL^{-1} BAP and 2.00 mgL^{-1}Kinetin. On this treatment only 40% explants showed shoot induction and 4.0 shoots are observed per explants on the other hand T5 found the most effective treatment exposing 63.3% explants proliferating shootlets (Table 4) with 6.30 shoots per explant (Table 2) on average within shortest time of 30 days (Table 3). These differences between the two treatments are significant settling T5 as the optimal treatment. The highest number of roots was observed when IBA was added to MS media at the concentration of 0.75mgL^{-1} i.e. 3.0 roots per shoot (Table 5). The rooted plantlets were harden and transferred to normal conditions successfully.

Table 3. The effect of different concentrations of Benzyl-amino-purine and Kinetin on shoot induction time of *Gossypium arboreum in vitro*

	Benzyl-amino-purine concentrations		
Kinetin concentrations	**0 mgL^{-1}**	**1.0 mgL^{-1}**	**2.0 mgL^{-1}**
0 mgL^{-1}	0.00d	43.80a	46.00a
0.5mgL^{-1}	36.20b	25.90c	45.00a
2.0 mgL^{-1}	43.50a	29.50c	40.20ab

Note: Means represent two replications with five explants in each. DMRT is applied to find the difference between means at p= 0.05. Means followed by same letters are not significantly different

Table 4. The effect of different concentrations of Benzyl-amino-purine and Kinetin on number of explants of *Gossypium arboreum* which induced shoots *in vitro*

	Benzyl-amino-purine concentrations		
Kinetin concentrations	**0 mgL^{-1}**	**1.0 mgL^{-1}**	**2.0 mgL^{-1}**
0 mgL^{-1}	0.00d	30.0bc	10.0cd
1.0 mgL^{-1}	26.7bc	63.3a	23.3bcd
2.0 mgL^{-1}	13.3cd	40.0b	16.7bcd

Table 5. The effect of different concentrations of Indol-butyric acid on root induction per shoot of *Gossypium arboreum in vitro*

Indol-butyric acid concentrations	**0 mgL^{-1}**	**0.75 mgL^{-1}**	**2.0 mgL^{-1}**
Mean roots number	0.00b	3.00a	1.30ab

Note: Means represent two replications with five explants in each. DMRT is applied to find the difference between means at p= 0.05. Means followed by same letters are not significantly different

Direct organ cultures are a safe option to generate plantlets of normal phenotype as compared to indirect cultures like callus cultures followed by somatic embryogenesis that could produce phenotypically and cytogenetically abnormal plants [30, 31]. In present study, the optimized protocol took only 3.5 months from seed germination to plantlets in soil planting (Figure 1) Cotton tissue culture by callus induction and indirect

somatic embryogenesis pathway need5-8 month for plant regeneration [9, 32]. The results of present study are in agreement to [33] they demonstrated the efficiency of shoot apex response at MS supplemented with 0.1 mgL^{-1}Kinetin irrespective of the genotypes. However, they observed the best rooting at MS+ 0.1 mgL^{-1} GA3 + 1.0 mgL^{-1} IAA. In contrast we used 1.0 mgL^{-1} IBA in MS basal media to induce roots. Although the number of roots was not very high, plantlets survived and showed good growth after transferring in pots. Shootlets induced during tissue culture need proper root system for survival. There are many reports demonstrating root induction in cotton tissue cultures as a major problem optimized a two stage rooting process first [34]. The 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 [35].

Regenerated shoots were cultured on MS media supplemented with 0.1 mgL^{-1}Kinetin, 2 mgL^{-1} NAA and 1gL^{-1} PVP however this medium stimulated weak rooting with browning. They excised weak roots after a week and transferred shoots to Woody Plant Medium (WPM) containing 1mgL^{-1} IBA. This new media regime stimulated rooting 70% in cotton variety Nazilli 84S and 80% in cotton variety ukurova1518 (Iranian *Gossypium hirsutum*). The rooting by planting *in vitro* generated shoots directly into the soil as they could not get rooting in MS media containing IBA [17, 36].

Figure 1. Shoot tip culture of *Gossypium arboreum*

Conclusion

It is concluded that shoot regeneration from shoot tips is quite simple as compared to somatic embryo genesis. It also needs less time to regenerate plantlets with very low rate of somaclonal variation. Among the tested concentrations 1.0 mgL^{-1} BAP and 0.5 mgL^{-1} KIN (T5) was optimal and provided shoot initiation within 26 days. This hormone combination revealed 6.3 shoots per explant on average. IBA at the concentration of 0.75 mgL^{-1}demonstrated 4.0 cm long 3.0 roots per plantlet on average. The in vitro generated healthy plantlets were acclimatized for 2weeks under high humidity with 100% survival. The *in vitro* plantlets can further be utilized for the *Inplanta* transformation experiments and/or molecular studies to overcome off season unavailability of plant material to explore genetic basis of diversity and organization of desirable traits in diploid germplasm.

Authors' contributions

Conceived and designed the experiments: S Yasin & A Yasmin, Performed the experiments: S Yasin, Analyzed the data: A

Yasmin, Wrote the paper: S Yasin & A Yasmin.

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