Direct shoot organogenesis of Indian cotton 

*G. arboreum* cv PA402 from embryonic axis explants

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Abstract

Cotton is multipurpose crop serving as an engine of economic growth in both developing and developed countries across six continents. India is the only country to grow all four cultivated (both diploid as well as tetraploid) species on commercial scale. The diploid cotton species can serve as valuable gene pool for the agronomically desirable tetraploid cultivars and offer better opportunities to study gene structure and function through gene knockouts. Development of transgenic lines is one of the ways for value addition in terms of transfer of different agronomic traits as well as study of different gene functions using different tetraploid and diploid germplasm backgrounds. Regeneration protocol through somatic embryogenesis is primary requisite to exploit valuable traits available through plant transformation. In present investigation successful direct shoot organogenesis was attempted in diploid cotton (*G. arboreum* cv PA 402) using MS media supplemented with Myo-inositol 100 mg/L Thiamine 10 mg /L Glucose 30 gm/L and agar 7 gm/L. The hormone kinetin was used in the range of 0.1–2.5 mg/L in five different levels. Growth of shoots on MS medium supplemented with Myo-inositol 100 mg/L thiamine 10 mg /L Glucose 30 g/L and NAA 0.1 mg/L resulted in regeneration of plantlets. Among different levels of kinetin 0.1 mg/L was found the most suitable concentration for direct shoot organogenesis from embryonic Axis explants.

Keywords: embryonic axis; organogenesis; MS media; regeneration; kinetin.

INTRODUCTION

The genus *Gossypium* consists of about 50 species, only four (*G. hirsutum* L., *G. barbadense* L., *G. arboreum* L., and *G. herbaceum* L.) of which are domesticated and produce spinnable fiber. (Fryxell, 1992). *G. hirsutum* and *G. barbadense* are tetraploid (AD genome) species and the remaining two are diploid, (A genome) species. *G. hirsutum* cultivars (Upland cotton) provide the bulk of the commercial cotton. The two diploid species are cultivated as rainfed crops in India, and where about 25–30% of cotton produced consists of *G. arboreum* (Choudhary and Laroia, 2001). *G. arboreum* accessions and land races could play a key role in cotton improvement because they have been reported to be resistant to pests and diseases, ranging from cotton bollworms (Dhawan et al., 1985) to fungal diseases (Wheeler et al., 1999). These two species are highly tolerant to drought and are fit for rainfed cultivation in low rainfall and poor soil areas in the country. These species also possess high structural uniformity of fibre with suitability for open end spinning (Banergee, 2000). In addition, diploid cotton cultivars offer a wider adaptability than the tetraploids. Transfer of desirable genes from *G. arboreum* to either *G. barbadense* or *G. hirsutum* could be achieved through interspecific hybridization but it also results in transfer of some undesirable traits. On the other hand, the recalcitrance of the *Gossypium* species, in particular *G. hirsutum* and *G. arboreum*, to tissue culture constitutes a major hindrance to the transfer of desirable traits to the cultivated tetraploid species through genetic engineering. An alternative to somatic embryogenesis approach is plant regeneration from explants having pre-existing meristems. Apical meristem culture of cotton was first reported by Chappel and Mauney (1967). Regeneration through tissue culture is limited to specific *hirsutum* cultivars. To exploit the genotypic potential of the old world cotton (*G. arboreum*), stable, reliable regeneration protocol is a prime requirement. Plant regeneration through organogenesis or somatic embryogenesis is a critical step for the success of any crop improvement program through biotechnological means. Therefore present investigation is aimed at standardization of
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protocol for regeneration of cotton (*G. arboretum*) through direct shoot organogenesis using embryonic axes as explants.

**MATERIALS AND METHODS**

The variety PA 402 (Vinayak) hirsutize quality arboreum cotton was used as an experimental material. It is the derivative of interspecific cross of *G. arboreum* and *G. hirsutum*. Polyploidy was induced in *G. arboreum* variety PA 85/86 by colchicine (Omran, 2008; Blakeslee et al., 1937) and crossed with *G. hirsutum* variety NH 239 (Poornima). This was backcrossed with autotetraploid PA 85/86 resulting in segregating population of backcross progeny. It was named as PA 402 by adapting pedigree method. General phenotypic characteristics are plant height 120-130 cm, sympodial, erect growing, medium broad serrated leaves and high response to fertiliser. Economic characteristics are big boll size (2.96-3.35 gm) and big flower size. The seeds of this variety were obtained from Cotton Research Station Mehboob bagh farm (Marathwada Agriculture University). Tissue culture grade chemicals and growth regulators were used from himedia and stock solutions for MS media including micro and macro nutrients were prepared and stored as per specific requirements. Stock solutions for different growth regulators were filter sterilized using 0.22µ size filter. Embryonic axes explants from three days old *in vitro* germinated seedlings were used in this experiment (Fig. 1).

**RESULTS AND DISCUSSION**

**Seed germination and preparation of explants**

The seeds of variety PA 402 were delinted using commercial grade Sulphuric acid which prepares seed for surface sterilization. The delinted seeds were initially rinsed 2-3 times with water for 10 minutes. Then seeds were transferred to the solution of liquid detergent for 15 min with continuous stirring. Thereafter seeds were washed three-four times thoroughly with double distilled water. This was followed by treatment with 0.1 percent solution of bavistin for 45 min. The flask was kept on orbital shaker and stirred at speed of 110 rpm. Seeds were then washed three-four times with sterile distilled water under laminar air flow to remove traces of bavistin. Seeds were then transferred in another autoclaved conical flask under laminar air flow. Then seeds were treated with 0.1 percent mercuric chloride solution for 5-7 min followed by washing with distilled water for 3-4 times. Lastly seeds (75-80 per plate) were inoculated on to half strength MS (Murashige and Skoog, 1962) agar medium for *in vitro* seed germination at 25±2°C for 72 hrs.

**Isolation of explants and direct shoot organogenesis**

Embryonic axes were excised under laminar airflow under aseptic conditions. Embryonic axis explants were used to standardize different combinations of MS media. Five different media combinations comprising myoinositol, kinetin thiamin were studied. After regeneration using different kinetin levels, shoots were grown on regular MS media.

**Figure 1:** Germination of seeds *in vitro.

**Figure 2:** Embryonic Axis explants growing on MS media.
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**Rooting**

After direct shoot organogenesis from explants, regenerated shoots were transferred to rooting medium containing basal MS medium supplemented with NAA 0.1 mg/L. Rooting was also tried on MS basal media without addition of growth regulator. Tissue culture regenerated plants were hardened in polyhouse providing controlled humidity and temperature conditions.

In present investigation cultivar PA 402 belonging to *G. arboreum* was used for regeneration through direct shoot organogenesis using embryonic axes. Healthy seeds from the common seed lot were selected and subdivided into 20 different seed lots. Delinted seeds of PA402 variety were surface sterilised as per procedure standardized by Nandeshwar *et al.* (2002). Total 2395 seeds were inoculated. Out of total 1837 seed were germinated into healthy seedlings. The average germination percentage of all the seed lots recorded was 76.70 percent. All the seed lots showed considerable variation in germination despite the similarities in germination conditions and growth medium which may be due to physical condition of individual seed. There are several reports available where germination of cotton seed has been reported by different methods. In one approach, seed coat was removed before seed was cultured in MS basal medium at 28 °C in light (Zapata *et al.*, 1999, Kumar *et al.*, 1998), while in other approach, surface sterilized seeds were directly placed on MS salts and B5 Vitamins for germination (Zhang *et al.*, 2000; Finer, 1988). Yet in another report, (Trolinder and Goodin, 1988; Davidonis and Hamilton, 1983) obtained cotton seed germination on moistened sterile filter papers in petri dishes. Different levels of kinetin were used along with thiamine to standardize levels of growth regulators. Primary objective of our experiment was determination of effect of kinetin on direct shoot organogenesis from embryonic axis explants of cotton with use of 0.1 mg/L kinetin and development of stable regeneration protocol. Average 1-2 shoots were regenerated from no of embryonic axes explants (Fig. 3). Increase in level of kinetin supplemented with thiamine increases no of shoots with stunted growth (Banerje, 2000). Cotyledonal node explants of cotton gives multiple shoot induction when media was supplied with BAP along with Kinetin (Nandeshwar *et al.*, 2002) Our results suggest shoot regeneration as well as induction of multiple shoots when kinetin was used along with Thiamine. Multiple shoot induction is also a common phenomenon which was observed during regeneration using MS medium supplemented with 0.1 mg/L of kinetin. Cultured shoot apices containing meristematic tissue at the apical and nodal region in MS medium with BAP and Kinetin combination also causes induction of multiple shoots (Nandeshwar *et al.*, 2002) Kinetin levels above 0.1 mg/L were found undesirable for direct shoot organogenesis as it produces stunted growth in regenerated shoots (Fig. 4A). Regeneration of plants via pre-existing meristems has been used as an alternative approach for development of true to type plants, independent of genotypes. *In vitro* culture of excised meristems of *G. hirsutum* L. was first reported by Chappel and Mauney (1967). Agrawal *et al.* (1997) reports induction of multiple shoots and plant regeneration from decapitated cotyledonary nodes in cotton. During our experiment phenolics were observed in some of the old cultures paired with browning of explants. Phenolics synthesized by the explants in any periods of organogenesis have important role on browning and rooting problems. Shoots derived from embryo axis were excised and transferred on medium consisting of half strength MS basal medium supplemented with NAA (0.1 mg/L) for rooting (Fig. 5).

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**Figure 3**: Direct shoots regeneration from embryonic Axis on MS media supplemented with kinetin myoinositol and Thiamine.

**Figure 4**: Shoot regenerates in to plantlets.

**Figure 5**: *In vitro* rooting of plant on MS media.
study (Table 1) show that kinetin at conc. 0.1 mg/l is appropriate. Rooted shoots were transferred to 8 cm pots containing 1:1 (vol/vol) mixture of soil and sand and kept under greenhouse conditions. Rooted shoots were hardened. Plants (20 each from embryonic axes) were transferred to field after 3-4 weeks of hardening in greenhouse. Embryonic axis derived shoots can be rooted on Tissue Culture media (MS) without addition of growth regulators like NAA (Hazra, 2001). Forty plants raised by this method survived and formed bolls on maturation in the field.

### Future prospects

The results will be of advantage in the ongoing efforts in genetic transformation of Indian cotton cultivars by particle bombardment and Agrobacterium mediated methods. Different level of other growth regulators like BAP needs to be tried which according to different reports also holds a promise for multiple shoot induction as well as direct shoot organogenesis. Cotyledonal node can also be tried for direct shoot organogenesis in desi (diploid) cotton cultivars. Different plant transformation methods can be studied using this protocol for regeneration. Low regeneration frequency, chimeras resulting from a group of cells, somaclonal variations and chromosomal abnormalities are some of the limitations that need to be addressed using direct organogenesis protocol. The method also may results in the development of true to type plants independent of genotype.

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