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HIGH FREQUENCY MULTIPLE SHOOTS INDUCTION AND PLANT REGENERATION IN SIX ELITE INDIAN COTTON CULTIVARS

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ABSTRACT

Direct multiple shoot induction and regeneration from the cotyledonary nodal explants of two Indian cultivars of upland cotton, *G. hirsutum* (hybrid H8 and Khandwa-2) and four cultivars of *G. arboretum* (BD-1, BD-6, Sarvottam and Jawahar Tapti BD) were investigated, using varying concentrations of BAP. An overall average of 5.5 shoots per explant was achieved in the study. The best multiple shoots formation (9 shoots per explant) was obtained from the two *G. hirsutum* cultivars cultured on 3.0 mg L-1 BAP. Shoots were harvested and elongated in the presence of 0.5 mg L^{-1} GA3. Root formation was achieved on hormone-free MS medium.

Keywords: Organogenesis, tissue culture, plant growth regulator.

INTRODUCTION

Cotton (*Gossypium* spp) is a worldwide multipurpose and high-valued crop of immense commercial importance as raw material for diverse industrial applications, ranging from food and feed, to textile and footwear, to automobiles and energy, to fertilizer and paper, and to medical and pharmaceutical. This all-important agricultural commodity has continued to be the backbone of rural economy, particularly the dry land areas of India, despite the dwindling production level due mainly to the narrow genetic base of the cultivated species**.** The cotton breeding strategies developed over the years for its genetic improvement have suffered major setbacks partly because of the constraint of its genetic base (Kumria *et al.*, 2003) and partly because of incompatibility problems between the cultivated and the wild type species (Fu *et al.*, 2009). Since the first report of the *in vitro* plant regeneration of cotton through somatic embryogenesis in the early 1980s (Davidonis and Hamilton, 1983), there have been substantial advances with respect to the range of explants used in cotton tissue culture, and the regeneration methods coupled to *Agrobacterium*-mediated transformation procedure, which have culminated in the production of herbicide- and insect resistant cotton lines. The cultivation of insect resistant (IR) cotton, in particular, in the US, Australia, China, India, Argentina, South Africa, Mexico and Brazil has revolutionized the cotton industry in these countries (James, 2008). The farm income benefit of cultivating IR cotton globally has been $12.58 billion, cumulatively since 1996 (Jin *et al.*, 2005). In spite of all these achievements, the crop is still not easily amenable to genetic transformation due to the genotype-dependent nature of its somatic embryogenesis-based *in vitro* plant regeneration system (Rauf *et al.*, 2004; Kouakou *et al.*, 2007). As such, its recalcitrance to tissue culture has not only slowed further development of transgenic cotton but has also narrowed its genetic base. Multiple shoot induction is the second method, which has been reported in many cultivars of cotton (Sun *et al.*, 2006; Özyiğit and Gözükirmizi, 2008). The direct multiple shoot induction offers a good alternative *in vitro* regeneration pathway for cotton, as it offers a preclusion from the current limitation of genotype-dependence in the existing system of cotton transformation. Additionally, the use of explants such as apical meristems and axillary buds has been found to be convenient as they are easy to regenerate, thereby avoiding the problems associated with long period of culture being experienced in the established cotton transformation system (Wilkins *et al.*, 2000; Wilkins *et al.*, 2004; Katageri *et al.*, 2007). There have been few reports on genetic transformation of Indian cotton cultivars through direct organogenesis pathway of plant regeneration, using shoot apical- and hypocotyl explants (Satyavathi *et al.*, 2002; Divya *et al.*, 2008).

In this study, we give a rapid and cost-effective protocol for the induction of multiple shoots by benzylaminopurine (BAP) and plant regeneration from the cotyledonary nodal explants of six elite Indian cultivars of cotton. This *in vitro* regeneration procedure can be coupled to genetic transformation either by particle bombardment or to *Agrobacterium*-mediated transformation, for the overall enhancement of cotton biodiversity.

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MATERIALS AND METHODS

Explant Preparation and culture conditions

Seeds of six elite Indian cotton were obtained from Cotton Research Centre, Bulandshahr (U.P) and Cotton Research Station, Khandwa (M.P) India. Four cultivars of *G. arboretum* (BD-1, BD-6, Sarvottam and Jawahar Tapti BD) and two cultivars of upland cotton *G. hirsutum* (hybrid H8 and Khandwa-2) were used in the present study. The seeds were washed in running tap water and then with sterilized distilled water. The seeds were, thereafter, treated with 70% ethanol for 5 min, followed by sodium hypochlorite (4% available chlorine), for 10 min followed by 0.1% mercuric chloride for 15 min. After each sterilization treatment, the seeds were washed three times with distilled water. Later the seeds were soaked for 5-6 hrs in distilled water to soften the seed coats. After removing the seed coats with the help of the forceps the seeds were then placed aseptically on 0.7% agar solidified medium, pH-5.8, containing Murashige and Skoog (MS) inorganic salts, vitamins and sucrose. For germination, the cultures were initially maintained in the dark and then incubated at 25±1ºC under cool white fluorescent light with an intensity of 40-60 μ $mols^{-1}m^{-2}$ with a photoperiod of 16h.

Induction of Multiple Shoots

For the induction of multiple shoots cotyledonary nodes were excised from 15 days old seedlings. The cotyledons and shoot meristem were excised and discarded. Cotyledonary nodes were cultured with the base in the shoot induction medium containing various varying concentrations of BAP. After 20-30 days small shoots started to emerge from the nodes, shoots of about 2-3 cm height were excised and transferred to shoot elongation medium. Elongated shoots were excised and cultured on half strength hormone free MS medium for the induction of roots. The plantlets regenerated were hardened and were transferred to pots containing sand and vermiculite.

Statistical analysis

All experiments were repeated three times. Data were statistically analyzed using SAS GLM procedure (SAS, 1993), using a completely randomized design and means were compared at the $p = 0.05$ level of significance using Duncan's new multiple range test.

Histological studies

For histological studies botanical microtechnique was used (Prasad and Prasad, 1975). Small pieces of organogenic callus and multiple shoots were embedded in wax and 10μm thin sections were cut through a microtome. These were then passed through an alcohol xylene series. The sections were then stained with saffranin and fixed in Canada balsam. The sections were observed under microscope.

RESULTS

Multiple shoot Induction

Direct multiple shoot induction and regeneration from the cotyledonary nodal explants of two Indian cultivars of upland cotton, *G. hirsutum* (hybrid H8 and Khandwa-2) and four cultivars of *G. arboretum* (BD-1, BD-6, Sarvottam and Jawahar Tapti BD) were investigated, using varying concentrations of BAP. It was striking to observe that BAP alone gave multiple shoot induction with a global average of 5.5 shoots per explant (Table 1). Another remarkable observation from the present study is a sort of pairing of the cultivars with respect to marked similarities in their responses to the different concentrations of the BAP used. Even though these similarities were not absolute, they were quite significant in some cases. Take for example cultivars BD-1 and BD-6, which formed an average of 6 shoots per explant on modified MS medium supplemented with 1.5 mg L^{-1} BAP (Table 1) but were less responsive to higher concentrations of BAP, even though their responses on 2.0 and 3.0 mg L^{-1} were significantly different. It should also be noted that the best response of this pair in the presence of 1.5 mg L^{-1} BAP was significantly better than the rest of the cultivars. Nonetheless, their poor responses on 2.0 and 3.0 mg L^{-1} BAP were not significantly poorer than the other cultivars. Also remarkable are the similar responses of another pair of cultivars Jawahar Tapti and Sarvottam, in that they produced a significantly higher average of 7.8 shoots per explants in the presence of 2.0 mg L^{-1} BAP, than the other cultivars, but likewise showed rather poor responses when cultured on 1.5 and 3.0 mg L^{-1} BAP-containing MS medium, even though their responses on 1.5 mg L^{-1} were significantly different (Table 1). Finally as well, the *G. hirsutum* cultivars Khandwa-2 and hybrid H8 gave rise to a significantly higher average of 9 shoots per explants when cultured on modified MS medium supplemented with 3.0 mg l^{-1} BAP, which represents the highest average multiple shoots induction in the study (Fig.1) but responded rather poorly on lower concentrations of the cytokinin (Table 1).

The shoots were harvested and elongated up to 4-5 cm within 30-40 days when cultured on MS medium supplemented with 0.5 mg L^{-1} GA3. They were then rooted in half strength hormone-free MS medium (Fig. 2).

Histology

Multiple shoots were observed to arise from the base of the nodal explant (Fig. 3). These shoots had a common origin and vascular connections were observed amongst them (Fig. 3).

DISCUSSION

The present study was originally motivated by our quest to investigate the induction of direct organogenesis from

Table 1. Number of multiple shoots induced from 10 days old cotyledonary nodes of different cultivars of cotton at different concentrations of BAP (The data represents an average of three replicates with three independent experiments in each case).

Cultivars	Concentration of BAP (mg L^{-1})		
	1.5*	2.0*	3.0*
BD-1	6.2±0.9 [a]	5.5±0.7 [b]	4.9±0.9 [b]
BD-6	5.8±1.0 [a]	3.7±0.8 [c]	2.7±0.8 [c]
Sarvottam	5.3±0.8 [a]	8.1±0.6 [a]	5.6±0.8 [b]
hybrid H8	3.7±0.3 [b]	5.4±0.8 [b]	8.9±1.5 [a]
Jawahar Tapti	3.5+0.7 [b]	7.5+0.9 [a]	5.5+0.9 [b]
Khandwa-2	3.3±0.6 [b]	4.8±0.8 [bc]	9.1±1.3 [a]

*Mean ± SE. Means having the same letter are not significantly different (p=0.05) according to Duncan's new multiple range test.

Fig.1. Induction of multiple shoot in *G. arboreum* cultivar Jawahar Tapti.

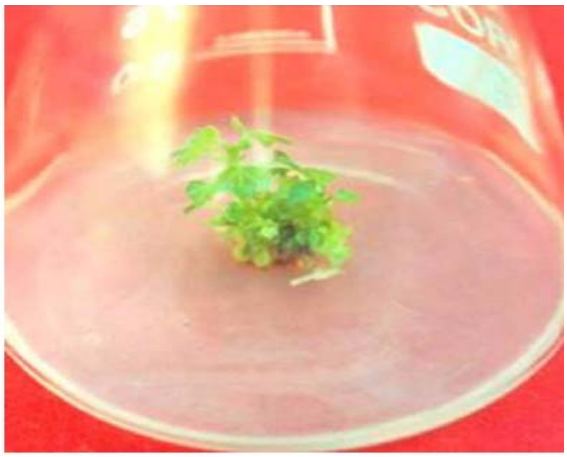

Fig. 2. Developed shoots of cultivar BD-1.

two upland cotton cultivars Hybrid H8 and Khandwa-2, which were found to be recalcitrant to *in vitro* regeneration through the somatic embryogenesis pathway (Khan *et al.*, 2006). Further more, we are of the viewpoint that all means should be employed to increase the range of cotton cultivars that are amenable to tissue culture regeneration and hence genetic transformation, which would pave way for broadening the fast eroding genetic diversity as well as ensure further development of the crop. We also shared same sentiment with Özyiğit and Gözükirmizi (2008) that the exploration of the alternative approach would serve a complementary purpose in the long-term development of cotton through genetic transformation. Hence, we deployed the direct organogenesis strategy to the two non-embryogenic cultivars and four embryogenic counterparts (for comparison).

It was remarkable to observe less pronounced genotype-specific responses to different concentrations of BAP (1.5, 2.0, 3.0 mg L^{-1}) used in the study, in that the six cultivars formed some sort of pairing clusters, with each cluster exhibiting somewhat similar responses in the presence of the three different concentrations of the BAP. A similar observation was reported for two India cotton (*Gossypium hirsutum* L. cv DCH-32 and NHH-44), which produced 5.1- and 4.3 multiple shoots per explants from nodal segments, in the presence of 2.22 µM BAP (Hazra *et al*., 2001). This sort of similar responses between two cotton cultivars signals a future possibility of developing genotype-independent transformation procedure for cotton genetic improvement. It was also remarkable that the two erstwhile recalcitrant (non-embryogenic) cultivars, Hybrid H8 and Khandwa-2 gave the overall best organogenic response (9 shoots per explant) on BAP as compared to others, even though this was achieved at a higher concentration 3 mg L^{-1}. The use of organogenesis indeed served a complementary purpose in this particular case. As such, this observation laid credence for the exploration of multiple approaches to broaden the range of cotton cultivars that are amenable to tissue culture regeneration and consequently transformation. BAP has been simply known to act by stimulating organogenesis from pre-existing meristematic tissue (preformed buds) (Hagio, 2002). The highest average shoot per explant ever reported is 10.6 but this was achieved in the presence of

two plant growth regulators, thidiazuron (TDZ) and naphthaleneacetic acid (NAA) (Divya *et al.*, 2008). The use of BAP alone is not only cost-effective but also provides a less-cumbersome multiple shooting and regeneration procedure. A recent report indicated that the use of BAP and kinetin together adversely affected multiple shoots formation from the cotyledonary nodal explants of a Sudanese upland cotton (*Gossypium hirsutum* L. cv Barac B- 67) (Abdellatef and Khalafalla, 2008), which was, however, contrary to the observation of Agrawal *et al.* (1997). Nonetheless, the same report also indicated that kinetin was better than BAP for cytokinin-induced multiple shooting, even though the best response in their study was 2.6 shoots per explants.

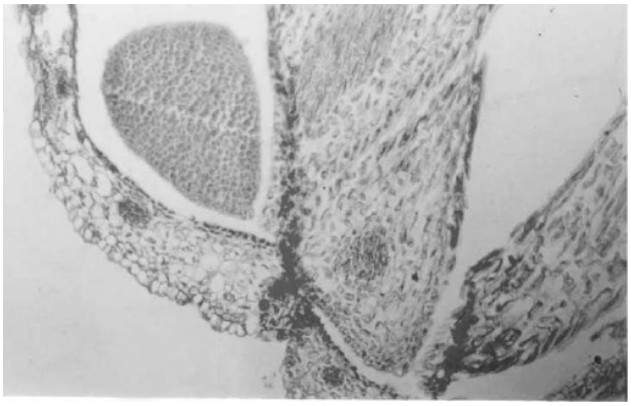

Fig. 3. Microtome section through the base of multiple shoots. (200X).

One additional remarkable observation from our study was that the six cultivars gave comparable multiple shoots formation (though at different concentrations of BAP) with the other reports, which culminated in an overall average of 5.5 shoots per explants. It could be objectively argued, however, that these responses may have been circumstantial after all, especially with respect to the use of fairly organogenic cultivars, even though this was unintentional. Nonetheless, these observations further strengthen the importance of trying out tons of different cultivars using different *in vitro* regeneration approaches, with a view to increasing the range of cultivars that are amenable to genetic transformation-mediated development, which in turn would increase the genetic base of the crop, reverse the dwindling trend in production, and finally ensure a safe future for the cotton industry.

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