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An Improved Protocol for Regeneration of *Sorghum bicolor* from Isolated Shoot Apices.

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Abstract

An improved and producible protocol for *in vitro* regeneration of sorghum [*Sorghum bicolor* (L.) Moench] from shoot meristem explant is reported. By striking an optimal balance between a weak auxin like naphthalene acetic acid (NAA) and the cytokinin thidiazuron (TDZ), the isolated shoot meristems were manipulated to follow either organogenic or embryogenic pathway. There was no intermediate callus formation. Multiple buds were induced on enlarged meristems using MS medium with 5.0 μM of TDZ, 17.72 μM Benzylaminopurine (BAP), and 1.074 μM NAA. To maximize the number of bud initials per explant, three parameters (seed size, germination technique, and age of explant) were studied. Five to 7-day-old shoot meristems responded best with $\geqq$ 80% induction of bud initials. Seed size was not significant in influencing induction potential of the shoot meristems. Six weeks after *in vitro* culture, each meristem produced 35-40 shoot buds. Direct somatic embryogenesis ($\geqq$ 80% induction) was accomplished following a two-step culture procedure consisting of induction of multiple buds and formation of somatic embryos. A high frequency (700-1000) of somatic embryos per explant was obtained on MS medium with 17.72 μM BAP and 2.69 μM NAA. Shoots from both organogenic and embryogenic pathways rooted on half-strength Murashige and Skoog (MS) medium with 8.28 μM Indole 3-butyric acid (IBA) and 1.14 μM Indole acetic acid (IAA). After one month on rooting medium, plants with well-developed roots were transferred to jiffy cups. Such plants were subsequently acclimatized in the glasshouse, and were grown till maturity; they showed normal seed set. Random amplified polymorphic DNA (RAPD) analysis of regenerants did not detect any DNA polymorphism.

Keywords: multiple buds, shoot apices, somatic embryos, *Sorghum bicolor* L., tissue stratum.

Abbreviations

BAP, 6-benzyladenine; MS, Murashige and Skoog; NAA, naphthalene acetic acid; TDZ, thidiazuron.

Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is a major cereal crop and ranks fifth in importance in the world. Until recently, genetic improvement of sorghum for agronomic and quality traits have been carried out using only traditional plant breeding methods. New biotechnological approaches that are now being developed can have a major impact on sorghum improvement for complex traits for which breeding by traditional methods is difficult. Genetic transformation with specific genes conferring disease and insect resistance provides an efficient tool to complement traditional breeding. Transgenic sorghums have been already reported using physical methods of gene transfer (Casas *et al.*, 1993). However, genetic transformation of sorghum is not yet routine and easy. Besides, existing protocols are not repeatable across different laboratories, and are to a significant extent genotype-specific.

A rapid and highly uniform regeneration system with high regeneration efficiency is a prerequisite for successful genetic transformation. Protocols have been established for *in vitro* plant regeneration of sorghum from different types of explants such as immature embryos, immature inflorescences, and shoot tips (Brar *et al.*, 1979; Boyes and Vasil, 1984; Elkonin *et al.*, 1984; Bhaskaran *et al.*, 1988; Bhas-

karan and Smith, 1988, 1989; Bhaskaran and Smith, 1990; Eapen and George, 1990; Lusardi and Lupotto, 1990; Nahdi and de Wet, 1995; Sairam *et al.*, 2000; Seetharama *et al.*, 2000) and also from cultured mesophyll protoplasts (Seetharama and Sairam, 1997; Sairam *et al.*, 1999). However, the frequency of plant regeneration reported so far does not seem to be high enough for genetic transformation on a routine basis. Highly uniform and meristematic tissues are desirable for genetic transformation to minimize chimeras and somaclonal variants. Cultured immature embryos and recently, shoot tips of cereals have been widely used for genetic transformation (Bajaj, 2000). In this study, we have refined the conventional shoot tip culture protocol reported earlier from our laboratory (Seetharama *et al.*, 2000). This is an improvement over the earlier reported technique of shoot tip culture in maize (*Zea mays* L.) (Zhong *et al.*, 1992) and sorghum (Zhong *et al.*, 1998), and involves isolation of meristematic tissue along with two pairs of primordial leaves from the shoot tip which is reprogrammed *in vitro* to produce multiple shoots or large number of somatic embryos.

In this paper, we report improvement in the rate of *in vitro* induction by use of thidiazuron (TDZ) and the trimming technique during first subculture. With this system, we could successfully control the pathway of regeneration (organogenesis and direct somatic embryogenesis) by striking an optimal balance between BAP and NAA concentrations. We also examined the effects of TDZ and BAP on shoot organogenesis, and the effect of BAP and two auxins, (NAA and 2,4-D) on somatic embryogenesis without any intermediate callus phase in both cases. We have further standardized different factors that influence the rate and rapidity of adventitious bud formation and their subsequent differentiation.

Materials and Methods

Plant material and method for seed germination

Two popular seed parents (BTx 623 and 296B) and a popular Indian cultivar (M35-1) of sorghum were used. Shoot apices were obtained from aseptically germinated seedlings. For sterilization, seeds were placed in 100 ml distilled water with 2 drops of Tween-20 and washed for 10 min. seeds were then rinsed thrice with sterile distilled water, and placed in 70% ethanol for 1 min. and then collected in a sterile flask. Later, seeds were rinsed again with sterile water and kept for 5 min. After decanting the water, seeds were placed in 0.1% mercuric chloride for 7 min. with continuous stirring. Seeds were then thoroughly rinsed twice for 10 min. with sterile water. Finally, seeds were allowed to germinate in dark in 15-mm petri dishes with 30-40 seeds per plate.

Isolation and in vitro culture of shoot meristems

Shoot apices were excised from hypocotyls of seedlings under the dissection microscope. A cut was made at the base of the apex below the attachment of the largest unexpanded leaf. Membranous sheath was then separated from the primordial leaf. Parts of the unexpanded primordial leaves were left in place (**Fig. 1A**). Shoot apices were cultured using induction medium in 15-mm petri dishes by placing them horizontally on MS medium (Murashige and Skoog, 1962). The petri dishes were sealed with parafilm and were incubated under light (16 h-day length) at 26 ℃ and 45% relative humidity.

Optimization of hormone combination for in vitro meristem culture

Protocol for direct organogenesis of shoot meristem was standardized using different hormone combinations for their induction and proliferation. Initially, four hormone combinations including a control (without BAP) in basal MS formulation along with three levels of BAP (4.43, 8.86, and 17.72 μM) were tested. Next, the above combinations were fortified with different concentrations of either NAA (0, 0.537, and 1.074 μM) or 2,4-D (0, 0.452 and 0.904 μM) and tested for induction of multiple buds. There were 3 replications, each with 20 explants per treatment. The experiment was repeated twice for each hormone combination. The induction response was recorded 14 days after initiation.

To study the effect of thidiazuron on multiple bud induction, 7 levels of TDZ (0.1, 0.5, 1.0, 3.0, 5.0, and 7.0 μM) along with control (no TDZ) were added to MS medium with 17.72 μM BAP + 1.07 μM NAA. Proliferating buds attached to the enlarged meristems were transferred to MS basal medium supplemented with 13.29 μM BAP, 1.07 μM NAA, and 3.0 μM TDZ. Three subcultures were done at weekly intervals. During each subculture, the concentration of TDZ was reduced. It was reduced from 5.0 μM to nil by the fourth passage (**Table 1B**).

Incubation of meristems on multiple bud induction medium, followed by two subcultures (passage 1 and passage 2) similar to organogensis (**Table 1A**) resulted in multiple bud formation. For induction of somatic embryos, meristem cultures with 35-40 *de novo* shoot buds on them, were cut into 3-4 pieces and transferred onto somatic embryo (SE) induction medium (MS +17.72 μM BAP + 2.69 μM NAA).

Table 1. Growth regulator combinations used with MS basal medium during each passage of plant regeneration from isolated shoot meristems for both organogenesis and somatic embryogenesis pathways.

A. Common culture conditions for both pathways

Weeks after culture initiation	Passage details	Type of response	Growth regulators used		
			BAP (μM)	TDZ (μM)	NAA (μM)
0	Induction medium	Primary leaves Unfolding; meristem enlargement	17.72	5	1.07
2	Passage 1	Formation of multiple leaf initials; further enlargement of meristem region	13.29	3	1.07
3	Passage 2	Formation of multiple bud initials	8.86	2	1.07

B. Organogenesis pathway

Weeks after culture initiation	Passage details	Type of response	Growth regulators used		
			BAP(μM)	TDZ(μM)	NAA(μM)
1	Passage 3	Appearance of more bud initials	8.86	1	1.07
2	Passage 4	Increase in the bud dimensions	8.86	0	1.07
3	Passage 5	Differential of buds into plantlets	8.86	0	1.07

C. Somatic embryogenesis pathway

Weeks after culture initiation	Passage details	Type of response	Growth regulators used		
			BAP(μM)	TDZ(μM)	NAA(μM)
1	SE induction medium	Appearance of translucent outgrowths; formation of somatic embryos	17.72	0.0	2.68
4	Germination medium	Germination and differentiation of somatic embryos	17.72	0.0	1.07

For germination and further differentiation of such somatic embryos, GA (0.26 μM) was added to the medium.

The elongated shoots derived from both organogenesis and somatic embryogenesis were rooted on half-strength MS medium with IBA (8.28 μM) and IAA (1.14 μM) as this combination was found to be optimal for routine rooting in our laboratory (unpublished data).

Optimization of factors affecting the organogenic response of shoot meristem

A factorial experiment was conducted with the genotype BTx 623 to determine the effect of seed size (large $\geq$ 3 mm; and small, < 3 mm), method of germination wet filter paper and MS semi-solid medium), and age of explant (4 to 10-day-old seedlings) on meristem proliferation. There were 28 treatments in all, with 20 explants per plate. The experiment was repeated once. Induction response was recorded after 2 weeks of culture. Twenty seedlings from each treatment were used for meristem isolation, from day four to day ten.

RAPD analysis of in vitro regenerants

DNA polymorphisms of shoot meristem derived plants were analyzed using the RAPD technique. Genomic DNA was extracted from 12 R_0 plants, along with a control (field grown) plant according to Saghai-Maroof *et al.* (1984).

DNA amplification: A total of 6 random decamer primers (OPA-01, OPA-03, OPD-01, OPE-01, OPL-03 and OPL-14) from *Operon Technologies* were used for detecting polymorphism in genomic DNA. PCR amplifications were performed following the protocol of Williams *et al.* (1990). Amplifi-

cation reactions were carried out in a thermocycler (*M J Research PTC-2000*) programmed for 38 cycles of amplification. Amplified products were analyzed on 1.8% agarose gels.

Results

The morphogenic potential of isolated shoot meristems of three sorghum genotypes (BTx 623, M35-1 and 296B) was evaluated on MS basal medium supplemented with various concentrations of BAP, NAA, and TDZ. The induction response (meristems producing multiple buds) and regeneration response (meristems that gave rise to plants), induction percentage was 80.0% for BTx 623, 72.5% in M35-1 and 54.5% in 296B; and the regeneration frequencies were 91.7%, 89.6%, and 84.6% respectively. After 6 weeks of culture covering 5 subcultures, 35-40 shoots per explant were obtained. Manipulation of BAP and NAA combinations after a common 4 weeks culture treatment resulted in direct somatic embryogenesis from the translucent outgrowths or tissue stratum of the multiple buds as described below. About 700-1000 germinating somatic embryos were observed on each explant.

Optimization of hormone combination for in vitro meristem culture

Initially, MS medium along with BAP and NAA was tried and the response was noted after 2 weeks of culture. Variation in BAP concentration (4.43-17.72 μM) had no significant effect on shoot development. Few explants produced fleshy abnormal shoots and the remaining explants turned dark brown. On the other hand, use of BAP along with NAA caused the explants to remain green and the frequency varied between 25 $\pm$ 3% (BAP 17.72 μM and NAA 0.537 μM) and 61 $\pm$ 8% (BAP 17.72 μM and NAA 1.07 μM). There was no enlargement of meristem and no callus formation on the above medium. BAP in combination with 2,4-D resulted in meristem enlargement, but was associated with some degree of callus formation. Upto 38 $\pm$ 5% of the meristems enlarged when BAP (17.72 μM) and 2,4-D (0.45 μM) combination was used and 65 $\pm$ 3 % meristems enlarged when BAP (17.72 μM) and 2,4-D (0.9 μM) was added to MS basal medium.

Based on the above results, the combination of BAP (17.72 μM) + NAA (1.07 μM) which could arrest browning of the meristem with no accompanying callus formation was selected. This was then fortified with different levels of TDZ (0, 0.1, 0.5, 1.0, 3.0, 5.0, and 7.0 μM) for induction (enlargement followed by formation of multiple leaf initials) and proliferation (formation of multiple bud initials) of the meristems. TDZ at concentrations of 0.1 to 1.0 μM initiated 1-2 buds in 2 weeks. However, beyond this concentration, the number of responding explants as well as the number of shoots formed per explant increased with concentration of TDZ. Use of 3.0 μM TDZ resulted in 4-5 buds and at a concentration of 5.0 μM formed 10-15 buds on bulged meristems. At a still higher concentration of TDZ (7.0 μM), the response declined to 8-10 buds. Thus, 5.0 μM TDZ was an optimal concentration for multiple bud formation.

After induction, the concentrations of BAP and TDZ were brought down gradually at each passage as shown in **Table 1**. From *passage 4*, TDZ was completely withdrawn from the culture medium. This resulted in about three-fold increase in the length and breadth of the bud initials within 1 week. The last subculture (*passage 5*) using the same media composition helped further differentiation of the buds into individual plantlets of approximately 2 cm in length.

Morphogenesis of shoot meristem

The first leaf unfolding was observed 48-72 hours after initiation of shoot apex cultures. At the end of first week of culture, 1-2 expanded leaf initials were seen. Unfolding of the primordial leaves followed by elongation of the leaf blade in the first week of culture was rapid. In addition, bulging of the lower meristem region was observed (**Fig. 1B**), and this became prominent in the second week. Size of the meristem tissue increased four to five folds after 9-10 days of culture initiation, which also coincided, with the appearance of additional leaf initials. These leaf initials were trimmed off when explants were 2 weeks old. This was followed by subculture onto fresh medium (*passage 1*), which resulted in further proliferation of the meristem mass with production of additional leaf initials. Trimming of leaf initials for the second time was carried out at the end of 3 weeks of culture and these meristem masses were subcultured on to fresh medium (*passage 2*). The production rate of leaf initials came down during this passage, with simultaneous appearance of dome-shaped buds all over the meristem mass (**Fig. 1C**). Four weeks after isolation, such responding meristematic masses containing multiple buds were dissected into 2 to 3 pieces and cultured (**Fig. 1F**) onto fresh medium (*passage 3*). Such meristems containing multiple shoots could root easily on rooting medium (**Fig. 1I**). Fully rooted plantlets transferred into jiffy cups established well (**Fig. 1J**) and were kept in incubator for further acclimatization. After 1 week,

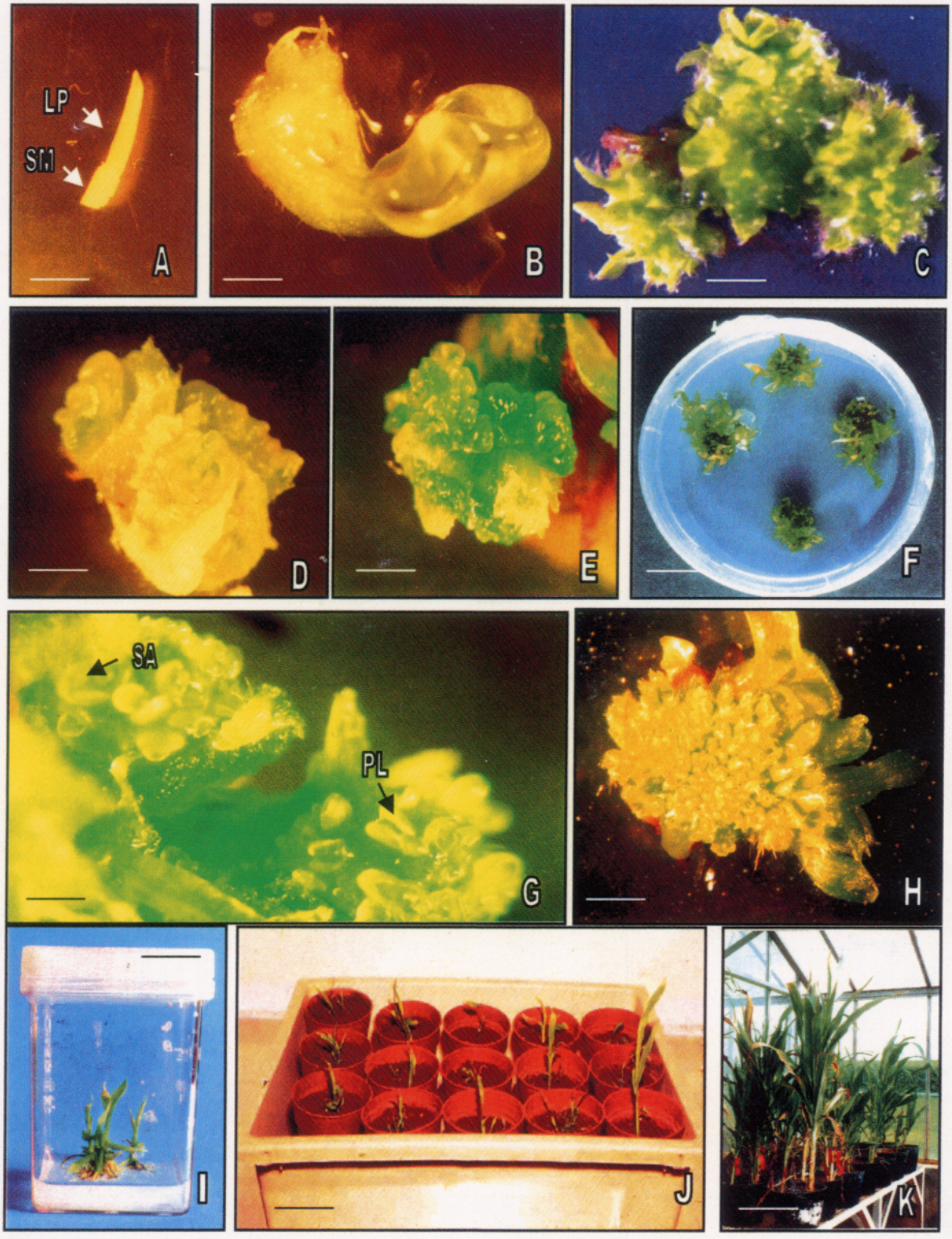

Fig. 1 Organogenesis and somatic embryogenesis from shoot meristem culturs. (A) One-day-old isolated shoot meristem with primordial leaves (***LP*** = *leaf primordia*, ***SM*** = *shoot meristem*) (Bar = 1.0mm). (B) One-week-old meristem showing bulged lower portion and expanded primordial leaves (Bar = 0.5mm). (C) Three-week-old meristematic mass showing multipul buds and leaf initials (Bar = 0.5mm). (D) Individual buds (shown in **Fig, 1C**) producting 2-8 translucent tissue strata (Bar = 1.0mm). (E) Each of the tissue stratum giving rise to many somatic embryos (Bar = 0.4mm). (F) Meristematic clumps showing differentiating buds (Bar = 18.0mm). (G) Germinating somatic embryos showing shoot apex (***SA***) surrounded by a pair of primary leaves (***PL***) (Bar = 0.26mm). (H) Differentiation of somatic embryos into plantlets (Bar = 1.0mm). (I) Plantlets with well-formed roots in magenta box (Bar = 16.5mm). (J) Acclimatization of plantlets in the growth chamber (Bar = 7.33cm). (K) Regenerated plants in greenhouse (Bar = 33.0cm).

hardened plantlets were transferred to the greenhouse and grown to maturity (**Fig. 1K**).

Direct somatic embryogenesis (without intermediary callus phase) from isolated shoot apices was possible by following a two-step culture protocol. The first step of 4-week culture period was same as that for organogenesis [incubation of explants on multiple bud induction medium followed by passage 1 and 2 (**Table 1A**)]. Shoot apex cultures with 35-40 *de novo* shoot buds were cut into 3-4 pieces and transferred on to SE induction medium (MS + 17.72 μM BAP + 2.69 μM NAA). This combination was favoured among all the media tried (**Fig. 2**). Maximum induction efficiency of 80% was observed on this phytohormone combination.

After 4 weeks of culture on SE induction medium, the differentiating meristem with shoot buds on it enlarged further and developed translucent outgrowths all over its surface (**Fig. 1D**). On these outgrowths, numerous somatic embryos developed at the end of the third week (**Fig. 1E**). Such somatic embryos could be maintained at this stage at least for 14 weeks by repeated subcultures at 2 weeks interval on the somatic embryo induction medium

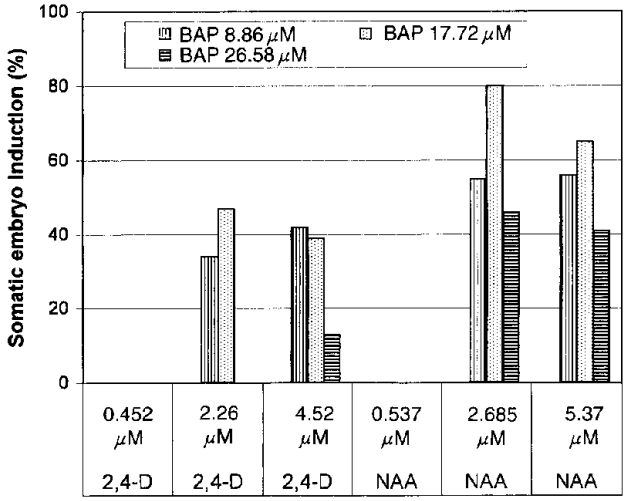

Fig. 2 Comparison of effects of 2,4-D and NAA for somatic embryo induction freguency with different concentrations of BAP.

described above. Alternately, these somatic embryos could be induced to germinate by transferring onto somatic embryo germination medium (MS medium + 17.72 μM BAP + 1.074 μM NAA) (**Fig. 1G**). During the process of embryo development into plantlets (**Fig. 1H**), only 5% of the embryos showed abnormal development.

Rooting of the shoots and transfer to greenhouse

Successful root initiation up to 95% (of the 500 plantlets transferred) of explants regenerating from both the morphogenic pathways (organogenesis and somatic embryogenesis) was possible on half-strength MS medium with IBA (8.28 μM) and IAA (1.14 μM) within 10 days of transfer. After 1 month of culture, rooted plantlets were removed from the magenta box, thoroughly rinsed with sterile water and transferred to jiffy cups containing autoclaved vermiculite mixture. After ten days of hardening in the culture room, plants were transferred to greenhouse. At maturity, all the 400 acclimatized plants obtained through *in vitro* culture (R_0) were uniform without any morphological variation and set normal seed (**Fig. 1K**).

Factors effecting the rate and efficiency of morphogenic induction

The effect of three different factors on *in vitro* morphogenic potential of shoot meristems was studied in genotype the BTx 623. The difference in the induction efficiency between explants derived from small and large seeds was statistically not significant (data not shown). In the experiments involving 7 developmental stages and the two methods of germination followed, the size of the seed proved to be of no importance to *in vitro* induction potential. Seed germination was better on filter paper (90.0%) than on MS medium (82.4%). Induction response of the explants isolated from seeds germinated on filter paper was better in 6 out of the 7 developmental stages tested (**Fig. 3**). Developmental stage of the explants at the time of meristem isolation influenced the induction effi-

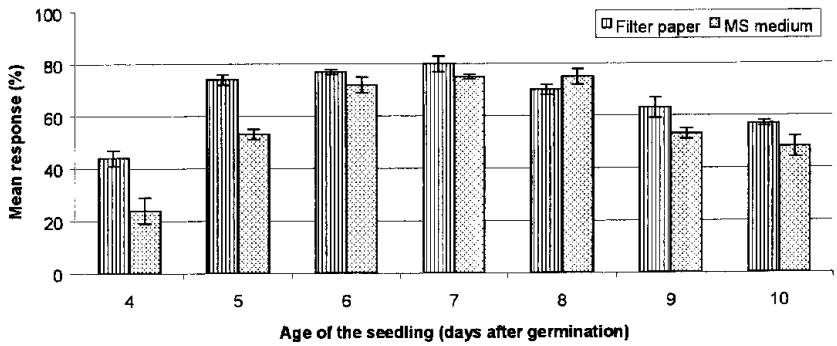

Fig. 3 Effect of germination technique and age of the seedling on induction of multiple shoot buds.

ciency. Percentage of induction increased with age of the explant from day 4 to day 7 (**Fig. 3**) and this response declined steadily when older explants were used for meristem isolation (tested up to tenth day after germination). Shoot tips showed gradual transformation from light yellowish on day 4 to pale green by day 7, which subsequently turned green by day 10. The size of the shoot tips varied from 2 mm to 5 mm during the course of experiment (day 4 to day 10). As color and size of the shoot tips were found to be reasonably good morphological indicators of differentiation stage, on each day shoot tips of average length with uniform color were chosen for meristem isolation. Overall, 5 to 8-day-old explants isolated from seeds germinated on filter paper showed the highest induction response (70-80%).

RAPD analysis of in vitro regenerants

RAPD profiles generated by using random decamers were monomorphic across all the plants tested. The profiles did not reveal any polymorphic bands among *in vitro* regenerated plants or between the *in vitro* regenerated plants and control plant (**Fig. 4**).

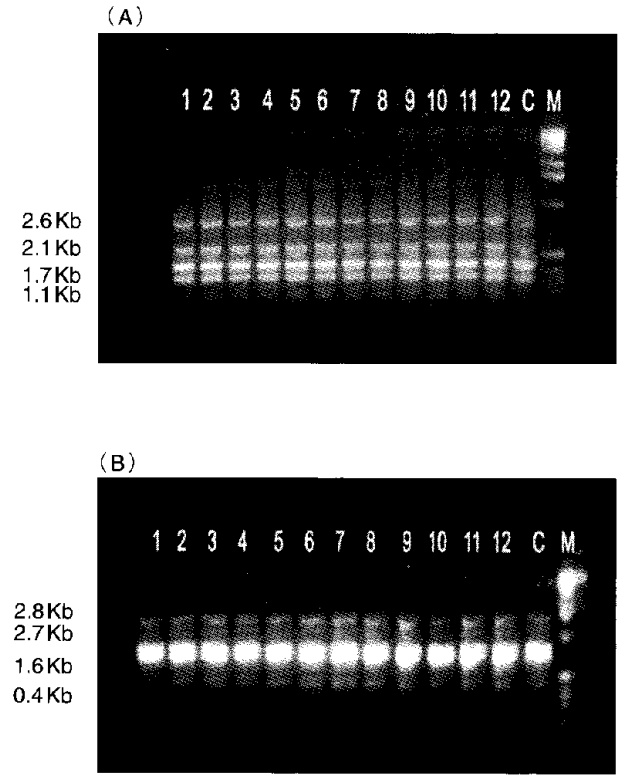

Fig. 4 RAPD analysis of regenerated plants from shoot meristems of sorghum genotype BTx 623, using the random primers from *Operon technologies, USA*. (A) OPA-03 and (B) OPL-14. Lanes 1-12 are the regenerated plants R_0. Lane 13 is the control plant "C", and lane "M" represents 1.0-kb ladder of DNA size marker.

Discussion

We have developed an improved and reproducible plant regeneration system from isolated shoot apices of sorghum. Use of shoot apices from *in vitro* germinated seedlings facilitates the year round availability of explants. The present system could be efficiently used for genetic transformation due to increased frequencies of multiple buds and regenerants from somatic embryo's capable of regeneration in a relatively short period.

By striking optimal balance between a weak auxin such as NAA (to avoid callus formation) and a strong cytokinin such as TDZ, the isolated shoot apices were manipulated to follow either organogenic or embryogenic pathway. Such morphogenic plasticity of the shoot meristem was reported by Zhong *et al.* (1998) by using shoot apices of sorghum seedlings along with a portion of mesocotyl, leaf primordia, leaf sheath, and one or two leaf pairs. However, improvement in the present system is reflected in minimizing such heterogeneity of the explant by further isolation of shoot apices devoid of much of mother tissues from shoot apices. Such trimming also resulted in enhancing the rate of shoot multiplication.

Plant growth regulators controlled the morphogenic competence, pathway, and speed of regeneration from isolated shoot meristems. The importance of auxin to cytokinin ratio in the control of regeneration is well known (Skoog and Miller, 1957). Such a process depends on cell or tissue competence (Christianson and Warnick, 1988). In earlier reports using shoot apex as an explant (El'konin *et al.*, 1984; Bhaskaran and Smith, 1988; Bhasakaran *et al.*, 1988; Lusardi and Lupotto, 1990; Seetharama *et al.*, 2000), embryogenic callus formation preceded the process of somatic embryogenesis. However, in order to minimize the generation of somaclonal variants commonly associated with callus-mediated regeneration, it is desirable to establish direct organogenesis or somatic embryogenesis with no callus formation. Also, a rapid and high frequency regeneration system will enhance the transformation potential. Our system meets the entire above-mentioned criterion. All the three genotypes used in the present study showed uniform regeneration frequencies and thus the system is genotype independent with a marginal difference in induction responses. This is in agreement with the finding of Zhong *et al.* (1998).

During the first 2 weeks of culture, there was a

significant expansion of shoot meristem region. At this stage, the bulged shoot meristem was trimmed and leaf primordia discarded. If the primordial leaves were not retained with the meristem during the first 2 weeks, meristems did not bulge, and there was no multiple bud formation. Earlier it has been demonstrated that if the apical dominance of existing buds is eliminated either physically or by using high cytokinin levels in the culture medium, a large number of shoots could arise *in vitro* (Polisetty *et al.*, 1997). In our study, use of BAP and TDZ (which shows strong cytokinin properties) when combined with the technique of trimming leaf initials during *in vitro* culture, leads to such a response.

Thidiazuron is a substituted phenyl urea developed primarily as a cotton (*Gossypium* sp.) defoliant, which also exhibited strong cytokinin like activity in various cytokinin bioassays (Mok *et al.*, 1982). TDZ also efficiently stimulated cytokinin-dependent shoot regeneration from a various plant species (Malik and Saxena, 1992; Huetteman and Preece, 1993). The precise mechanism of action of TDZ is not known yet. However, two hypotheses are presented (Huetteman and Preece, 1993). First, TDZ could directly promote growth due to its own biological activity in a way similar to that of cytokinins. Second, it might effect accumulation of endogenous cytokinins (by reduction the rate of degradation) or increase the synthesis of endogenous cytokinins.

Our study demonstrated that the addition of TDZ in the induction medium was effective for multiple bud formation from bulged meristems. The multiple bud formation was a branch-point from where it can lead to either organogenic or embryogenic pathway depending on the manipulation of plant growth regulators. Zhong *et al.* (1998) obtained direct somatic embryogenesis from shoot meristems on medium supplemented with BAP and 2,4-D. In our experiments with isolated shoot meristems, use of the above medium was always accompanied with a certain degree of callus formation. In our improved protocol, with isolated shoot meristems, it was observed that a weak auxin such as NAA was more effective; in fact, it was more favoured for signaling somatic embryogenesis, than a strong auxin such as 2,4-D (**Fig. 2**). Moreover, replacement of 2,4-D with NAA induced somatic embryogenesis without any callus formation. Specific combinations of NAA and BAP were found to be highly effective for inducing somatic embryogenesis in previous studies as well (Tetu *et al.*, 1990; Gill and Saxena, 1993).

TDZ altered the shoot regeneration process from organogenesis to embryogenesis in leaf disc cultures of tobacco (*Nicotiana tabacum* L.) (Gill and Saxena, 1993). In the present study, though we did not use TDZ in the somatic embryo induction medium (containing only BAP and NAA), it was used during the initial 4 weeks of culture, up to which a common route for both organogenic and embryogenic pathways was followed. This suggests that probably the carry-over effect of TDZ (present during initial 4 weeks) must have promoted the induction of direct somatic embryogenesis. Similar role of TDZ in somatic embryogenesis has also been reported in woody species such as *Rubus* (Fiola *et al.*, 1990) and *Vitis vinifera* L. (Matsuta and Hirabayashi, 1989).

The age of the seedling at the time of meristem isolation significantly influenced the induction potential of the isolated meristems (**Fig. 3**). When we compared *in vitro* response from explants at seven stages (derived from 4 to 10 day-old seedlings), *in vitro* response showed a steep increase between day 4 and day 5 and the response was maximum with 7-day-old explants, after which it declined (**Fig. 3**). Therefore, 5 to 7-day- old explants are optimum for isolated shoot meristem culture of sorghum. Age-dependent variation in *in vitro* responses was linked to difference in endogenous auxin levels (Cassels *et al.*, 1982) or endogenous cytokinin levels (Josphina *et al.*, 1990). It was reported that genetic and environmental factors alter the levels of endogenous hormones, which determine the *in vitro* responses (Narasimhulu and Chopra, 1988).

In this study, the uniformity of the field grown and *in vitro* derived plants and the resemblance of their RAPD profiles (with no polymorphism) with the control plants (in the field) indicates that there is no somaclonal variation in these regenerants (**Fig. 4**). Similar results on absence of DNA variation of plants regenerated from callus, cell and protoplast cultures in poplar (*Populus sps.*) (Qiao *et al.*, 1998), wheat (Triticum aestivium L.) (Chawdhury *et al.*, 1994) and rice (Oriza sativa L.) (Saleh *et al.*, 1990) were reported earlier.

The uniformity of the explants, rapidity of regeneration, and minimized or no chances of formation of somaclonal variants, makes the system described in this paper useful for both developmental studies involving shoot meristem and genetic transformation.

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