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BRIEF COMMUNICATION

Efficient plant regeneration from shoot apices of sorghum

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Abstract

An efficient and rapid regeneration protocol was developed using shoot apices from germinating seedlings of two cultivars of sorghum, SPV-462 and M35-1, as explants. A vertical slit given from the base of each dissected apex enhanced the efficiency of callusing response by two fold. MS medium containing 0.5 mg dm^{-3} each of 2,4-D and kinetin was most effective in producing friable and embryogenic calli. Scanning electron microscopy of these calli detected somatic embryogenesis. Calli thus induced gave rise to approximately 42 green shoots per callus in both the genotypes when transferred to regeneration medium containing 1.5 mg dm^{-3} kinetin.

Additional key words: 2,4-dichlorophenoxyacetic acid, kinetin, plant growth regulators, *Sorghum bicolor* (L.) Moench.

Until recently, improvement of sorghum for agronomic and quality traits such as tolerance to biotic and abiotic stresses and grain protein quality was carried out largely by traditional plant breeding methods. The application of biotechnology for the genetic improvement of this crop has been lagging behind that of other cereal crops. Sorghum has been categorized as one of the more difficult plant species to manipulate for tissue culture and regeneration (Zhu *et al.* 1998, Maqbool *et al.* 2001). The major bottlenecks include *1*) genotype dependent response, *2*) very low regeneration, *3*) production of phenolics and *4*) problems in acclimatization. Nevertheless, there are reports of successful regeneration of sorghum from certain explants. In general, immature inflorescence (Brettell *et al.* 1980, Boyes and Vasil 1984, Murty *et al.* 1990, Cai and Butler 1990, Eapen and George 1990) and immature embryo (Ma *et al.* 1987, Oldach *et al.* 2001) are considered to be excellent explants. However, production of these explants is a space, time and labour consuming task. Explants such as mature embryos from seed (Botti and Vasil 1983, MacKinnon *et al.* 1987, Zapata *et al.* 2004) shoot tips or meristems (Bhaskaran *et al.* 1992, Zhong *et al.* 1998) are definitely more convenient. In this paper, we report a highly efficient, rapid and season independent protocol for plant regeneration from shoot apices of sorghum.

Mature seeds of *Sorghum bicolor* (L.) Moench cv. SPV-462 and M35-1 were used for generating the shoot apex explants. SPV-462 is a popular sorghum cultivar grown predominantly during the rainy season (June - October) whereas, M35-1 is a land race grown quite extensively during the post rainy season (November - March). Seeds were washed with *Tween-20*, rinsed 2 - 3 times with distilled water and then surface sterilized with 0.1 % $HgCl_2$ for 10 min. Subsequently, seeds were washed 5 - 6 times with sterile distilled water and were aseptically germinated on wet cotton in culture bottle (50 seeds per bottle) in dark at 27 °C. After 48 h of germination, 3 mm sections of shoot apices were carefully dissected from the germinating seedlings. A vertical slit was given from the base of each dissected apex for enhancing the efficiency of callus induction. The sections were then cultured on Murashige and Skoog (MS) basal medium supplemented with various concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D; 0.25, 0.5, 0.75 and 1.0 mg dm^{-3}) and kinetin (6-furfuryl-aminopurine; 0.25, 0.5, 1.0 and 1.5 mg dm^{-3}) and solidified with 0.8 % agar. Ten explants were horizontally buried in the medium in each Petri dish. A total of 600 explants were tested for each treatment. Callus

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initiation was observed typically, 10 d after inoculation. Transfers were carried out at every 13 d interval and at the time of each transfer, leaves, coleoptiles and elongated shoots were removed from the explant so as to expose the shoot apices.

Thirty-five days after inoculation, each of the cultures was transferred to regeneration medium, which contained different concentrations of kinetin (0.0, 1.0, 1.5 and 2.0 mg dm^{-3}). The cultures were then shifted to light (85 μmol m^{-2} s^{-1}) and maintained under a 12-h photoperiod. The relative efficiency of multiple shoot regeneration was evaluated by visually scoring the number of shoots 15 d after sub-culturing into regeneration medium. Individual shoots were then separated and placed on MS half-strength medium with 1.0 mg dm^{-3} naphthalene acetic acid (NAA). Subsequently, plantlets were transferred to a substrate comprising of 1:1 peat and *Soilrite* for further development in greenhouse. Each treatment had 3 replications and was repeated twice.

For histological studies under light microscope, samples were collected from 35-d-old cultures growing on MS medium supplemented with 2,4-D and kinetin and processed as per standard procedures using hematoxylene and eosine staining and viewed under an inverted transmitted light microscope (*Leitz Labovert FS*, Wetzlar, Germany). For scanning electron microscopy, the samples were fixed in 3 % glutaraldehyde and post fixation was done using 2 % aqueous osmium tetroxide according to Dunstan *et al.* (1978). The samples were coated using platinum sputtering (*JEOL JFC 1600*) followed by dehydration and drying and observed under scanning electron microscope (model *JEOL-JSM 5600*, Tokyo, Japan).

The leaves and mesocotyls of shoot apices started to elongate rapidly after inoculation on media containing 2,4-D and kinetin. Continuous removal of the leaves and mesocotyls from shoot apices led eventually to the retardation of growth of these within 15 d. The vertical slit given on shoot apex at the time of inoculation resulted in enhancing the callusing response by two fold (Fig. 1*A*, Table 1). At lower concentrations of 2,4-D (0.25 and 0.5 mg dm^{-3}) in combination with kinetin, callus induction was slower and shoot growth was profuse, whereas, callus induction and its growth were better under relatively higher concentrations (0.75 and 1.0 mg dm^{-3}) of 2,4-D (Table 1). Nevertheless, the potential subsequent regenerability of the calli induced at higher 2,4-D concentrations was relatively lower. The shoot growth, callusing and ability to regeneration of the calli were also dependent on the concentration of kinetin in the medium. Kinetin concentration of 0.25 mg dm^{-3} in the callus induction medium resulted in profuse rooting, while kinetin above 0.5 mg dm^{-3} induced more shoots (without roots) instead of callus initiation. Calli growing on MS medium containing 0.5 mg dm^{-3} each of 2,4-D and kinetin gave about 42 shoots per callus, whereas, other combinations of 2,4-D and kinetin resulted in 2-fold lower shoot number (Fig. 1*D*, Table 2). Hence the combination of 2,4-D and kinetin at 0.5/0.5 mg dm^{-3} was found optimum for callus induction.

Table 1. The effect of 2,4-D and kinetin on efficiency of callusing response in two genotypes of sorghum five weeks after inoculation of shoot apices. Callus diameter in cm: A - 0.2 and below, cream to green; B - 0.2 to 0.5, cream to green; C - 0.2 to 0.5, white to green; D - 0.5 to 1.0, cream to green; E - 0.5 to 1.0, white to green; F - 0.5 to 1.0, white puffy; S - no callusing, development of adventitious shoots. Vertical incision from the base of each dissected shoot apex resulted in values ≥ 100 %. Every treatment had 20 plates each containing 10 calli. The values are means of results from 3 individual experiments.

2,4-D + kin [mg dm^{-3}]	SPV462 size	frequency [%]	M35-1 size	frequency [%]
0.10+0.50	S	--	S	--
0.10+1.00	S	--	S	--
0.25+0.25	A	145 ± 2.9	A	145 ±5.8
0.25+0.50	A	135 ± 2.9	A	135 ± 2.9
0.25+1.00	A	130 ± 5.8	A	130 ± 2.9
0.25+1.50	A	130 ± 5.8	A	130 ± 7.6
0.50+0.25	B	140 ± 5.8	B	140 ± 5.0
0.50+0.50	C	127 ± 3.3	C	125 ± 2.9
0.50+1.00	C	110 ± 2.9	C	110 ± 1.7
0.50+1.50	B	125 ± 7.6	B	130 ± 4.6
0.75+0.25	C	145 ± 5.0	C	145 ± 2.9
0.75+0.50	E	155 ± 2.9	E	155 ± 2.9
0.75+1.00	E	130 ± 7.6	E	135 ± 5.0
0.75+1.50	E	130 ± 1.2	E	130 ± 2.3
1.00+0.25	F	100 ± 5.0	F	100 ± 4.0
1.00+0.50	F	125 ± 2.9	F	125 ± 5.0
1.00+1.00	E	140 ± 5.8	E	140 ± 2.9
1.00+1.50	E	120 ± 2.9	E	115 ± 5.0

The kinetin concentration in the regeneration medium controlled the frequency and efficiency of regeneration. The number of shoots produced per callus varied under different kinetin concentrations (Fig. 1*E*,*F*, Table 2) with the highest number of shoots at 1.5 mg dm^{-3}. Scanning electron microscopy of 20-d old calli revealed shoot-apical domes indicative of somatic embryogenesis (Fig. 1*J*,*K*) Light microscopic observation of 35-d-old calli growing on regeneration medium clearly revealed multiple shoot apex (Fig. 1*L*).

On the whole, shoot apices cultured on 0.5/0.5 mg dm^{-3} 2,4-D and kinetin produced friable and embryogenic calli (Fig. 1*B*,*C*). The callus formation and efficiency were similar in both the genotypes tested. After two subcultures, calli transferred on to regeneration medium containing 1.5 mg dm^{-3} kinetin created approximately 40 green shoots per each callus. Finally, plantlets developed at the end of 50-d culture. NAA 1 mg dm^{-3} was found to be optimal for efficient rooting (Fig. 1*G*) which was achieved within 2 weeks. Over 90 % of rooted plantlets grew well and were fertile after transfer to a net house. The developmental morphology of these plants was similar to that of seed derived plants in the net house and they flowered normally after 70 d and produced

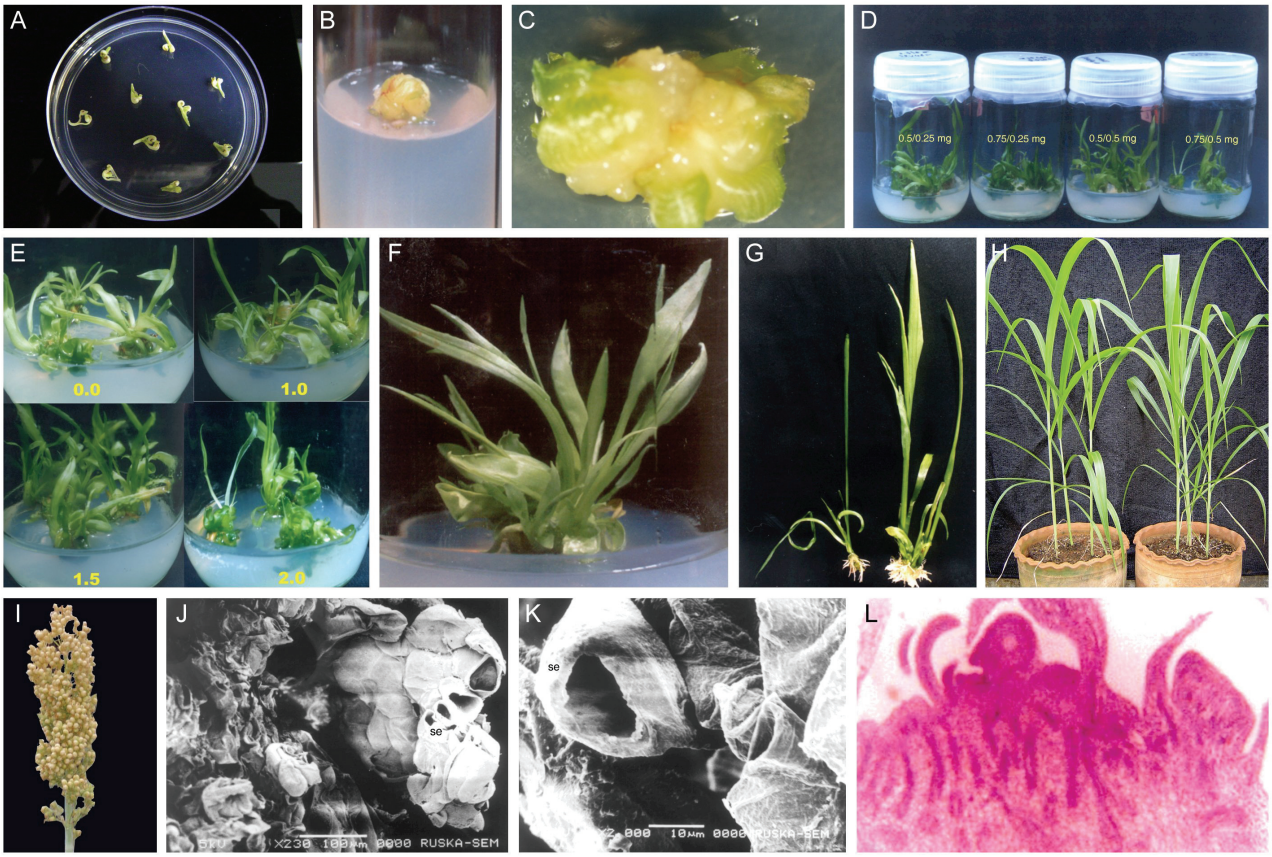

Fig. 1. *A* - Explants on the callus induction medium showing callusing from either side of the vertical slit given from the base of each dissected shoot apex; *B* - 25-d-old calli on induction medium; *C* - Somatic embryos differentiating to shoots after transfer on to regeneration medium; *D* - Plate depicting the effect of various concentrations of 2,4-D and kinetin in the callus induction medium on subsequent regeneration efficiency of sorghum shoot apical explants; *E* - Plate depicting the effect of various concentrations of kinetin in regeneration medium on efficiency of regeneration of sorghum shoot apical explants; *F* - Regeneration of plantlets; *G* - Rooting; *H* - Regenerated plants growing in pots; *I* - Panicle of a regenerated pot-grown plant; *J* - Scanning electron micrograph of differentiation of shoot apical domes from 20-d-old calli cultured on MS medium comprising 0.5 mg dm^{-3} each of 2,4-D and kinetin and regenerating somatic embryos, *bar* = 100 μm; *K* - Enlarged view of a somatic embryo, *bar* = 10 μm, se - somatic embryo; *L* - Multiple shoot apex viewed under light microscope in regenerating 35-d-old calli.

Table 2. The effect of various concentrations of plant growth regulators in the callus induction and regeneration media on regeneration efficiency of sorghum shoots. * - Observations on shoot development were recorded on subsequent transfer of calli to regeneration medium containing 1.5 mg dm^{-3} kinetin. ** - The calli were cultured on the callus induction media containing 0.5 mg dm^{-3} each of 2,4-D and kinetin prior to transfer on to regeneration media.

	Induction medium* 2,4-D+kinetin [mg dm^{-3}]				Regeneration medium** kinetin [mg dm^{-3}]			
	0.50+0.25	0.75+0.25	0.50+0.50	0.75+0.50	0.00	1.00	1.50	2.00
Number of shoots [$callus^{-1}$]	18 ± 0.60	13 ± 0.33	42 ± 0.58	19 ± 0.33	4 ± 0.00	8 ± 0.40	42 ± 0.58	10 ± 0.30

mature viable seeds (Fig. 1*H,I*).

The morphogenetic response of the shoot apices depended on the concentration of 2,4-D and kinetin in our experiments. Our results revealed that a combination of 2,4-D and kinetin in lower concentrations (0.5/0.5 mg dm^{-3}) led to apical domes and ridges indicative of somatic embryogenesis. Morphogenetic plasticity of the shoot apices was earlier reported in maize, oats and sorghum (Zhong *et al.* 1992, 1998, Zhang *et al.* 1996). 2,4-D in combination with BAP had also been reported to induce *in vitro* morphogenesis in rice (Nhut *et al.* 2000), maize (Zhong *et al.* 1992) and sorghum (Zhong *et al.* 1998). The significant enhancement of callus growth by 2,4-D could be due to the delay or prevention of the cytokinin-induced development of shoots and roots. However, calli growing on concentrations greater than 0.5 mg dm^{-3} of

2,4-D were found to be relatively white, puffy with less greening and less regenerable upon transfer to regeneration medium. Higher 2,4-D has been reported to reduce chlorophyll content (Bhaskaran *et al.* 1992), resulting in a loss of shoot forming potential in sorghum. Also in barley, 2,4-D in the induction medium was less effective than dicamba in somatic embryogenesis and regeneration efficiency (Halámková *et al.* 2004).

The concentration of kinetin in the regeneration medium controlled the regeneration efficiency. Low (0.5 mg dm^{-3}) during the callus induction phase and a high (1.5 mg dm^{-3}) for regeneration clearly improved the efficiency. The optimal combination of 2,4-D and kinetin for callus induction and for efficient regeneration is probably a reflection of endogenous contents of these phytohormones.

Browning of the callus and root initiation were the two main factors which hindered callus growth and eventually led to death of the tissue. The release of dark brown phenolic pigments into the medium, observed in most of the media combinations inhibited callus growth and proliferation of the shoot clumps resulting in complete necrosis of the cultures. This accumulation of phenolics was predominant in light, which could be partly overcome by maintaining the cultures in dark until 3 subcultures and shifting to light (85 µmol m^{-2} s^{-1}) with a 12-h photoperiod only while culturing on to regeneration media. Maintaining the cultures in the dark was also absolutely essential for retardation of shoot growth in the callus induction phase in the present study. Culture under darkness was found to stimulate somatic embryogenesis than light in the presence of 2,4-D (Nhut *et al*. 2000).

In conclusion, the results of this study have clearly demonstrated establishment of an efficient and season independent protocol for plant regeneration *via* induction of embryogenic callus in sorghum. Our regeneration system is also relatively rapid as the regenerated and rooted plantlets can be obtained within 65 - 67 d. The choice of shoot apex explants has the following advantages: *1*) several hundred explants could be used at any given time without limitation of time and space, *2*) it is season independent unlike the other explants such as immature inflorescence or immature embryos. Further, the tissue culture and regeneration system described above was almost equally efficient in two diverse genotypes SPV-462 and M35-1. This kind of a robust regeneration system is critical for efficient genetic transformation of sorghum.

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