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RESEARCH ARTICLE

SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM SORGHUM MERISTEM EXPLANTS WITHOUT INTERVENING CALLUS PHASE

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ABSTRACT

Sorghum bicolor (L. Moench.) is considered as one of the crop species highly recalcitrant to tissue culture and genetic manipulation studies. During the last two decades several studies were done on sorghum somatic embryogenesis (SE) and plant regeneration, but the rate of plant regeneration was not sufficiently high and practical difficulties still exist in establishing and regenerating plants in a relatively shorter time period. The approach to regenerate the plantlets from somatic cells via intervening callus phase is time consuming and laborious which takes about 15-20 weeks. Hence an improved protocol using direct somatic embryogenic route of regeneration from shoot apices explants was developed. The shoot tips were cultured in the presence of Benzylaminopurine (BAP) and 1-Napthaleneacetic acid (NAA) at varying concentrations in Murashigeand Skoog (MS) media to determine the optimal media for SE as indicated by bulging of explants in the meristematic nodal region. The highest frequency of bulging (80%) due to SE was obtained on MS medium supplemented with 5.0 mgl^{-1} of BAP and $1mgl^{-1}$NAA.Further,plant regeneration could be directly induced from the bulged regions when supplemented half-strength MS media with Indole Butyric acid (IBA) and Indole acetic acid (IAA). Nearly 90% plant regeneration was achieved by using $2mgl^{-1}$of IBA and 0.5 mgl^{-1} of IAA. The well rooted plants are subjected to hardening and acclimatization in the glasshouse for 4 weeks. This rapid and efficient sorghum plant regenerative protocol described in this study is more useful for various genetic transformation studies.

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INTRODUCTION

Sorghum [*Sorghum bicolor* (L.). Moench], an African grass related to sugar cane and maize, is grown for food, feed, fiber and fuel, and ranks fifth among the cereals in global food production. It is a self-pollinated diploid (2n=2x=20) C4 grass with its smaller genome size (730 Mbp) is fully sequenced and makes sorghum an attractive model for functional genomics of C4 grasses (Paterson *et al.,* 2009). Although conventional breeding approaches have greatly augmented sorghum yields, transgenic technology which follows Somatic Embryogenesis (SE) pathway offers advantages of a more directed approach to introduce target specific and *de novo* traits in a single generation (Madhu *et al.,* 2015).Somatic Embryogenesis is an *invitro* a sexual reproduction process in which somatic cells give raise to somatic embryo sunder favorable experimental conditions. Since most somatic cells are not naturally embryogenic, an induction phase is required for the cells to acquire embryogenic competence (Dodeman *et al.,* 1997), which is provided by the optimal combinations of plant growth regulators (PGR). Generally sorghum tissues or explant shows highly recalcitrance nature towards any tissue culture and genetic manipulation studies (Zhu *et al.,* 1998; Grootboom *et al.,* 2010), so for the successful generation of transgenic sorghum is mainly dependent on the availability of an efficient tissue culture and regeneration protocol (Girijashankar *et al.,* 2005). Sorghum tissue culture and regeneration protocols mostly follow either organogenesis (Maheswari *et al.,* 2006) or direct somatic embryogenesis pathway (Harshavardhan *et al.,* 2002). The regeneration of sorghum has been achieved from a variety of explants like immature embryos (Gupta *et al.,* 2006; Jogeswar *et al.,* 2007; Dora *et al.,* 2014), Shoot apices (Bhaskaran and Smith 1989; Maheswari *et al.,* 2006; Saikishore *et al.,* 2006; Sharma *et al.,* 2007; Polumahanthi *et al.,* 2014) and from mature embryos of sorghum (McKinnon *et al.,* 1986). However, the rate of plant regeneration is not sufficiently high in sorghum and practical difficulties still exist in establishing and propagating regenerable cultures for long periods (Jogeswar *et al.,* 2007).

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The process of developing a complete plant from a single somatic cell is mainly depends upon several factors, such as species, type of explants, genotype and composition of culture media used for regeneration (type, ratio and concentration of plant hormones).Indirect SE pathway results in faster and efficient for plant regeneration compared to the time taken to regenerate the plantlets via intervening callus phase which is a laborious process and completes in 15-20 weeks (Synman *et al.,* 2000). The present study aimed at developing a rapid and reproducible protocol for plant regeneration from somatic embryos without intervening callus phase using shoot apex explants of sorghum.

MATERIALS AND METHODS

Plant Material

For this study, shoot apices containing meristematic region were used as main explant material and obtained from aseptically germinated seedlings (Fig.1A) of sorghum 296B Genotype. To surface sterilize, the seeds were rinsed in 100 ml of water supplemented with 2-3 drops of Tween-20 (Sigma) for 15 minutes followed by 3-5 washes using sterilized water. Then the seeds were treated with 70% ethanol for 1 min and finally sterilized by rinsing in 0.1% mercuric chloride (Sigma) for 6-8 min with continuous stirring. Subsequently the seeds were thoroughly rinsed with sterile water and placed on Petri dishes containing pre-moistured filter papers and were allowed to germinate in dark at 28°C in a growth chamberfor 4-5 days.

Culture Medium and conditions

In all the tissue culture experiments on direct somatic embryo induction, shoot apiceswere cultured on MS (Murashige and Skoog, 1962) basal medium containing 30mg l^{-1}sucrosewas used. The pH of the medium was adjusted to 5.8 with NaOH (1N) / HCl (0.1N) prior to addition of 0.8% agar and was autoclaved at 15 psi pressure for 20 min.

Two step culture for induction of somatic embryos and plant regeneration

In the first step of direct SE, shoot apices containing meristem along with small portions of coleoptile and stem was excised aseptically from a 5-day old seedling (Fig.1B). Such isolated shoot apex was cultured on the SE induction medium supplemented with various concentrations of BAP and NAA growth hormones for 4 weeks, with one subculture in the same medium after 2 weeks (Table 1).

In the second step of direct SE, the proliferated shoot forming explants were transferred to half strength MS medium for the initiation of roots which is supplemented with various concentrations of IBA and IAA growth hormones for 3 weeks (Table 2).

All the cultures were maintained at 25±2°C under the 16 h light and 8 h dark cycle with 60% relative humidity. Plant regeneration with good root formation was observed after 4-6 weeks in the regeneration medium, with 1-2 subcultures. Healthy plants were carefully removed from media and washed with tap water to remove the traces of adhered media with agar, then transferred into pots containing the mixture of sterilized soil and sand (3:1) and kept for a week in tissue culture room. These pre-hardened plants were finally transferred to greenhouse and grown in pots till maturity.

RESULTS AND DISCUSSION

In this study the feasibility of replacing intervening callus phase by direct somatic embryo regeneration into plantlet from shoot apices as an explant material was assessed. Cell suspension and protoplasts (Battrawand Hall, 1991), callus from immature embryos (Zhu et al.1998; Zhao *et al.,* 2000) and shoot meristem explants from germinated seeds (Gray *et al.,* 2004; Girijashankar *et al.,* 2005) were commonly used, of which the latter two have resulted in highertransformation efficiency. Of these two, shoot meristem explants are easy to generate and are season-independent, ensuring yearround supply of explants (Harshavardhan *et al.,* 2002).

To fulfill this aim shoot apices comprising a part of leaf primordial, shoot region, coleoptiles were excised from 5-7 day old seedlings and placed in MS induction medium supplemented with three different combinations and concentrations of hormones BAP and NAA for 3 weeks (Table 1). Between first 24-48 hours the size of the meristematic tissue increases with emerging leaf outgrowth is observed. About 80% bulging is achieved when shoot apices were treated with 5 mg l^{-1}BAP and 1 mg l^{-1}NAA (Table 1, IM2 media). The elongated primary leaves were excised out to avoid suppression of somatic embryo development. The induction and proliferation of shoot meristem as a bulged structure of shoot meristem (Fig.1C) were clearly observed indicating formation of somatic embryos at the end of this step (4 weeks of culture). The occurrence of SE at that stage has been documented in sorghum (Harshavardhan *et al.,* 2002; Aparna *et al.,* 2004) and pearl millet (Devi *et al.,* 2000).

Table 1. Effect of BAP and NAA on sorghum shoot apices bulging

Media	BAP (mg l^{-1})	NAA (mg l^{-1})	Proportion of explants showing bulging (%)
IM1	4	0.5	49.5
IM2	5	1	80.0
IM3	5	0.5	46.7

Table 2. Effect of IBA and IAA in inducing regeneration from sorghum somatic embryos

Media	IBA (mg l^{-1})	IAA (mg l^{-1})	Percentage explants showing regeneration (%)
RM1	1.2	0.19	61.3
RM2	2	0.5	90.7
RM3	4	0.5	49.7

The second step was to optimize the process of direct plant regeneration from the bulged portions of shoot apices tissue which already having 1-2 leaf primordia after 4 weeks of first phase of culture. The main purpose of this step was to induce shoot elongation and root induction by placing the explants in half strength MS medium for 3 weeks supplemented with three different combinations of hormones IBA and IAA (Table 2). Among the combinations tested, 90.7% rooting is achieved from regenerated plants when supplemented with IBA and IAA at a concentration of 2 mg l^{-1} and 0.5 mg l^{-1} respectively (RM2 media, Fig.1D). The optimal levels of PGR combinations and concentrations broadly agree with Harshavardhan *et al.* (2002) for their ratio, but different growth regulators were used. Moreover they outlined more elaborate steps for plant regeneration subsequent to SE, which have been cut down to save time in the present study. One possible limitation of the protocol developed in this study may be the reduced number of plants developing from each explant. However, as generating a large number of explants is not a limitation owing to excellent seed multiplication ratio in sorghum, this bottleneck is easily overcome using a large number of explants.

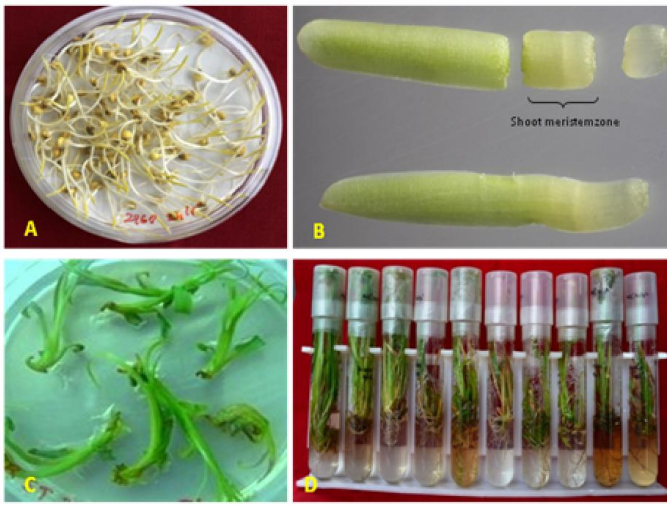

Fig. 1. Somatic embryogenesis and plant regeneration from sorghum shoot meristem explants

A. Aseptically germinated 4 days seedling were used to source the explants
B. Explant with shoot meristem zone shown
C. Explants after two weeks of culture showing bulged regions with somatic embryos. Primary leaves were excised out to allow shoot regeneration from somatic embryos
D. Rooted plants obtained by direct regeneration from somatic embryos

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