Effect of Kanamycin on Explant Selection in Genetic Transformation Experiments of Sorghum
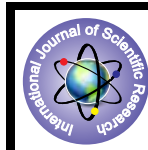
Biotechnology

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ABSTRACT

Efficient genetic transformation of sorghum plant requires an effective selection marker system thatprecisely differentiates transformed cells from non-transformed ones. Hence the present study was undertaken to optimize the selection marker antibiotic kanamycin to screen transformed cells in sorghum transformation experiments with vectors harbouring nptII gene. Shoot meristem explants of sorghum were grown in somatic embryo induction media containing various concentrations of kanamycin. At lower concentrations of kanamycin (20mg/L and less), the growth of shoot apices was unaffected and the survival rates were very high (87.5–100%) during the three sub-culture passages. At a kanamycin concentration of 50 mg/L the percentage of survival decreased gradually with three subcultures - 62.5, 25 and 0% survival respectively, while at concentrations of 200 and 800 mg/L, these were higher mortalities of explants in the first subculture itself (only 31.3-37.5% survival). The least concentration of kanamycin at which all susceptible explants died was 50 mg/L (after three subcultures). Therefore it is suggested that 50 mg/L of kanamycin may be used as the optimum concentration for the selection of sorghum shoot meristem explants transformed with vectors harbouring the nptII gene.

1. Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is an African grass related to sugar cane and maize, is grown for food, feed, fiber and fuel. Globally as a major cereal it occupies fourth place in area after wheat, rice and corn and is the dietary staple of more than 500 million people in 30 countries. Although conventional breeding approaches have greatly augmented sorghum yields, transgenic technology offers advantages of a more directed approach to introduce target specific and *de novo* traits in a single generation.

Sorghum is categorized as one of the highly recalcitrant plant species for the tissue culture and genetic transformation studies (Zhu *et al.*, 1998; Grootboom et al. 2010). The first report of genetic transformation of sorghum by Battraw and Hall (1991) deployed introduction of DNA into protoplasts by electroporation. *Agrobacterium*-mediated genetic transformation in sorghum was first reported by Zhao *et al.*, (2000). For generating successful transgenic sorghum, it requires an efficient identification or selection system (selectable marker genes) which clearly differentiates transformed cells from non-transformed ones. So far three selection systems were employed, which includes selection on the antibiotics like kanamycin (*nptII*), Hygromycin (*hpt*), and herbicide phosphinothricin (Bar). The kanamycin system is commonly found in transformation vectors which are used for selection of *Agrobacterium* cultures harbouring transgene vector. In this present study our main objective was to determine the effects of the antibiotic kanamycin on initiation, proliferation and development of somatic embryos in sorghum as one of the best selection and screening agent for transformed cells in sorghum transformation experiments with vectors harbouring *nptII* gene.

2. Materials and methods
Plant material

Shoot apices were obtained from aseptically germinated seedlings of sorghum 296B Genotype were collected. To surface sterilize, the seeds were rinsed in 100 ml of water supplemented with 2 drops of Tween-20 (Sigma) for 15 minutes followed by 4-5 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 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 for 4-5 days.

Kanamycin solution

The stock solution of Kanamycin (50 mg/ml) was made by dissolving Kanamycin sulphate monohydrate (Duchefa) and was filter sterilized. It was added to the autoclaved and cooled MS media at 40-50°C at appropriate concentrations.

Induction and maintenance of somatic embryos

Shoot apices (2-4mm length) were excised from the hypocotyls of 5-7 day-old germinated seedlings and placed on a medium containing 4 mg/L Benzyl adenine, 0.5 mg/L 2,4-D and 0.5 µg/L thidiazuron, to induce somatic embryogenesis. The petri dishes were sealed with parafilm and incubated in light for 16 h and 8 h dark per day at 26 C and 45% relative humidity. Subsequently, the explants were sub-cultured on to a fresh media every week, up to 3 weeks.

Screening somatic embryos for tolerance to kanamycin

After induction of somatic embryos, the explants were placed on MS media supplemented with 4 mg/L Benzyl adenine,0.5 mg/L 2, 4-D, and 0.5 µg/L thidiazuron with various concentrations of kanamycin (0, 3, 7.5, 20, 50, 200 and 800 mg/L). A completely randomized design was used for these experiments. Each experiment was repeated four times using 16 explants for each treatment, for a period of 3 weeks followed by sub-culturing each plate every week in to fresh media.

3. Results

Various explants have been used in sorghum for successful development of transgenics. Cell suspension and protoplasts (Battraw and 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 higher transformation efficiency. Of these two, shoot meristem explants are easy to generate and are season-independent, ensuring year-round supply of explants. Therefore, in this study it was attempted to optimize the kanamycin based selection of shoot meristem explants from germinated seeds to assist sorghum transformation.

Effect of Kanamycin on shoot meristem explants

Incubation of shoot apices containing meristematic regions on kanamycin containing media resulted in aborted growth of shoot apices and bleaching (tissues lacking chlorophyll) to various degrees (Table 1; Fig. 1). At lesser concentrations of kanamycin (3mg, 7.5mg and 20 mg/L), the growth of shoot apices was unaffected and the survival rates were very high (87.5 – 100%) during the three sub-cultures (in the time period of 21 days) respectively. Explants at these concentrations performed similar to control plants (without kanamycin) giving raise to bulged somatic embryos and chlorophyll pigment was unaffected indicating that these three concentrations (3mg, 7.5mg, and 20mg/L) were not ideal for selecting sorghum transgenics, as they are chances of selecting non-transformed cells along with transformed ones.

At higher concentrations of kanamycin (50, 200 and 800 mg/L), the survival rate greatly varied along the three subcultures. At a kanamycin concentration of 50 mg/L the percentage of survival decreased gradually with three subcultures - 62.5, 25 and 0% survival respectively, while at concentrations of 200 mg/L these were 37.5, 12.5 and 0%. Evidently at the highest concentration of 800 mg/L, survival rate drastically reduced with 31.3, 6.3 and 0% after subsequent subcultures. The explants were turned into achlorophyllus and growth was aborted and finally because of tissue necrosis explants survival rate is diminished, as it is one the desirable phenomenon for selecting transformed tissues (having resistance gene for kanamycin, *nptII*) from non-transformed ones in any kill curve experiments. The least concentration of kanamycin at which all susceptible explants died was 50 mg/L (after three subcultures) which may be useful for precise screening of resistant explants vis-à-vis susceptible (non-transformed) ones in transformation experiments.

4. Discussion

The main objective of this study was to evaluate potential of kanamycin as a selectable marker were the transgenic plants carrying *nptII* gene. The amino glycoside kanamycin, acting as a selective agent, has been commonly used in plant genetic engineering (Bao-Hong Zhang *et al.*, 2001).The main mode of action or effect of this particular antibiotic is by inhibiting the growth of plant cells by binding to the 30S ribosomal subunit, thereby inhibiting initiation of plastid translation (Moazed D *et al.*, 1987) and inhibiting ribosomal protein synthesis (Kohanski *et al.*, 2010).
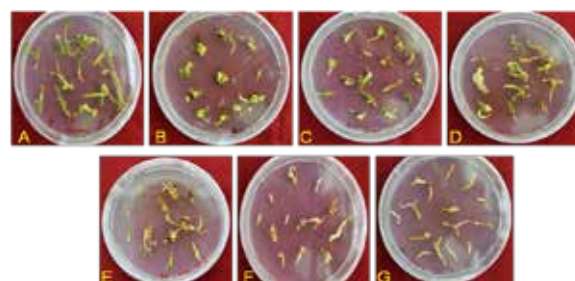
Several transformation vectors come with *nptII* gene and optimization of kanamycin based selection in sorghum would be useful to readily use these vectors without subcloning. As many monocots such as cereals and grasses possess endogenous resistance to kanamycin (Hauptmann *et al.*, 1988), high concentrations are required to be effective. Hence a wide range of concentrations of kanamycin were tried.

Of the series of concentrations tried in our experiment, 50mg/L was the least one at which non-resistant shoot meristem explants (do not contain kanamycin resistance conferring *nptII* gene) completely died after 3 cycles of sub-culturing. It may be safely assumed that at this concentration the resistant explants would have survived in large numbers, similar to control explants in media without kanamycin. In *bar* gene based selection systems in sorghum transformation (e.g., see Zhu *et al.*, 1998; Zhao *et al.*, 2000), the concentration of selection agent (with bar gene) used were - 8-10 mg/l of glufosinate or 2-3 mg/l of bialophos or 2-5 mg/l phosphinothricin. Higher concentration of kanamycin has been necessitated by the high endogenous resistance of sorghum tissue to kanamycin (Hauptmann *et al.*, 1988).

Therefore it can be concluded that, a 50 mg/L of kanamycin is the optimum and reliable concentration for *in vitro* selection of sorghum transgenic shoot meristem explants carrying *nptII* gene after transformation. This concentration of kanamycin will be useful for screening transformed sorghum explants and eliminate the non-transformed explants with precision, thus reducing the need to characterize a large number of putative transgenic plants after regeneration. This would also increase the efficacy of transgenic plant development in sorghum.

Table 1: Effect of kanamycin on survival of shoot apex explants

Conc. of kanamycin used in mg/L	No. of explants incubated	% explants surviving after first subculture	% explants surviving after second subculture	% explants surviving after third subculture
0	16	100.0	100.0	100.0
3	16	100.0	100.0	100.0
7.5	16	93.8	93.8	93.8
20	16	87.5	87.5	87.5
50	16	62.5	25.0	0.0
200	16	37.5	12.5	0.0
800	16	31.3	6.3	0.0

Fig. 1: Effect of Kanamycin on sorghum shoot apices.

A. Control – without kanamycin, Kanamycin concentration (mg/L) - B. 3, C.7.5, D. 20,   E. 50, F. 200, and G. 800.

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