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RESEARCH ARTICLE

EFFECT OF HYGROMYCIN ON EXPLANT SELECTION IN GENETIC TRANSFORMATION EXPERIMENTS OF SORGHUM (*SORGHUM BICOLOR* (L.) MOENCH)

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ABSTRACT

Efficient genetic transformation of sorghum plant requires an effective selection marker system that precisely differentiates transformed cells from non-transformed ones. Hence the present Hygromycin study was undertaken to optimize the selection marker antibiotic Hygromycin to screen transformed cells in sorghum transformation experiments with vectors harbouring *hpt* gene. Shoot meristem explants of sorghum were grown in somatic embryo induction media containing various concentrations of Hygromycin. At lower concentrations of Hygromycin (0.2 mg/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 Hygromycin concentration of 0.5 mg/L the percentage of survival decreased gradually with three subcultures - 62.5, 25 and 0% survival respectively, while at concentrations of 1.0 and 2.0 mg/L, these were higher mortalities of explants in the first subculture itself (only 31.3-37.5% survival). The least concentration of Hygromycin at which all susceptible explants died was 0.5 mg/L (after three subcultures). Therefore it is suggested that 0.5 mg/L of Hygromycin may be used as the optimum concentration for the selection of sorghum shoot meristem explants transformed with vectors harboring the *hpt* gene.

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INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Monech) is a graminaceous crop 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 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 Hygromycin (*hpt*), kanamycin (*nptII*) and herbicide phosphinothricin (Bar). The Hygromycin system is commonly found in transformation vectors which are used for selection of *Agrobacterium* cultures harbouring transgene vector. Hygromycin is an antibiotic produced by the bacterium *Streptomyces hygrocopicus*. In this present study our main objective was to determine the effects of the antibiotic Hygromycin on initiation, proliferation and development of somatic embryos in sorghum as one of the best selection and screening agent for selecting transformed cells in sorghum transformation experiments with vectors harbouring *hpt* gene.

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MATERIALS AND METHODS

Plant material

Shoot apices were obtained from aseptically germinated seedlings of sorghum Genotype 296B 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.

Hygromycin solution

The stock solution of hygromycin (0.5 mg/ml) was made by dissolving Hygromycin B powder (Sigma) in the distilled water and was filter sterilized. It was added to the autoclaved and cooled MS media at 40- 50°C at appropriate concentrations (Table I).

Induction and maintenance of somatic embryos

Shoot apices (2-4mm length) were excised from the hypocotyls of 4-5 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 photo period for 16 h and 8 h dark per day at 26°C with 45% relative humidity. The explants were sub-cultured on fresh media every week.

Screening somatic embryos for tolerance to Hygromycin

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 Hygromycin (0, 0.05, 0.1, 0.2, 0.5, 1.0 and 1.2 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.

RESULTS

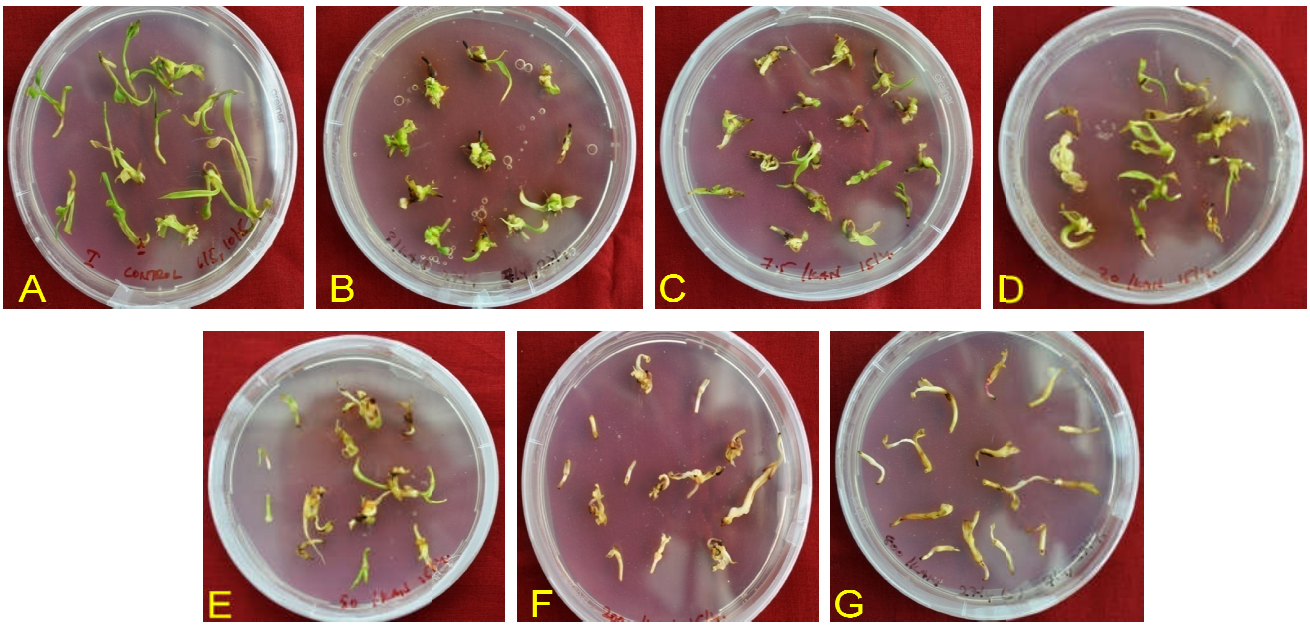
Various explants have been used in sorghum for successful development of transgenics. Cell suspension and protoplasts (Battraw and Hall, 1991), callus from immature embryos (Zhu *et al.* 1998; Zhao *et al.*, 2000) and shoot meristem explants from germinated seeds (Gray et al., 2004; Girijashankar *et 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 hygromycin based selection of shoot meristem explants from germinated seeds to assist sorghum transformation.

Effect of hygromycin concentration on shoot meristem explants

Incubation of shoot apices containing meristematic regions on hygromycin containing media resulted in aborted growth of shoot apices and bleaching (tissues lacking chlorophyll) to various degrees at higher concentrations (Table 1; Fig. 1). At lesser concentrations of a hygromycin 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 hygromycin) giving raise to bulged somatic embryos and chlorophyll pigment was unaffected indicating that these three concentrations (0.05mg, 0.1mg, and 0.2mg/L) were not ideal

Table 1. Effect of Hygromycin on survival of shoot apex explants

Conc. of Hygromycin 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
0.05	16	100.0	100.0	100.0
0.1	16	93.8	93.8	93.8
0.2	16	87.5	87.5	87.5
0.5	16	62.5	25.0	0.0
1.0	16	37.5	12.5	0.0
2.0	16	31.3	6.3	0.0



A.Control – without Hygromycin, Hygromycin concentration (mg/L) - B. 0.05, C.0.1, D. 0.2, E. 0.5, F. 1.0, and G. 2.0.

Fig. 1. Effect of Hygromycin on sorghum shoot apices

for selecting sorghum transgenics, as they are chances of selecting non-transformed cells along with transformed ones. At higher concentrations of hygromycin (0.5, 1.0 and 2.0 mg/L), the survival rate greatly varied along the three subcultures. At a hygromycin concentration of 0.5 mg/L the percentage of survival decreased gradually with three subcultures - 62.5, 25 and 0% survival respectively, while at concentrations of 1.0 mg/L these were 37.5, 12.5 and 0%. Evidently at the highest concentration of 2.0 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 Hygromycin, *hpt*) from non-transformed ones in any kill curve experiments. The least concentration of Hygromycin at which all susceptible explants died was 0.5 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.

DISCUSSION

The main objective of this study was to evaluate potential of Hygromycin as a selectable marker were the transgenic plants carrying *hpt* gene. The amino glycoside Hygromycin, acting as a selective agent, has been commonly used in plant genetic engineering (Padilla and Burgos 2010). 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 *et al*., 1987) and inhibiting ribosomal protein synthesis (Kohanski *et al*., 2010). Several transformation vectors come with *hpt* gene and optimization of Hygromycin 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 hygromycin (Hauptmann *et al*., 1988), high concentrations are required to be effective. Hence a wide range of concentrations of Hygromycin were tried. Of the series of concentrations tried in our experiment, 0.5 mg/L was the least one at which non-resistant shoot meristem explants (do not contain Hygromycin resistance conferring *hpt* 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 Hygromycin. 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 Hygromycin has been necessitated by the high endogenous resistance of sorghum tissue to Hygromycin (Hauptmann *et al*., 1988).

Therefore it can be concluded that, a 0.5 mg/L of Hygromycin is the optimum and reliable concentration for *in vitro* selection of sorghum transgenic shoot meristem explants carrying *hpt* gene after transformation. This concentration of Hygromycin 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.

REFERENCES

Battraw, M. and Hall, T.C. 1991. Stable transformation of Sorghum bicolor protoplasts with chimeric neomycin phosphotransferase II and β-glucuronidase genes. *Theor. Appl. Genet*., 82: pp.161–168.

Girijashankar, V., Sharma, H.C., Sharma, K.K., Swathisree,V., Sivarama Prasad, L., Bhat, B.V., Monique Royer, Blanca San Secundo, Lakshmi Narasu M, Altosaar I & Seetharama, N. 2005. Development of transgenic sorghum for insect resistance against the spotted stem borer *(Chilo partellus*). *Plant Cell Reports*, 24(9): pp.513-522.

Gray, S.J., Zhang, S., Rathus, C., Lemaux, P.G. and Godwin I.D. 2004. Development of sorghum transformation: Organogenic regeneration and gene transfer methods. In: Sorghum Tissue Culture and Transformation (Eds. Seetharama, N. and Godwin, I.D), *Oxford Publishers*, New Delhi. pp.35-43.

Groot boom, A.W., Mkhonza, N.L., O'Kennedy, M.M., Chakauya, E., Kunert, K.J. and Chikwamba, R.K. 2010. Biolistic mediated sorghum (*Sorghum bicolor* L. Moench) transformation via mannose and bialaphos based selection systems. *Intl J Bot.,* 6: pp.89-94.

Hauptmann, R.M., Vasil, V., Ozias-Akins, P., Tabaeizadeh, S.G., Rogers, S.G., Fraley, R.T., Horsch, R.B. and Vasil I.K. 1988. Evaluation of selectable markers for obtaining stable transformants in the Gramineae. *Plant Physiology,* 86: pp.602-606.

Kohanski, M.A., Dwyer, D.J. and Collins, J.J. 2010. How antibiotics kill bacteria: from targets to networks. *Nature Reviews Microbiology,* 8: pp.423-435.

Moazed D. and Noller HF. 1987. Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature*, 327(6121): pp.389-94.

Zhao, Z. Cai, T., Tagliani, L., Miller, M., Wang, N., Pang, H. and Pierce, D. 2000. Agrobacterium-mediated sorghum transformation. *Plant Molecular Biology,* 44: pp.789–798.

Zhu, T., Mettenburg, K., Peterson, D.J., Tagliani, L. and Baszczynski C. 2000. Engineering herbicide resistant maize using chimeric RNA/DNA oligonucleotides. *Nature,* 18: pp.555–558.

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