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Identification of Selective Agents Concentrations for Optimal Plant Regeneration from Transformed Calli and Immature Embryos in Wheat

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A B S T R A C T

Success rate in selection of putative transformants is mainly dependent upon the optimum concentration of selective agent. Effect of antibiotic concentrations of hygromycin (10, 20, 30 and 50 mg/L) and paromomycin (10, 20, 30 and 50 mg/L) in callusing and regeneration media on immature embryo-derived calli was evaluated using wheat variety PBW621. Hygromycin at 10 mg/L and paromomycin at 15 mg/L gave about 50% regeneration from calli (compared to more than 90% in control) leading to identification of these concentrations as optimal. In a second experiment, seedlings derived from immature embryos were subjected to hygromycin, kanamycin and paromomycin at 5, 10, 15, 20 and 25 mg/L. Hygromycin and paromomycin gave 100 percent killing of seedlings at 20 and 25 mg/L concentrations, respectively, and were identified as optimal concentrations for screening of seedlings. Kanamycin treated seedlings turned albino even at lowest concentration of 5 mg/L. A similar response had been observed in case of calli, showing relative unsuitability of kanamycin as a selective agent.

Introduction

The target tissue for plant genetic transformation such as embryogenic calli or embryos is generally made up of thousands of cells. A small percentage of the explants and just a fraction of the cells within the transformed explants actually get transformed by virtue of transgene integration in the genome. Thus it is important to provide a preferential *in vitro* regime to transformed cells in order to avoid their loss and enhance their contribution to subsequent cell growth and plant regeneration. About 50 selectable marker genes have been explored or used in transgenic plant research (Miki *et al*., 2004). *NptII* (*neomycin phosphotransferase II*), a widely used marker gene was isolated from *E. coli* strain Tn5 (Bevan *et al*., 1983) and confers resistance to kanamycin, geneticin G-418, paromomycin and neomycin.

NptII catalyses phosphorylation and thereby inactivation of the antibiotics. In genetic transformation studies on wheat, *nptII* gene has been widely used as effective selectable marker (Cheng *et al*., 1997; Xue *et al*., 2004; Verma *et al*., 2011). *Hpt* (*hygromycin phosphotransferase*) gene, another important selectable marker, was isolated from *E. coli* (Gritz *et al*., 1983). Hygromycin has been used as a selection agent for producing transgenic monocots (Hauptmann *et al*., 1988), including gramineae species such as

rice (Christou *et al*., 1991), maize (Weymann *et al*., 1993) and wheat (Ortiz *et al*., 1996; Brisibe *et al*., 2000; Chugh *et al*., 2003).

Large number of regenerants or survivors coupled with optimal selection regimes are a pre-requisite for obtaining adequate number of transformants involving several independent transformation events. Inappropriately high antibiotic concentrations drastically reduce plant regeneration frequencies and run the risk of decimating or even eliminating the recovery regenerants, including the confirmed transformants. The issue is particularly relevant for wheat lines other than model genotypes such as Bobwhite. A currently cultivated, agronomically desirable variety PBW621 was used in the present study. The available information on concentrations of selective antibiotics hygromycin and paromomycin, in context of *hpt* and *nptII* marker genes proved inadequate. This study addresses these gaps and aims at identifying concentrations which optimize the output of the transformation system by achieving a balance between selection efficiency and regeneration related parameters.

Materials and Methods

Hexaploid wheat variety PBW621 cultivated in North Western plains of India, was used for the experiments. Immature seeds were collected approximately two weeks post anthesis and sterilized in 1% Bavistin (Carbendazim fungicide, BASF India Limited) solution for 30 min, followed by 0.1% $HgCl_2$ (Hi-Media, Mumbai, India) for 7 min and finally with 100% ethanol for 1 min, followed by three washes in sterilized water after each treatment. The immature embryos were isolated from the seeds and precultured on callus induction medium containing MS salts (Murashige *et al*., 1962) supplemented with 4% maltose, 2.2 mg/L picloram, 0.5 mg/L 2,4-D, 500 mg/L proline, 100 mg/L casein hydrolysate, 500 mg/L glutamine, 100 mg/L ascorbic acid and solidified with 8 g/L agar and on MS (MS + 3% sucrose + 8 g/L agar) medium for germination.

For preparing stock solutions of antibiotic hygromycin-B, kanamycin acid sulphate and paromomycin sulphate (Himedia)**,** 100 mg of each was dissolved in distilled water with a final volume of 5 ml. This gave an antibiotic stock concentration of 20 mg/mL. The stock solutions were filter sterilized using 0.2 µm Millipore^{TM} nylon filter.

To determine the optimum concentration of antibiotics for calli, 10 immature embryo-derived calli of wheat variety PBW621 grown on callusing medium were subcultured on to same callusing medium (control) and callusing media containing different concentrations of the antibiotics hygromycin (30 to 60 mg/L) and kanamycin (30 to 60 mg/L). The cultures were incubated in the dark for four weeks with one subculturing on same medium after two weeks. Average fresh weight of callus was calculated by weighing ten calli from each concentration of both antibiotics and from control samples. Percent somatic embryogenesis was recorded for all treatments including control calli. Regeneration on medium (MS + 4% maltose + 0.2 mg/L 2,4-D + 500 mg/L proline + 100 mg/L ascorbic acid) with same concentrations of antibiotics was also recorded. In a second experiment employing lower concentrations, 20 calli were cultured on to callusing media containing 10 to 50 mg/L of hygromycin and 10 to 50 mg/L of paromomycin and incubation in the dark for three weeks. After that calli were divided into two groups and transferred to regeneration medium containing different concentrations of the antibiotic hygromycin (10 to 50 mg/L) and paromomycin (10 to 50 mg/L) besides regeneration medium not supplemented with antibiotics.

To determine the optimum concentration of antibiotics for seedlings, 10 seedlings (two days old) from immature embryos of wheat variety PBW621 germinated on MS medium were transferred to MS medium containing 5 to 25 mg/L of hygromycin, 5 to 25 mg/L of kanamycin and 5 to 25 mg/L of paromomycin and incubation in the light for four weeks with one subculturing on same media after two weeks. In case of experiment with paromomycin, incubation was extended to eight weeks with regular subculturing on same media every two weeks.

Results and Discussion

In this study hygromycin (for *hpt* selectable marker gene) and kanamycin and paromomycin (for *nptII* selectable marker gene) were evaluated separately. Previously wheat variety PBW621 had been selected as best genotype for callus induction and regeneration from three varieties under study (Jhinjer *et al*., 2012). It was used in optimization of antibiotic concentration and subsequently taken up for transformation experiments.

It was observed that with increase in concentration of hygromycin, average fresh weight of calli decreased from 195.0 mg at 0 mg/L to 42.8 mg at 60 mg/L (Table 1, Figure 1A). Somatic embryogenesis was seen in 30 mg/L concentration of hygromycin only and increase in concentration of hygromycin totally inhibited the embryogenesis in calli. Hygromycin concentration at 30 mg/L gave 26.67% embryogenesis compared to 93.33% in control calli cultured in absence of hygromycin. With increase in concentration of kanamycin from 0 and 60 mg/L, average fresh weight of calli decreased from 195.0 to 62.6 mg (Table 1, Figure 1B). Somatic embryogenesis was seen only at 30 and 40 mg/L concentration of kanamycin and increase in concentration of kanamycin totally inhibited embryogenesis. Kanamycin at concentration of 30 and 40 mg/L gave 16.67 and 3.33% embryogenesis, respectively. It was however observed that all regenerated plantlets from kanamycin treated calli were albino (Figure 2D). These plantlets could be grown in *in-vitro* conditions but remained totally albino and died when transferred to soil. Thus practically no viable plants could be regenerated at these concentrations.

Considering the almost complete inhibition of regeneration at the antibiotics concentrations discussed above a new set of treatments including lower doses of 10 and 20 mg/L was taken up (Table 2). In control conditions when calli were incubated on antibiotics free callusing and regeneration medium, 90.0% regeneration was observed. Both antibiotics gave no regeneration when selection was carried out with 50 mg/L in callusing medium only and both callusing and regeneration media (Table 2). In case of hygromycin when selection was carried out in callusing medium only, regeneration frequency was observed to be 66.7, 43.3 and 23.3% at 10, 20 and 30 mg/L respectively. When hygromycin was used in both call using and regeneration media, regeneration was 53.3, 26.7 and 3.3% at same concentrations. In case of paromomycin when selection was carried out in callusing medium only, regeneration was seen as 76.7, 60.0 and 33.3% at 10, 20 and 30 mg/L respectively. When paromomycin was used in both call using and regeneration media, regeneration frequency was observed to 63.3, 40.0 and 16.7% respectively at the three concentrations.

When effect of hygromycin on germinated seedlings was studied, it was observed that seedlings started to turn yellow within a week (Table 3). After four weeks of incubation, two types of seedlings were obtained, partial yellow or complete yellow. In control medium (not supplemented with hygromycin)

all seedlings were green after four weeks of culture but in hygromycin containing media, with increase in concentration of hygromycin, yellow seedlings increased from 20% at concentration of 5 mg/L to 100% at concentration of 20 and 25 mg/L. In case of kanamycin also, seedlings were started to turn albino within a week. Partial albino or complete albino seedlings were obtained after four weeks of incubation. At each concentration of kanamycin (5-25 mg/L), all seedlings turned to albino (100%). In case of paromomycin, seedlings started to turn yellow after six weeks. Unlike hygromycin and kanamycin, complete yellow seedlings were obtained after eight weeks. Seedlings started to turn yellow at 15 mg/L of paromomycin. With increase in concentration of paromomycin, yellow seedlings increased from 20% at concentration of 15 mg/L to 100% at concentration of 25 mg/L.

When compared with control seedlings after four weeks, both shoot and root length was affected with increase in concentration of hygromycin in medium (Figure 2A), in case of kanamycin, shoot length was more dramatically inhibited than root length (Figure 2B). With increase in concentration of paromomycin, both shoot and root length was affected even when seedlings remained green at 5 and 10 mg/L concentration of paromomycin (Figure 2C). Shoot length was inhibited to a greater extent than root length.

Table.1 Average weight of callus (mg), percent embryogenesis and regeneration of wheat calli on media supplemented with different concentrations of hygromycin and kanamycin

Antibiotics concentration (mg/L)	Average weight of callus (mg) after 4 weeks		Percent embryogenesis of calli		Percent regeneration	
	Hygromycin	Kanamycin	Hygromycin	Kanamycin	Hygromycin	Kanamycin
0	195.0a	195.0a	93.3a	93.3a	92.7a	92.7a
30	118.8b	105.4b	26.7b	16.7b	4.3b	2.3b
40	67.9c	85.6c	0.0c	3.3c	0.0c	0.0c
50	51.3d	63.6d	0.0c	0.0d	0.0c	0.0c
60	42.8e	62.6d	0.0c	0.0d	0.0c	0.0c

Means followed by different letters are significantly different at P = 0.05

Table.2 Percent regeneration in wheat calli on media supplemented with different concentrations of hygromycin and paromomycin

Antibiotic concentration (mg/L)	Percent regeneration in one step selection (only callusing medium contained antibiotic)		Percent regeneration in two steps selection (both callusing and regeneration medium contained antibiotic)	
	Hygromycin	Paromomycin	Hygromycin	Paromomycin
0	90.00a	90.00a	90.00a	90.00a
10	66.67b	76.67b	53.33b	63.33b
20	43.33c	60.00c	26.67c	40.00c
30	23.33d	33.33d	3.33d	16.67d
50	0.00e	0.00e	0.00e	0.00e

Means followed by different letters are significantly different at P = 0.05

Table.3 Percent killing of wheat seedlings on media supplemented with different concentrations of hygromycin, kanamycin and paromomycin

Antibiotics concentration (mg/L)	Percent killing of seedlings (2 weeks)			Percent killing of seedlings (4 weeks)			Percent killing of seedlings (8 weeks)
	Hygromycin	Kanamycin	Paromomycin	Hygromycin	Kanamycin	Paromomycin	Paromomycin
0	0.00a	0.00	0.00	0.00a	0.00	0.00	0.00a
5	20.00b	100.00	0.00	20.00b	100.00	0.00	0.00a
10	53.33c	100.00	0.00	53.33c	100.00	0.00	0.00a
15	73.33d	100.00	0.00	73.33d	100.00	0.00	20.00b
20	100.00e	100.00	0.00	100.00e	100.00	0.00	80.00c
25	100.00e	100.00	0.00	100.00e	100.00	0.00	100.00d

Means followed by different letters are significantly different at P = 0.05

Fig.1 Effect of different concentrations (0, 30, 40, 50 and 60 mg/L) of hygromycin (A) and kanamycin (B) growth of wheat calli

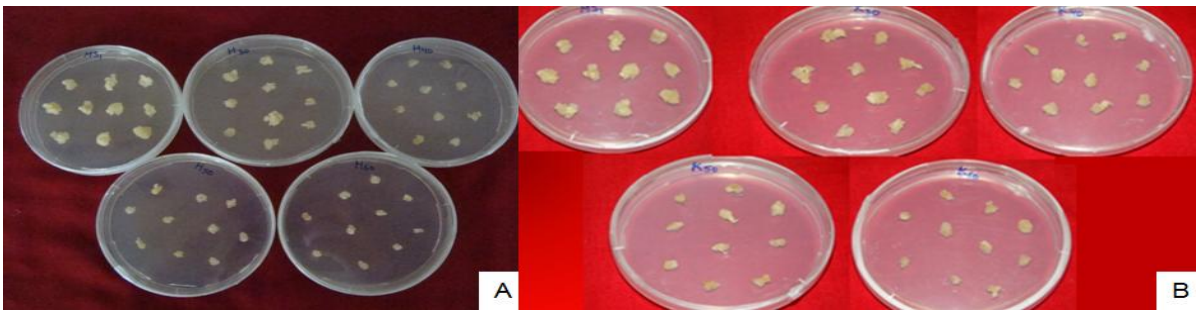
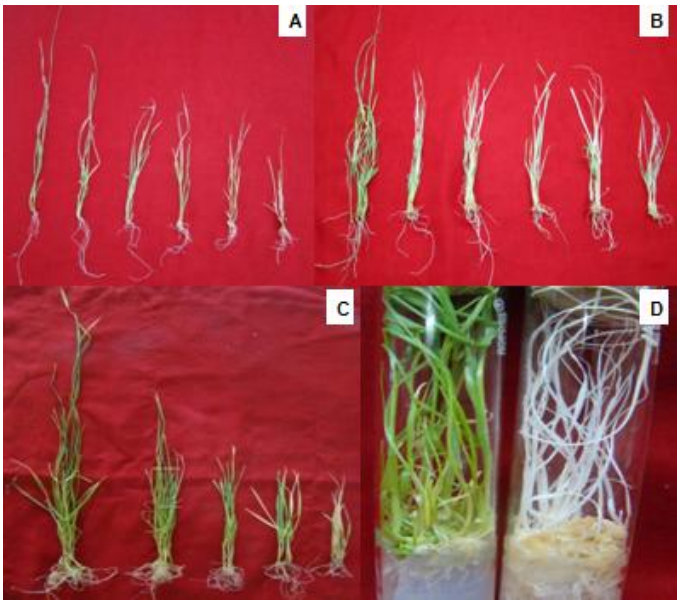


Fig.2 Effect of different concentrations (0, 5, 10, 15, 20 and 25 mg/L) of hygromycin (A) kanamycin (B) and paromomycin (C) on growth of germinated seedlings of wheat. D) Albinism effect of kanamycin on regenerated plantlets of wheat



When we compared two antibiotics for *nptII* gene, it showed that at low concentrations (5-10 mg/L), paromomycin inhibits only the vegetative growth of germinated seedlings. At higher concentration of 15 mg/L, the chlorophyll content started depleting. But in contrast to it, kanamycin caused depleted chlorophyll content at very low concentration (5 mg/L).

The concentration of selective agents need to be carefully chosen to avoid either being too low and thereby allowing undesirable number of escapes of plants to develop, or too high so that transformants expressing moderate level of resistance are lost. Strong selection at early stages may reduce the number of viable shoots while delayed selection may increase the number of escapes. Transformation procedures also cause stress to tissues and these stresses such as in case of calli, bombardment, osmoticum stress, vacuum pressure and injuries by microcarrier particles further decrease the regeneration of calli. In case of *Agrobacterium*-mediated transformation, *Agrobacterium* inoculation and infectious growth as well as subsequent antibiotics used for eliminating *Agrobacterium* growth add to to the stress.

Hygromycin at 10 mg/L for *hpt* gene and paromomycin at 15 mg/L for *nptII* gene in two step selection (in callusing as well as in regeneration medium) gave about 50% regeneration of non-transformed calli of wheat variety PBW621. These concentrations are proposed as optimal for selection of transformed calli. In previous studies hygromycin was used at concentrations from 5 to 25 mg/L and paromomycin at concentration from 50 to 100 mg/L (Patnaik *et al*., 2006) in selection of transformed wheat calli. Dose rate optimization of the selective agent is highly tissue and species specific as described by Parveez *et al*., (1996). Differences are also expected at the genotypic level due to genetic variation in the endogenous resistance.

When embryos are used as such for transformation, the transgene integrates into the already differentiated cells of apical meristem giving chimeric plants. The T_0 plants may thus not be appropriate material for screening against antibiotics. T_1 plants or subsequent generations should be used for selection as they would not be chimeric. For seedlings selection, hygromycin for *hpt* gene at 20 mg/L and paromomycin for *nptII* gene at 25 mg/L gave hundred percent yellow plants, thus considered as optimum concentrations for selection of T_1 and next generations of transgenics. In one of the previous study, hygromycin was used at concentration of 25 mg/L in screening of T_1 sorghum seedlings germinated from immature embryos (Carvalho *et al*., 2004). It was also used at concentrations of 60 mg/L[18] and 30 mg/L (Zale *et al*., 2009) in screening of T_2 and T_1 of wheat seeds respectively. Paromomycin was applied as spray (2% solution) to screen T_1 young plants (Zale *et al*., 2009; Patnaik *et al*., 2003). Kanamycin did not turn out to be suitable selective agent because of albinism in both regenerated and germinated plantlets at even low concentration.

The optimization of concentrations of hygromycin and paromomycin in this study has been achieved for calli at fifty percent regeneration ability and for seedlings at hundred percent mortality when untransformed plant material is used.

References

Bevan, M.W., Flavell, R.B. and Chilton, M.D. 1983. A chimearic antibiotic resistance to marker gene as a selectable marker

for plant cell transformation, *Nature*, 304: 184-187.

Brisibe, E.A., Gajdosova, A., Olesen, A. and Andersen, S.B. 2000. Cytodifferentiation and transformation of embryogenic callus lines derived from anther culture of wheat, *J. Exp. Bot.*, 51: 187–196.

Carvalho, C.H.S., Zehr, U.B., Gunaratna, N., Anderson, J., Kononowicz, H.H., Hodges, T.K. and Axtell, J.D. 2004. *Agrobacterium*-mediated transformation of sorghum: factors that affect transformation efficiency, *Genetics and Mol. Biol.*, 27: 259-269.

Cheng, M., Fry, J.E., Pang, S., Zhou, H., Hironaka, C.M., Duncan, D.R., Conner, T.W. and Wan, Y. 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*, *Plant Physiol.*, 115: 971-980.

Christou, P., Ford, T. and Kofron, M. Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos, *Biotech.,* 9: 957-962.

Chugh, A. and Khurana, P. 2003. Regeneration via somatic embryogenesis from leaf basal segments and genetic transformation of bread and emmer wheat by particle bombardment, *Plant Cell Tiss. Org.*, 74: 151–161.

Gritz, L. and Davies, J. 1983. Plasmids-encodes hygromycin B resistance: the sequence of hygromycin B phos-photransferase gene and its expression in *Escherichia coli* and *Sacchoromyces cerevisiae*, *Gene,* 25: 179-188.

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 Physiol.*, 86: 602-606.

Jhinjer, R.K., Bains, N.S. and Gosal, S.S. 2012. Establishment of somatic embryogenesis and induction of differentiation in bread wheat (*Triticum aestivum* L.), in *International Conference on Sustainable Agriculture for Food and Livelihood Security*, held on 27-29 Nov, 2012 (Punjab Agricultural University, Ludhiana, India) 313-314.

Miki, B., and McHugh, S. 2004. Selectable marker genes in transgenic plants: applications, alternatives and biosafety. *J. Biotech.*, 107: 193-232.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol. Plantarum*, 15: 473–97.

Ortiz, J.P.A., Reggiardo, M.I., Ravizzini, R.A., Altabe, S.G., Cervigni, G.D.L., Spitteler, M.A., Morata, M.M., Elias, F.E. and Uejos, R.H.V. 1996. Hygromycin resistance as an efficient selectable marker for wheat stable transformation, *Plant Cell Rep.*, 15: 877-81.

Parveez, G.K.A., Chowdhury, M.K.U. and Saleh, N.M. 1996. Determination of minimal inhibitory concentration of selection agents for oil palm (*Elaeis guineensis* Jacq.) transformation, *Asia Pacific J. Mol. Biol. Biotech.*, 4: 219-228.

Patnaik, D. and Khurana, P. 2003. Genetic transformation of Indian bread (*T. aestivum*) and pasta (*T. durum*) wheat by particle bombardment of mature embryo-derived calli, *BMC Plant Biol.*, 3.

Patnaik, D., Vishnudasan, D. and Khurana, P. 2006. *Agrobacterium*-mediated transformation of mature embryos of *Triticum aestivum* and *Triticum durum*, *Curr. Sci.*, 91: 307-317.

Supartana, P., Shimizu, T., Nogawa, M., Shioiri, H., Nakajima, T., Haramoto, N., Nozue, M. and Kojima, M. 2006. Development of simple and efficient *in planta* transformation method for wheat (*Triticum aestivum* L.) using *Agrobacterium tumefaciens*, *J. Biosci. Bioeng.*, 102: 162–170.

Verma, L., Jhinjer, R.K. and Gosal, S.S. 2011. *Biotechnology: A New Approach*, edited by P C Trivedi (Agrobios Publisher, Jodhpur, India), 91-144.

Weymann, K., Urban, K., Ellis, D.M., Novitzky, R., Dunder, E., Jayne, S. and Pace, G. 1993. Isolation of transgenic progeny of maize by embryo rescue under selective conditions, *In Vitro Cell Dev. Biol.*, 29: 33-37.

Xue, Z.Y., Zhi, D.Y., Xue, G.P., Zhang, H., Zhao, Y.X. and Xia, G.M. 2004. Enhanced salt tolerance of transgenic wheat (*Triticum aestivum* L.) expressing a vacuolar Na+/ H+ antiporter gene with improved grain yields in saline soils in the field and a reduced level of leaf Na+, *Plant Sci.*, 167: 849.

Zale, J., Agarwal, S., Loar, S. and Steber, C.M. 2009. Evidence for stable transformation of wheat by floral dip in *Agrobacterium tumefaciens. Plant Cell Rep.*, 28: 903–913.

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