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# Mutagenesis of embryogenic cultures of soybean and detecting polymorphisms using RAPD markers

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## Abstract

Embryogenic suspension cultures of soybean (*Glycine max* L. cv. Iroquois) were subjected to mutagenesis using varying concentrations (1, 3, 10, and 30 mM) of ethyl methanesulfonate (EMS). Depending on the concentration of EMS used, the mean survival rate of embryogenic cultures decreased from 74 % (1 mM EMS) to 43 % after 30 mM EMS treatment. Random amplified polymorphic DNA (RAPD) analysis was used to determine whether induction of genetic variability in embryogenic cultures in response to the different EMS treatments may result in identification of polymorphic markers. Two of 35 'core' primers tested revealed polymorphisms. One of the primers, OPO-01/1150, revealed polymorphism in tissue treated with 10 mM EMS, while the other primer, OPO-05/1200, revealed polymorphism in tissue treated with either 1 or 30 mM EMS. These results suggest that RAPD markers are useful in detecting mutations in embryogenic cultures of soybean.

*Additional key words*: chemical mutagenesis, *Glycine max*, molecular markers, somatic embryogenesis.

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## Introduction

Soybean is a major source of edible protein and oil, and it is one of the most important agronomic crops in the world (Smith and Huyser 1987). Tissue culture techniques are valuable tools for conducting genetic manipulation studies (Parrott *et al.* 1994). Molecular marker techniques, such as random amplified polymorphic DNA (RAPD) analysis, have been used to assess somaclonal variability (Hashmi *et al.* 1997, Al-Zahim *et al.* 1999, Rout and Das 2002, Zucchi *et al.* 2002) as well as evaluate effects of growth regulators (Mangolin *et al.* 2002) and chemical mutagens (Teparkum and Veilleux 1998) on *in vitro*-grown cultures. The RAPD assay is efficient for screening for nucleotide sequence polymorphism among individuals as each primer (on average) will direct the amplification of several discrete loci within a genome (Samal *et al.* 2003). As primers modified by even a single nucleotide produce different banding profiles, the RAPD technique can generate polymorphisms between very closely-related genotypes (Deng *et al.* 1995).

Although ethyl methanesulfonate (EMS) has been used to induce mutations in mature seed and cell suspension cultures of soybean (Sung 1976, Wilcox *et al.* 1984, Fuji and Tano 1986), there are no reports on inducing mutations in embryogenic cultures of soybean. In this study, embryogenic cultures of soybean were subjected to chemical mutagenesis, evaluated for survival, and RAPD markers were used to detect DNA polymophisms in these cultures.

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*Abbreviations*: 2,4-D - 2,4-dichlorophenoxyacetic acid; EMS - ethyl methanesulfonate; MS - Murashige and Skoog (1962) medium; PCR - polymerase chain reaction; RAPD - random amplified polymorphic fragment; TBE - Tris borate EDTA.
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## Materials and methods

Immature soybean pods were harvested from greenhouse grown plants of soybean (*Glyine max* L. cv. Iroquois). Pods were surface-sterilized by immersion in 1.09 % sodium hypochlorite solution (20 % *Clorox*® in commercial bleach) containing 3 to 4 drops of *Tween-20* for 25 min followed by three rinses in sterile double-deionized water. Immature cotyledons of 3 to 6 mm in length were excised, and the embryonic-axis removed. Cotyledons were placed with the adaxial surface upward on *ca*. 35 $cm^3$ of a solidified initiation medium in 15 × 120 mm Petri dishes. The initiation medium consisted of MS (Murashige and Skoog 1962) salts, B5 vitamins (Gamborg *et al.* 1968), 40 mg $dm^{-3}$ 2,4-dichloro-phenoxy-acetic acid (2,4-D), 3 % sucrose, and was solidified with 0.2 % *Gelrite*. Prior to autoclaving, the pH level was adjusted to 7.0 with 1 M NaOH. Cultures were incubated for 4 weeks under a 23-h photoperiod (irradiance of 30 µmol $m^{-2}$ $s^{-1}$) at 25 ± 1 °C in a controlled environment.

Following initiation, somatic embryos were transferred to a fresh solid medium as described above, but containing 20 mg $dm^{-3}$ 2,4-D and the pH was adjusted to 5.8 to proliferate somatic embryos for 4 - 12 weeks with monthly subculture to fresh medium. Somatic embryos were inoculated into 35 $cm^3$ of a liquid medium containing MS salts, B5 vitamins, 6 % sucrose, 15 mM glutamine, and 5 mg $dm^{-3}$ 2,4-D, and pH adjusted to 5.8. Cultures were maintained in the liquid medium under irradiance of 30 µmol $m^{-2}$ $s^{-1}$ with continuous shaking (1200 rpm) on a gyratory shaker (*Lab-line Instruments*, Melrose Park, USA) and a temperature of 26 ± 1 °C. Embryogenic cultures were subcultured to fresh medium once every two weeks.

Embryogenic suspension cultures that had been maintained in liquid culture for at least 6 months were treated with the chemical mutagen EMS (*Sigma*, St. Louis, USA) by inoculating 35 $cm^3$ of a liquid culture medium in a 250 $cm^3$ flask (as described above) containing 12 embryogenic clumps with 0, 1, 3, 10, or 30 mM concentrations of EMS. A total of three flasks (12 embryogenic clumps/flask) per EMS treatment was used. Cultures were incubated with EMS for 4 h on a gyratory shaker at 120 rpm at 28 ± 1 °C. Following incubation, explants were rinsed three times with the liquid culture medium. Three to four days post-treatment, embryogenic clumps were placed individually into 12-well microplates, and were subcultured biweekly. Each 12-well microplate represented one replication per EMS treatment, and a total of three replications per EMS treatment were used in a randomized complete block design. This experiment was repeated twice over time. Data on survival of embryogenic cultures, based on presence of yellowish-coloured friable and proliferating cultures, were recorded after 60 d, and these were subjected to regression analysis ($P < 0.05$) using the *SAS* statistical analysis package.

DNA was isolated from 16-month old somatic embryogenic tissue grown in suspension cultures subjected to each of the five EMS treatments described above. A 0.3 $cm^3$ sample of sedimented embryogenic tissue was collected, vacuum dried for 2 h, and stored at -20 °C until DNA isolation. Tissue was ground into a fine powder in liquid nitrogen in a 1.5 $cm^3$ Eppendorf tube using an ice pick. Immediately following grinding, 0.7 $cm^3$ of 2X CTAB [1.4 M NaCl, 100 mM Tris base, pH 8.0, 2 % cetyldimethyl ethylammonium bromide (CTAB), 20 mM ethylenediaminetetraacetic acid (EDTA), 0.5 % sodium bisulfite, 1 % 2-mercaptoethanol] was added into each tube. Tubes were incubated at 65 °C for 2-h with occasional swirling. The mixture was emulsified with 0.6 $cm^3$ chloroform:isoamyl alcohol (24:1) by shaking on an orbital shaker for 30 min, and then centrifuged at 5 000 *g* for 10 min. The aqueous phase was removed, treated with 10 µg RNAase A (R 5503, *Sigma*) in a 37 °C water bath for 1-h, and then emulsified as above. The aqueous phase was removed, and DNA was precipitated in 0.8 vol. of isopropanol chilled at -20 °C. DNA was pelleted at 5 000 *g* in a microcentrifuge for 5 min. The supernatant solution was discarded, the pellet was washed three times in 70 % ethanol, and any residual ethanol was removed by pipetting. The pellet was air-dried overnight, resuspended in 0.05 $cm^3$ of 0.1X TE (1.0 M Tris, 0.1 M EDTA, pH 8.0), and stored at -20 °C. The DNA concentration was determined using a UV-VIS spectrophotometer (*Shimadzu*, Tokyo, Japan), diluted to 10 ng $cm^{-3}$ in sterile-deionized water, and used for PCR analysis.

Thirty-five decamer primers previously identified as 'core' primers for soybean (Thompson and Nelson 1998) were used for RAPD analysis. This 'core' set of primers has been selected based on their high reproducibility and their high polymorphism information content (PIC) scores of 30 or above. The PCR amplification reaction contained the following: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$ (*Gibco BRL*, Bethesda, USA), 0.2 mM of dNTP mix (*Sigma*), 0.65 U of *Taq* DNA polymerase (*Gibco BRL*), 50 ng of template DNA, and 10 µM of primer. The reaction mixture was overlaid with a drop of mineral oil (*Sigma*) to avoid evaporation, and placed in a *Hybaid* thermocycler (*Hybaid*, Hampton Hill, UK). Amplification was conducted using the following cycles: 94 °C for 3 min, 40 cycles of 94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min, with a ramp of 3 s $°C^{-1}$ between annealing and extension steps. Reactions were maintained at 72 °C for 4 min and then at 25 °C for at least 5 min. A 0.005 $cm^3$ solution (50 % glycerol, 5X TBE, 0.02 M EDTA, 0.2 % SDS, 0.05 % bromophenol) was added to each tube, and stored at 4 °C until

electrophoresis. PCR amplification reactions were done in duplicates to check for reproducibility. Amplified products were electrophoretically resolved on 1.5 % agarose gels immersed in a 1X Tris borate EDTA (TBE) buffer, stained with ethidium bromide, visualized under ultraviolet light, and documented using an *Eagle Eye II* Gel Documentation System (*Stratagene*, La Jolla, USA). DNA isolation and PCR amplification from samples from the five different EMS treatments was replicated once over time.

## Results and discussion

A decrease in survival of embryogenic cultures was observed as the concentration of EMS in the medium increased (Fig. 1). Significant differences were observed

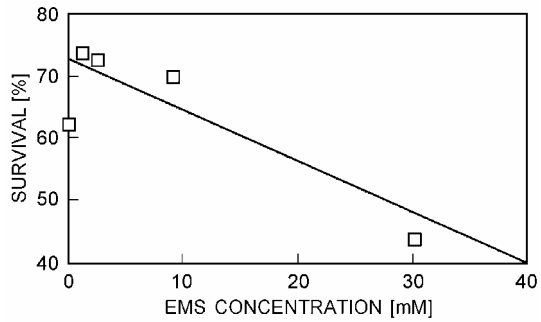


Fig. 1. Linear regression analysis ($P < 0.05$) of different EMS concentrations on survival of somatic embryogenic suspension cultures of soybean cv. Iroquois.

in the survival rate of embryogenic cultures subjected to 30 mM EMS compared to all other concentrations of EMS (1, 3, and 10 mM) used as well as control treatment (0 EMS). Previously, Sung (1976) reported a decrease in survival of soybean cell suspension cultures with increasing concentrations of EMS. Carroll *et al.* (1986) subjected soybean seeds to three mutagens including γ-rays, sodium azide, and EMS, and found all mutagens resulted in reduced germination frequency in mutagenized rather than in non-mutagenized seed. The results obtained with embryogenic cultures of soybean in this study were similar to those previously reported for both cell suspension cultures and seeds of soybean (Sung 1976, Carroll *et al.* 1986).

Subjecting embryogenic cultures to EMS provides an alternative strategy for inducing soybean mutants with useful genetic traits as these cultures can be more readily regenerated into whole plants than soybean cell suspension cultures. In this study, embryogenic cultures from various EMS treatments have been readily grown into whole plants (20 - 25 % frequency of regeneration) following standard protocols of maturation, desiccation, and germination for soybean (Ko *et al.* 2003). To our knowledge, this is the first study whereby embryogenic cultures of soybean have been subjected to mutagenesis, and then grown into whole plantlets.

Thirty-five primers assayed revealed a total of 124 PCR fragments over all embryogenic culture tissues used. Among those, 122 fragments were monomorphic

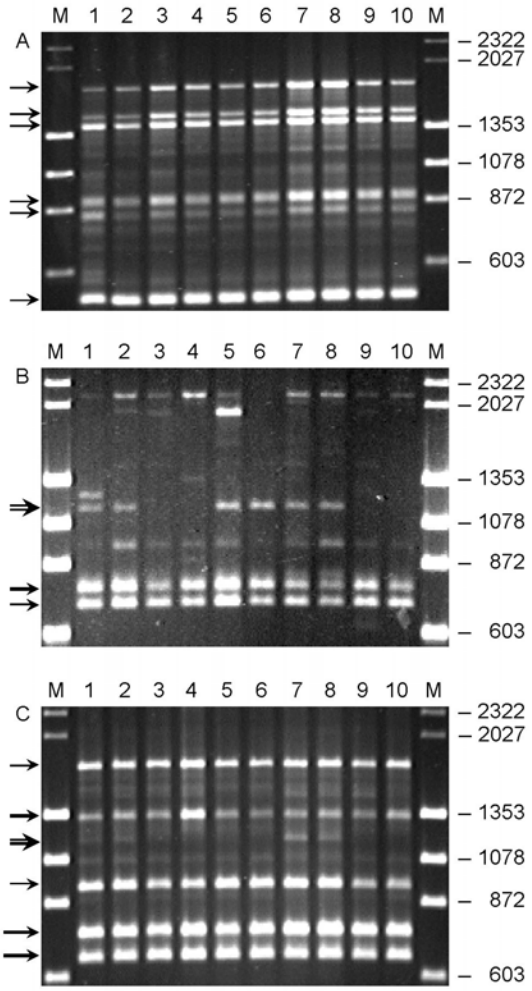


Fig. 2. Amplification of genomic DNA from somatic embryos of soybean cv. Iroquois treated with various concentrations of EMS using various RAPD primers. For each of the three panels, *lanes M* correspond to a l kb DNA ladder; *lanes 1* through *10* correspond to DNA from embryogenic cultures treated with either 0, 1, 3, 10, or 30 mM EMS (two consecutive lanes per EMS concentration). *A* - Amplification profiles of the different samples using one of the core primers, OPH-13; *arrows* depict markers that were consistently both reproducible and scorable; note all bands were monomorphic. *B* - Amplification profiles of samples using primer OPO-1/1150; *solid arrows* point to markers that were reproducible and scorable, while the *blank arrow* points to the polymorphic marker. *C* - Amplification profiles of samples using OPO-5/1200; *solid arrows* depict markers that were reproducible and scorable, while the *blank arrow* points to the polymorphic marker.

across all five treatments (including control), and generated electrophoretic profiles (Fig. 2*A*). However, primers OPO-01 and OPO-05 generated polymorphic fragments which were also confirmed in a repeated experiment. The polymorphism generated by OPO-01 revealed a fragment ~1150 bp in size (Fig. 2*B*). Soybean cultures subjected to EMS concentrations of either 0, 3, or 10 mM (Fig. 2B) showed a band that was absent in cultures subjected to EMS concentrations of either 1 mM or 30 mM. Similarly, the primer OPO-05 generated a fragment ~1200 bp in size which was polymorphic (Fig. 2*C*). Soybean embryogenic cultures subjected to EMS concentration of 10 mM showed a band not observed in cultures subjected to concentrations of either 0, 1, 3, or 30 mM EMS.

Changes in DNA caused by mutagens result in genetic variation detected by RAPD analysis (Rani *et al*. 1995, Teparkum and Veilleux 1998). However, it has to be noted that direct-acting alkylating agents such as EMS primarily cause point mutations which are the result of a single base pair deletion, addition, or substitution (Schy and Plewa 1989). Polymorphic amplification products which represent one allele per locus can result from changes in either the sequence of the primer binding site, such as point mutations, or from changes altering the size or preventing successful amplification of a target DNA such as insertions, deletions, and inversions (Rani *et al*. 1995). Since RAPD markers are resolved on low-resolution agarose gels, point mutations caused by an alkylating agent occurring within the amplified region will go undetected. Mutations resulting in polymorphisms are those occurring on primer binding sites, leading to an increase or decrease in the total number of primer binding sites, and consequently the number of amplified fragments.

In the present study, two RAPD primers have revealed polymorphic fragments among soybean embryogenic cultures that are most likely due to treatment with the chemical mutagen EMS (Table 1). These polymorphisms indicate presence of genetic differences in embyrogenic tissues of the soybean cv. Iroquois treated with various concentrations of EMS. It is possible that these mutations have occurred in different loci, although it is not yet known whether these mutations have resulted in alterations of agronomically useful traits. RAPD polymorphisms observed in this study are likely due to alterations in the number of primer binding sites following mutagenesis. Point mutations at other regions of the genome that fall within the amplified fragments are likely to go undetected. Al-Zhaim *et al.* (1999) have reported that although RAPDs are capable of detecting polymorphism in somaclonal variants of garlic derived from *in vitro*-grown embryogenic callus cultures, no association has been found between the frequency of cytological changes and the frequency of RAPD polymorphisms observed. Similarly, Zucchi *et al.* (2002) have detected polymorphisms among somaclonal variants of sugarcane plants derived from rhizome and meristem cultures, but these polymorphisms have not been correlated with number of subcultures. In this study, two polymorphisms detected out of 124 fragments assayed indicate a high level of mutation due to chemical mutagenesis as RAPD markers tend to underestimate the level of polymorphisms observed.

Thus, RAPD markers are useful in detecting polymorphisms in embryogenic cultures of soybean subjected to chemical mutagenesis as they provide sufficient numbers of DNA fragments for conducting assays. This approach can then be used to rapidly screen large numbers of embryogenic cultures in efforts to detect mutants with desirable agronomic traits.

Table 1. Primers that produced RAPD polymorphisms (pm) in somatic embryogenic tissues of soybean cv. Iroquois treated with five concentrations of EMS. Other primers that generated only monomorphic (mm) bands included OPA-20, OPE-01, OPF-04, OPG-06, OPH-02, OPH-12, OPH-13, OPH-15, OPK-01, OPK-03, OPK-10, OPK-16, OPL-09, OPL-18, OPN-03, OPN-08, OPN-09, OPN-18, OPO-04, OPO-14, OPO-19, OPP-07, OPP-09, OPP-11, OPR-07, OPR-10, OPR-12, OPR-13, OPS-01, OPS-03, OPS-05, OPS-11, and OPS-14.

| Primer | Sequence | Number of bands | | |
|---|---|---|---|---|
| | | total | mm | pm |
| OPO-01 | GGCACGTAAG | 5 | 4 | 1 |
| OPO-05 | CCCAGTCACT | 6 | 5 | 1 |

## References


Al-Zahim, M.A., Ford-Lloyd, B.V., Newbury, H.J.: Detection of somaclonal variation in garlic (*Allium sativum* L.) using RAPD and cytological analysis. - Plant Cell Rep. **18**: 473-477, 1999.

Carroll, B.J., McNeil, D.L., Gresshoff, P.M.: Mutagenesis of soybean (*Glycine max* (L.) Merr.) and the isolation of non-nodulating mutants. - Plant Sci. **47**: 109-114, 1986.

Deng, Z.M., Gentile, A., Nicolosi, E., Domina, F., Vardi, A., Tribulato, E.: Identification of *in vivo* and *in vitro* lemon mutants by RAPD markers. - J. hort. Sci. **70**: 117-125, 1995.

Fujii, T., Tano, S.: Mutagenic activities of EMS on somatic (M1) and recessive (M2) mutations in the soybean test system. - Environ. exp. Bot. **26**: 191-195, 1986.

Gamborg, O.L., Miller, R.A., Ojim, K.: Nutrient requirement of suspension cultures of soybean root cells. - Exp. Cell Res. **50**: 151-158, 1968.

Hashmi, G., Huettel, R., Meyer, R., Krusberg, L., Hammerschlag, F.: RAPD analysis of somaclonal variants derived from embryo callus cultures of peach. - Plant Cell

Rep. **16**: 624-627, 1997.

Ko, T.-S., Lee, S., Krasnyanski, S.F., Korban, S.S.: Two factors are critical for transformation of zygotic embryos of multiple soybean cultivars: *Agrobacterium* strain and orientation of explant. - Theor. appl. Genet. **107**: 439-447, 2003.

Mangolin, C.A., Ottoboni, L.M.M., Machado, M.F.P.S.: RAPD markers to evaluate callus tissue of *Cereus peruvianus* Mill. (*Cactaceae*) maintained in different growth regulator combinations. - Biochem. Genet. **40**: 351-358, 2002.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Parrott, W.A., All, J.N., Adang, M.J., Bailey, M.A., Boerma, H.R. Stewart, C.N., Jr.: Recovery and evaluation of soybean plants transgenic for a *Bacillus thuringiensis* var. Kurstaki insecticidal gene. - *In Vitro* cell. dev. Biol. Plant **30**: 144-149, 1994.

Rani, V., Parida, A., Raina, S.N.: Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoides* Marsh. - Plant Cell Rep. **14**: 459-462, 1995.

Rout, G.R., Das, G.: An assessment of genetic integrity of micropropagated plants of *Plumbago zeylanica* by RAPD markers. - Biol. Plant. **45**: 27-32, 2002.

Samal, S., Rout, G.R., Nayak, S., Nanda, R.M., Lenka, P.C., Das, S.: Primer screening and optimization for RAPD analysis of cashew. - Biol. Plant. **46**: 301-304, 2003.

Schy, W.E., Plewa, M.J.: Molecular dosimetry studies of forward mutation induced at the *yg2* locus in maize by ethyl methanesulfonate. - Mutat. Res. **211**: 231-241, 1989.

Smith, K.J., Huyser, W.: Soybeans: Improvement, Production and Uses. 2nd Ed. - American Society of Agronomics, Madison 1987.

Sung, Z.R.: Mutagenesis of cultured plant cells. - Genetics **84**: 51-57, 1976.

Teparkum, S., Veilleux, R.E.: Indifference of potato anther culture to colchicine and genetic similarity among anther-derived monoploid regenerants determined by RAPD analysis. - Plant Cell Tissue Organ Cult. **53**: 49-58, 1998.

Thompson, J.A., Nelson, R.L.: Core set of primers to evaluate genetic diversity in soybean. - Crop Sci. **38**: 1356-1362, 1998.

Wilcox, J.R., Cavins, J.F., Nielsen, N.C.: Genetic alteration of soybean oil composition by a chemical mutagen. - J. amer. Oil Chem. Soc. **61**: 97-100, 1984.

Zucchi, M.I., Arizono, H., Morais, V.A., Fungaro, M.H.P., Vieira, M.L.C.: Genetic instability of sugarcane plants derived from meristem cultures. - Genet. mol. Biol. **25**: 91-96, 2002.