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## Mutagenic effect of sodium azide on somatic embryo regeneration and root growth of cotton (*Gossypium hirsutum* L. CV. SVPR2)

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Ganesan, M., Bhanumathi, P. and Jayabalan, N. (2005). Mutagenic effect of sodium azide on somatic embryo regeneration and root growth of cotton (*Gossypium hirsutum* L. CV. SVPR2). Journal of Agricultural Technology 1(2): 365-380.

The mutagenic effects of different concentrations of sodium azide (SA) on somatic embryo germination and survival percentage of somatic embryo derived plantlets of cotton (*Gossypium hirsutum* L. cv SVPR 2) were examined. The root number and root length of SA treated somatic embryos were significantly increased to  $5.5 \pm 0.4$  and  $39.5 \pm 2.4$  cm respectively when compared with the control ( $1.2 \pm 0.15$  and  $4.2 \pm 0.7$  respectively). After 30 days of root induction, in SA (10mM) treated cultured, the number of primary, secondary and tertiary roots were  $5.5 \pm 0.4$ ,  $20.2 \pm 2.9$  and  $11.2 \pm 0.2$  respectively, but in control somatic embryos the number of primary, secondary and tertiary roots were  $1.2 \pm 0.06$ ,  $6.3 \pm 0.5$  and  $4.2 \pm 0.8$  respectively. The percentage of somatic embryo germination was increased from 44.6 to 50.9 in 10 mM SA treated matured somatic embryos. Our study also proved that the duration of exposure of somatic embryos to SA influences percentage of germination; of all the treatments, SA 10 mM for 180 seconds proved most effective and increased root number and length. At the same time, the percentage of maturation was affected by SA treatment in all the concentrations.

**Key words:** B5 vitamins, induced root number and length, isozymes, MS medium, mutants

### Abbreviations:

T.HCl – Thymine hydrochloride

BA - Benzyl adenine;

KT - Kinetin;

2-ip – 2-isopentenyl adenine

PO - Peroxidase;

B5 - Gamborg medium;

PIC – Picloram,

NAA - Naphthaleneacetic acid;

GA<sub>3</sub> - Gibberellic acid;

SA – Sodium azide

MS - Murashige and Skoog medium;

SA – Sodium Azide

### Introduction

The uses of tissue culture techniques are important tools for varietal improvements of plants *via* genetic engineering, *in vitro* mutagenesis and

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somaclonal variation induction. Among the various techniques, somatic embryogenesis of plants through callus induction is widely used in many plant species for genetic transformation, mutant regeneration and somaclonal variation induction. Cotton (*Gossypium hirsutum* L.) is an economically important fibre crop and it has been estimated that 180 million people depend on cotton production for textile industry and seed oil (Benedict and Altman, 2001). A polyphenolic binaphthyl dialdehyde compound produced from the cotton pigment glands is gossypol, and is involved in resistance. Gossypol has several biological properties including anti-cancer, antimicrobial, anti-HIV, anti-oxidation and male contraceptive (PICMA, 1995).

Efficient reproducible protocols for enhanced regeneration of somatic embryos have been developed, which includes stress induced germination, haemoglobin-mediated germination (Jayabalan *et al.*, 2004; Ganesan and Jayabalan, 2004) and supplementation of oxygen to the growing embryos (Shimazu and Kurata, 1999). In this present investigation, we examined SA mediated *in vitro* mutagenesis to improve the somatic embryogenesis frequency of cotton. Mutation breeding methodologies have proven their potential for generating useful variability in crop improvement. Mutant cotton regeneration was not reported due to *in vitro* cotton regeneration difficulties (Rajasekaran *et al.*, 1996). *In vitro* mutagenesis is an important tool to induce phenotypic and genotypic variations. Spontaneous variation found in regenerated plants has been termed as "somaclonal variation" and this is a common phenomenon in callus-derived plantlets. Both somaclonal variation and induced mutations result in the production of new genotypes with limited change in their original genome (Maluszynski *et al.*, 1995). Compared with spontaneous mutation, mutagen induced mutations provide tools for the rapid generation of variability in crops. The influence and impact of mutation techniques for crop improvement has already been presented clearly (Maluszynski, 1990; Micke and Donini, 1990; Rutger, 1992). We have been attempting to enhance the variation through the application of mutagens to somatic embryos for successful regeneration. Compared with plant breeding techniques, *in vitro* mutagenesis can overcome some of the limitations such as lack of screening of effective mutant treatment techniques, production time for mutated plant regeneration and calculated on the basis of an expected frequency of mutation for a variety with desired characters (Maluszynski *et al.*, 1995). This research therefore focuses on the regeneration of mutagen treated somatic embryos.

In the present investigation, mutagen sodium azide (SA) was considered because of its most efficient mutagenic characters (Konzak *et al.* 1972; Nilan,

1981) and its easy handling and the low cost (Nilan and Pearson, 1973; Lundqvist, 1992; Olsen, 1993).

## **Materials and methods**

### ***Plant material and seed germination***

Delinted cottonseeds, SVPR2 (Parentage – TSDT 22 × JR 36), were collected from Cotton Research Institute, Tamil Nadu Agricultural University, Srivilliputhur, Tamil Nadu, India. Surface sterilization of seeds and *in vitro* regeneration of seeds were carried out by our previous method (Ganesan and Jayabalan, 2004).

### ***Embryogenic callus induction***

Horizontally sectioned hypocotyl explants (0.4-0.6 cm in length) from 6-day old seedlings, were placed horizontally on medium comprising full strength MS salts ( $\text{mg l}^{-1}$ ) (Murashige and Skoog, 1962), myo-inositol ( $50 - 200 \text{ mg l}^{-1}$ ), T.Hcl ( $0.1 - 1 \text{ mg l}^{-1}$ ),  $8.0 \text{ g l}^{-1}$  agar and glucose  $30 \text{ g l}^{-1}$  and plant growth regulators like PIC ( $0.1 - 0.5 \text{ mg l}^{-1}$ ) and KT ( $0.05 - 0.2 \text{ mg l}^{-1}$ ). After 3 weeks of culture, proembryogenic calluses were isolated and subcultured on the same medium for another 2 weeks at weekly intervals for the induction of embryogenic calli.

### ***Embryoid induction and maturation***

500 mg of embryogenic callus was separately transferred to 500 ml conical flasks (Borosil, India), which contained 50 ml of liquid medium fortified with MS salts,  $0.1-2 \text{ g l}^{-1} \text{ NH}_3\text{NO}_4$ ,  $0.5-2.5 \text{ g l}^{-1} \text{ MgCl}_2$ ,  $50-150 \text{ mg l}^{-1}$  myo-inositol,  $0.1-0.4 \text{ mg l}^{-1}$  T. HCl,  $30 \text{ g l}^{-1}$  glucose,  $1-3.0 \text{ mg l}^{-1}$  PIC,  $0.05-0.2 \text{ mg l}^{-1}$  KT and  $0.1-0.4 \text{ mg l}^{-1}$  2-ip for embryoid induction. For embryoid induction through suspension cultures, weekly subculture was carried out, removing half of the liquid medium and replacing with fresh medium containing the desired amount of hormones. The liquid cultures were maintained at 125 rpm at a light intensity of 2500 lux. After 6-7 weeks of subculture batches of proembryogenic tissues were formed (these tissues only induced the pro-embryoid formation) and after another 6 weeks of subculture of pro-embryoids, whitish transparent, visible globular embryoids were formed in clusters on the surface of embryogenic tissues. Immature globular embryoids

were subcultured from liquid to semisolid medium (0.6 % agar – w/v) for complete maturation.

### ***Plantlet regeneration***

After 4 to 6 weeks, matured green somatic embryos were identified by the presence of cotyledonary stage and transferred to plantlet regeneration medium fortified with MS basal salts (half strength), B5 vitamins (Gamborg *et al.* 1968), 30 g l<sup>-1</sup> glucose along with GA<sub>3</sub> (0.1-0.5 mg l<sup>-1</sup>) and BA (0.1-0.5 mg l<sup>-1</sup>). After complete regeneration of plantlets (35 days), the regenerated plants were transferred to plastic pots containing sand, soil and vermiculite (1:1:1 ratio). Hardened plants were completely covered with plastic bags for 2 weeks to maintain humidity, the bags progressively removed to allow adaptation to normal environmental conditions. Then plantlets were transferred to earthen pots for further growth and development.

### ***Mutagen treatment***

Five week old embryoid clusters were selected for mutagenic treatment. The embryoids were dispersed in sterilized 0.1 M phosphate buffer at pH 3 with different concentrations of SA (1-20 mM) (Himedia, India) for the period of 30-500 seconds. After treatment, explants were washed with MS basal liquid medium and transferred to embryoid maturation medium. Mutagen treatment was carried out in aseptic condition. The plantlets regenerated through normal somatic embryogenesis were considered as control plantlets.

All the hardened plants were maintained in normal green house condition. During germination from somatic embryos to whole plantlets, phenotypic characters were carefully observed for variation. The characters studied include variation in root and shoot size and number, plant colour and size and chlorophyll deficiencies (albina, xanta, viridis and chlorine mutants).

### ***Isozyme analysis***

Variations in phosphoglucosmutase (PGM) activities was analysed in this study by using poly acrylamide electrophoresis (PAGE). The leaf samples (0.2 g) obtained from the 30 days old regenerated plants were extracted in 1 ml of 0.2 M Tris-HCl buffer (pH-8.5) containing 1 M sucrose, 15 mg ml<sup>-1</sup> dithiotheritol (DTT) and 10% (w/v) insoluble polyvinylpyrrolidone (PVP-6755). The procedures of Wetter and Dyck (1983) were followed for gel

preparation and electrophoresis. After electrophoretic separation, staining was achieved by the method of Arulsekhar and Parfitt (1986).

### ***Statistical analysis***

Means and standard errors were used throughout the study and the values were assessed using a parametric Moods median test. ( $n = 6$ ) (Snedecor and Cochran, 1989). As appropriate, the data were analyzed for variance by Duncan's multiple range test (DMRT) using the SAS program (SAS Institute, Cary, N.C.).

## **Results and discussion**

### ***Embryogenic callus induction***

Highly friable, creamy white embryogenic callus was obtained from the medium fortified with MS salts, 100 mg l<sup>-1</sup> myo-inositol, 0.3 mg l<sup>-1</sup> T.HCl, 0.1 mg l<sup>-1</sup> KT, 0.3 mg l<sup>-1</sup> PIC, 30 g l<sup>-1</sup> glucose and 8.0 g l<sup>-1</sup> agar. During the formation of embryogenic callus the non-embryogenic callus formation was observed. From the obtained callus, embryogenic tissues were isolated and sub-cultured on the same medium to improve the quantity of embryogenic callus. The embryogenic callus was identified by the presence of small, less vacuolated and densely filled cytoplasmic cells with creamy white colour (Sakhanokho *et al.* 2000).

### ***Embryoid induction and maturation***

The well-developed pro-embryoids were induced from liquid medium fortified with MS salts, 100 mg l<sup>-1</sup> myo-inositol, 0.2 mg l<sup>-1</sup> T.HCl, 30 g l<sup>-1</sup> glucose, 0.2 mg l<sup>-1</sup> 2,4-D, 0.2 mg l<sup>-1</sup> 2iP, 1.0 g l<sup>-1</sup> NH<sub>3</sub>NO<sub>4</sub> and 100 mg l<sup>-1</sup> MgCl<sub>2</sub>. The pro-embryoids were induced only after 6 weeks of subculture and the cell cluster formation was observed before pro-embryoid induction. During the ontogeny, cell cluster formation was clearly identified by the formation of double-celled stage to multi-celled stage and embryo head formation. The embryogenic single cells and cell clusters were identified by the presence of densely filled cytoplasm with more amounts of starch granules. The cell clusters with proembryoids were sieved by 100 µm mesh and transferred to semisolid embryo maturation media fortified with 0.6 % agar.

2, 4-D has widely been used for the induction of embryogenic callus and embryoids (Zhang, 2000). Zeatin (Zhang *et al.* 2000), and a combination of

NAA with KT have also been used for induction and proliferation of embryoids (Sakhanokho *et al.*, 2000). In our study combination of PIC, KT, T.HCl, MgCl<sub>2</sub> and NH<sub>3</sub>NO<sub>4</sub> produced required greenish-white healthy embryoids after 6 weeks of subculture. The induced embryoids were subcultured for another 2 weeks for complete maturation and the formation of globular, heart and green torpedo shaped embryos was observed. Our study also shown that the embryoid induction rate was improved by supplementation of NH<sub>4</sub>NO<sub>3</sub> and MgCl<sub>2</sub> (Kumria *et al.*, 2003).

### ***Plant regeneration from somatic embryos***

Mature embryoids were transferred to somatic embryo regeneration medium fortified with MS salts, B5 vitamins, GA<sub>3</sub> (0.2 mg l<sup>-1</sup>) and BA (0.2 mg l<sup>-1</sup>). In this concentration only 44.6% of plant regeneration from matured somatic embryos was observed. The regenerated plants with primary, secondary and tertiary roots were separately transferred to plastic pots for hardening. 79.5% of survival percentage of hardened plants was observed in the earthen pots at green house condition. The survival percentage of hardened plants dependent on root number and length. Per somatic embryo, 1.2 ± 0.06 primary roots, 6.2 ± 0.5 secondary roots and 4.2 tertiary roots per secondary root were formed.

### ***Effect of mutagen on embryoid induction, maturation and plant regeneration***

Both beneficial and harmful effects on somatic embryo regeneration were observed by mutagen treatment. Maturation of somatic embryos was affected by the treatment of SA in all the concentrations. At the same time, regeneration percentage of somatic embryos was increased by 10 mM SA treatment. In control cultures, 53.6% of maturation was noticed from the 98 somatic embryos tested. But in the case of mutagen treated cultures, the maturation percentage was decreased and it ranged from 10.4 to 48.8%. During normal somatic embryogenesis, 52.5 ± 3.2 out of 98 somatic embryos regenerated into plantlets i.e. 44.5% regeneration was noticed from the matured somatic embryos. In 10 mM SA treated cultures, a maximum of 51% of somatic embryo regeneration was observed from the matured somatic embryos (Table 1). Except in 10 mM, all other concentrations of SA treated cultures showed decreased plant regeneration compared to the untreated control. In higher doses, at 12, 14 and 16 mM SA treated cultures, a reduced percentage of

**Table 1.** Effect of different concentrations of SA on regeneration percentage and variant induction in cotton (*Gossypium hirsutum* L. cv. SVPR 2).

Parameters	Concentrations of sodium azide (SA) mM						
	0	6	8	10	12	14	16
Number of embryos matured	52.5±3.2a (98)	35.2±1.8a (78)	36.4±2.4a (80)	33.0±0.9a (70)	35.2±0.4a (72)	21.2±1.9a (75)	8.5±2.1a (81)
Percentage of Somatic embryo maturation	53.6	45.1	45.5	47.14	48.8	28.2	10.4
Number of embryos germinated	23.4±2.1 b	14.8±1.1b	15.3±2.1b	16.8±2.2b	11.2±1.6b	2.2±0.2c	NR
Percentage of somatic embryo germination	44.5	42.0	42.0	50.9	31.8	10.3	NR
Number of embryos rooted (without shoot)	2.3±0.2bc	4.2±1.0bc	4.8±0.6c	7.2±1.2c	8.5±3.2c	10.2±3.5b	6.8±3.0a b
Number of shooted embryos (without root)	1.2±0.15c	1.6±0.6c	2.0±0.2cd	1.4±0.15c d	1.4±0.4d	0.6±0.1cd	NR
Abnormalities in germination	--	--	--	--	++	+++	++++

Values within parenthesis indicated the number of embryoids tested.

Values are means ± standard error of three repeated experiments.

Each treatment consisted of four replicates.

Means within a column followed by the same letters are not significant at P=0.05 according to DMRT

NR – Nil Response.

somatic embryo conversion was observed. At 16mM SA treatment, only maturation of somatic embryos was noticed and the whole plant regeneration was completely absent due to browning and death of somatic embryos. Abnormal embryoid formation is usual process in which the somatic embryo will not produce the whole plantlet; instead it will produces only shoots or roots or sometimes callus. Abnormal embryoid formation and production of shoots or roots germination was observed at 12 and higher concentrations of SA treated cultures. Treatment of higher concentrations (more that 12 mM SA)

showed lethal effect to the somatic embryos. The regenerated control R0 plants were phenotypically similar to *in vivo* seed derived plantlets.

### ***Effect of mutagen on variations in phenotypic characters in regenerated plants***

The results of this experiment proved that all the regenerated plants showed greater variation in the phenotypic characters compared with control plantlets. Higher percentage of root length increased variant was significantly regenerated in this study and the mutants with increased root number and dwarf variants were also obtained. In the first variants, the root length of regenerated somatic embryo reached upto 38.5 cm (range 36-40 cm) after 20 days of germination (Fig. 1a) and 3 plantlets showed increased root length variation. Usually cotton regeneration through somatic embryos is quite difficult and requires optimized plant growth regulators and media (Wilkins *et al.*, 2000). Earlier reports (Gould *et al.*, 1991) have been shown that root initiation could be difficult in cotton during *in vitro* regeneration. In some cases the elongated shoots were transferred to rootone containing soil for root induction (Hemphill *et al.*, 1998). In other crop plants also different difficulties in the simultaneous production of root and shoot induction was noticed (Prem *et al.*, 2000). For successful regeneration of plantlets from matured somatic embryos, simultaneous induction of roots and shoot from the somatic embryos is essential (Varisai *et al.*, 2004) and it has been accepted that the regeneration of somatic embryos with both root and shoot is very recalcitrant (Choi *et al.*, 1999). Hence, the studies on somatic embryos for successful regeneration become valuable. In this present investigation, the percentage of root induction and elongation was enhanced by the treatment of SA (10 mM) in cotton. The increased root length significantly influenced the survival percentage of the regenerated somatic embryos and in these plantlets 100 % survival rate was observed compared with control (Table 2). The number of roots per somatic embryo also increased in some of the mutants. About 5.5 primary roots were regenerated from single somatic embryos (Fig. 1b) with the length of 38.5 cm after 180-second treatment (Fig. 2). Highest of  $20.2 \pm 2.9$  secondary roots per primary root and  $11.2 \pm 0.2$  tertiary roots per secondary root were observed in the 10 mM SA treated somatic embryos. Generation of a single primary root per germinated somatic embryo is common in cotton (Ganesan and Jayabalan, 2004). Our previous experiments proved that survival percentage of regenerated somatic embryos reached up to 65% during normal condition with one primary root. The survival percentage was contrastingly increased up to 78% when plants were hardened after formation of secondary and tertiary

roots. Formation of secondary and tertiary roots was clearly observed during maintenance of regenerated somatic embryos up to 30 days without any disturbance at normal culture room condition. These results also proved that no specialized plant growth regulators were required for secondary and tertiary root formation.

The treatment of SA (10 mM) to matured somatic embryos increased the number and length of roots. Our observation also proved that the percentage of plant regeneration from matured somatic embryos was also increased by SA treatment at 10 mM level for 180 seconds exposure. In 10 mM SA treated somatic embryos increased percentage (50.9) of somatic embryo regeneration was observed from matured somatic embryo when compared to control. In other concentrations of SA treated somatic embryos reduced percentage of somatic embryo conversion was observed. The replications and repeated experiments confirmed that treatment of 10mM SA for 180 seconds accelerated the somatic embryo conversion percentage with high frequency of root induction. Our studies on mutant plant regeneration *via* somatic embryogenesis produced the mutants with increased root length and root number without affecting the germination percentage of somatic embryos (10 mM SA treatment), which was significant and it becomes important for successful acclimatization of regenerated cotton somatic embryos.

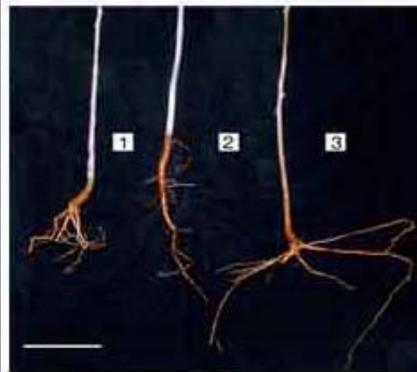
At that same time, after 40 days of germination of hardened plants, variation in plant height and leaves were clearly observed. Similar types of variants were observed in several plants including strawberry (Nehra *et al.*, 1992), Barley (Ruiz *et al.*, 1992) and Phalaenopsis (Chen *et al.*, 1998). A few ( $2.5 \pm 0.2$ ) germinated somatic embryos showed plant regeneration without branching. This unbranched mutant exhibited all other characters of normal cotton plantlets except branching. The flowers and boll formations were observed on the top of the plantlet with very small branching (Fig. 3a). In some of the variants ( $1.75 \pm 0.25$  plants), high concentration of mutagen (15 mM) treated somatic embryos showed slow growth with significantly reduced germination percentage and they reached only half of the total height of the parental lines during flowering. In these dwarf variants, reduced leaf size, petiole length and reduction in number of serrations were clearly observed (Fig. 3b). These types of variation in plant height, particularly dwarf plantlets, were observed in strawberry plants during the treatment of different concentration of plant growth regulators (Nehra *et al.*, 1992).

In our study, chlorophyll mutants (xantha) were also obtained by using 10 mM SA concentration (Fig. 3c). These types of chlorophyll and early flowering mutants were also regenerated through mutation breeding techniques (Kechagia *et al.*, 1994; Reddy *et al.*, 1992; Kuzin, 1990) and this is the first

report via *in vitro* somatic embryogenesis (Fig. 3d). The above said mutants showed some of the economically important characters (early flowering). Hence, our further research studies will be focused on the characterization of above mentioned mutants.



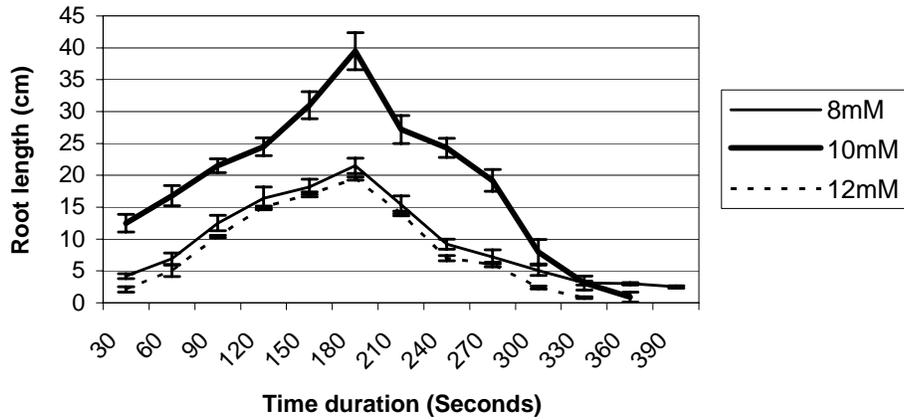
**Fig.1a**



**Fig.1b**

**Fig. 1** Effect of SA (10 mM) on root length and number of regenerated somatic embryos.

- a) Plantlets with elongated roots up to 39.5 cm (Bar = 5 cm).
  - 1 – Control plantlet derived from matured somatic embryos
  - 2, 3 – Mutants regenerated from the mutagen treated somatic embryos.
- b) Plantlets with more primary roots (Bar = 5 cm).
  - 2 – Control plantlet derived from matured somatic embryos
  - 1, 3 – Mutants regenerated from the mutagen treated somatic embryos.



**Fig. 2.** Effect of SA treatment time duration on the root length of regenerated somatic embryos.

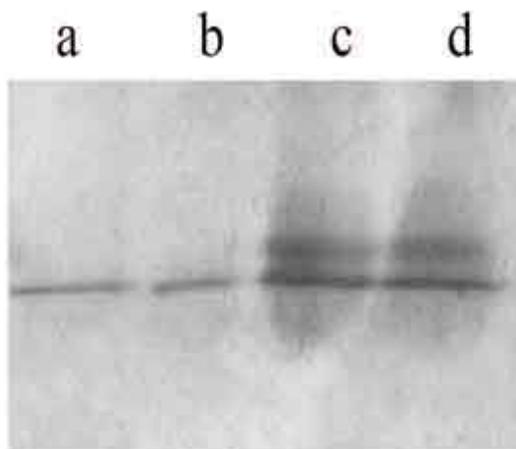


**Fig. 3.** Mutants with variation in phenotypic characters.

- a) Tall mutant with small branches and flower heads in the top the plant (Bar = 25 cm)
- b) Dwarf plant (Bar = 30 cm)
- c) Chlorophyll mutant (Bar = 1.8 cm)
- d) Early flowering plant (Bar = 9 cm)

In our experiments, PGM isozyme showed variations. In the control plants only one band was observed, where as in the obtained mutants 2 to 3 zymograms were observed (Fig. 4). This result proved that, variation in banding pattern is observed due to SA treatment, indicating altered gene expression in the mutants. The variation in the isozyme pattern supported the phenotypic and genotypic variation occurrence. The studies on isozyme banding are vastly increased for the analysis of variants and genetic diversity (Oja *et al.*, 2003). By using this isozyme studies through electrophoresis, the genetic variation in populations of Scots pine (*Pinus sylvestris* L.), one of the species covering large areas in Turkey, have been studied (Turna, 2003). Production of improved protocols for cotton somatic embryogenesis was vastly increased to rectify the regeneration problems and unfortunately, the survival percentages of regenerated plants was very low (Chaudhuri *et al.*, 2004).

This experiment proved that SA treatment (10 mM) for 3 minutes, greatly increased the percentage root number and length. This enhanced root length and number leads to the improved survival percentage of regenerated somatic embryos. Occurrences of phenotypic and genotypic variations may trigger the production and yield and it will be useful for cotton germplasm improvement. Presently, our experiment was targeted to evaluate this protocol for transgenic somatic embryo regeneration, somaclonal variation and mutant induction studies.



**Fig. 4.** Variation in isozyme pattern of phosphoglucomutase (PGM) in the control and mutagen treated plants.

Lanes a, b - normal plantlets and

Lanes c, d - mutagen treated plans

**Table 2.** Effect of different concentrations of SA on number of roots, root length and survival percentage of regenerated plants of cotton.

Parameters	Concentrations of sodium azide (SA) mM					
	0	6	8	10	12	14
Number of Primary roots per somatic embryo	1.2±0.06d	2.3±0.12c	3.4±0.4b	5.5±0.4a	2.3±0.2c	0.8±0.2de
No. of secondary roots per primary root (30 days)	6.3±0.5e	9.5±0.23d	14.3±1.4b	20.2±2.9a	11.4±1.6c	4.2±0.8f
No. of tertiary roots per secondary root (30 days)	4.2±0.8d	6.3±1.2c	8.9±0.9b	11.2±0.2a	5.3±0.4cd	3.0±0.2e
Primary root length (cm) (30 days)	8.5±0.8d	15.5±0.9c	21.5±1.4b	39.5±2.4a	19.5±1.4bc	5.5±0.7e
Survival percentage (after two months of hardening)	79.5±2.8d	88.4±4.3c	100±00a	100±00a	95.2±2.9b	42.0±4.5e

Values are means ± standard error of four repeated experiments.

Each treatment consisted of three replicates.

Means within a column followed by the same letters are not significant at P=0.05 according to DMRT

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(Received 13 June 2005; accepted 29 November 2005)