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Full Length Research Paper

Effect of various amino acids on shoot regeneration of sugarcane (*Sacchrum officinarum* L.)

Shaheen Asad*, Muhammad Arshad*, Shahid Mansoor and Yusuf Zafar

Plant Biotechnology Division, National Institute for Biotechnology and Genetic Engineering [NIBGE], P.O.Box-577, Jhang Road, Faisalabad, Pakistan.

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An efficient regeneration protocol from sugarcane callus has been developed through inclusion of amino acids in regeneration medium. Sugarcane (*Saccharum officinarum* L.) SP-241 callus was induced from meristematic explants cultured on Murashige and Skoog medium supplemented with B5 vitamins containing 13.6 µM 2-4, dichlorophenoxyacetic acid, 0.05% (w/v) casein hydrolysate, 10% (v/v) coconut water and 3% glucose. Five levels (0.1, 0.25, 0.5, 0.75, 1.0 mM) of five different amino acids (glutamine, asparagine, glycine, cysteine and arginine) were tested on the same medium containing 6.8 µM 2, 4-D to compare their ability to induce somatic embryogenesis and shoot regeneration from six week old callus. Among the tested amino acids, glycine (0.75 mM), arginine (0.5 mM) and cysteine (0.25 mM) showed significant effect on smatic embryogeensis (94%) and shoot production as compared to non-amino acid medium. Of the evaluated amino acids, glycine was most effective to promote somatic embryogeensis and maximum shoot regeneration. Except low callus growth index (G.I) amino acid treatment resulted in high percentages of somatic embryogenesis and shoot regeneration as compared to non-amino acid medium. Regenerated shoots when transferred to same medium supplemented with 19.7 µM IBA, grew normal and developed roots. These results indicate the efficacy of amino acids in stimulating sugarcane plant regeneration from non embryogenic callus, and may be suitable for future use in genetic transformation studies to enhance regeneration of transgenic sugarcane plants.

Key words: *Saccharum officinarum* L, amino acids, embryogenic calli, plant regeneration, 2, 4 dichlorophenoxyacetic acid.

INTRODUCTION

More than 100 countries produce sugar, 74% of which is made from sugarcane (World Sugar Statistics, 2006). Due to its importance in agricultural industry, concerted efforts are being made for its improvement using conventional and biotechnological techniques. High ploidy level, specific climatic requirements for flowering and seed setting coupled with longer life cycle are major limitations for improvement through conventional breeding. Furthermore, since many sugarcane varieties do not produce fertile seeds (Sugarcane encyclopedia, 1994), and due to difficulties in selfing and crossing, the crop is one of those plant species that could not survive without human intervention (Helen, 1939). However, plant biotechnology has the potential to overcome these problems and bring about sugarcane improvement through tissue culture techniques and genetic engineering. The development of tissue culture protocols for embryogenic callus induction and plant regeneration is essential for crop improvement through genetic engineering. The first report on sugarcane tissue culture was published in 1961 (Liu, 1993). Subsequently callus initiation and whole plant regeneration directly or indirectly has been reported. Several protocols for embryogenesis and organogenesis have been developed using callus derived from various explants. Rapid callus has been obtained mostly from young expanding leaves or immature inflorescences. Somatic embryogenesis and direct plant regeneration was reported from young leaves (Ahloowalia and Maretz-

*Corresponding author: E-mail: arshadchbt@yahoo.com or sasad@nibge.org.

Abbreviations: Gln, Glutamine; Asn, asparagines; Cys, cysteine; Arg, arginine; Gly, glycin; CW, coconut water; ca, approximately; ctr, control; 2,4-D, dichlorophenoxyacetic acid; GRPs, glycine-rich proteins.

ki, 1983), as well as organogenesis from young leaves (Chengalrayam and GalloMeagher, 2001; Fitch and Moore, 1990; Gossal et al., 2006). In most of these studies, callus was induced in the presence of auxin, either 2,4-dicholorophe-noxyacetic acid (2, 4 -D) or picloram. To promote regeneration, callus was transferred to medium with either a reduced auxin concentration or containing no auxin (Ahloowalia and Maretzki, 1983). Callus has also been transferred to 9.3 µM kinetin and 22.3 µM NAA to obtain regeneration (Irvine and Benda, 1987; Irvine et al., 1991).

Tissue culture techniques are widely used in sugarcane improvement programes. Somatic embryogenesis in cell and callus cultures has also become the choice for high volume propagation systems and setting up such a large pathogen free delivery system would be desirable in multiplying new sugarcane varieties. Production of transgenic plants through any transformation method (Physical, chemical or biological) requires highly efficient and reliable plant regeneration systems (Arencibia et al., 2000).

Exogenously added amino acids play an important role in plant tissue culture but culture media of existing regeneration protocols are scarcely supplemented with amino acids. Specific media components involving amino acids have been found to play an important role on tissue culture systems of certain species (Benson, 2000; Liu, 1993). Amino acids have been used as organic nitrogen source in *in vitro* cultures of several species as alfalfa, maize, sorghum, pineapple, rice and other monocots to enhance somatic embryogenesis and regeneration (Skokut et al., 1985; Claparols et al., 1993; Rao et al., 1995; Hamasaki et al., 2005; Grewel et al., 2006). It has been suggested that positive effect of organic nitrogen, in comparison to that of inorganic sources is associated to enhanced mobility of the former at a lower energy cost than the later (Kim and Moon, 2007). Therefore, the objective of the present study was to conduct detailed and systematic studies on five different amino acids to determine optimum amino acid concentration to develop the most efficient sugarcane regeneration system. Five different amino acids, glutamine, asparagines, cysteine, arginine and glycine were evaluated for their ability to induce shoots in sugarcane. To our knowledge, this is the first report in which a number of different amino acids have been compared for their effects on sugarcane regeneration.

MATERIALS AND METHODS

Sugarcane variety, SP-241 provided by Directorate of Sugarcane, Ayub Agricultural Research Institute, Faisalabad, Pakistan was used in this study for callus induction and regeneration. Apical portion of healthy shoots were stripped to the terminal bud and attached immature leaf rolls were immersed in a solution of 10% commercial bleach (0.5% sodium hypochloride) for 20 min. Excess bleach was removed by repeated rinsing (five times) with sterile deionized distilled water. Leaf rolls were peeled under sterile conditions to cylinder (inner whorls) approximately 5 mm in diameter. Serial slices, 3 mm thick, were removed from the part immediately above the apical meristem. Three to six slices were taken from each cylinder and cultured on callus induction medium consisting of MSB (Murashige and Skoog, 1962) basal salts plus B5 vitamins (Gamborg et al., 1968) supplemented with 3% glucose, 13.6 µM 2, 4-D, 0.05% (w/v) casein hydrolysate and 10% coconut water (v/v). Fresh nodular callus formed on callus induction medium was cultured on MSB medium containing 3% glucose, 6.8 µM 2,4-D 0.05% (w/v) casein hydrolysate and 10% CW (v/v), with either glutamine, asparagines, cysteine, arginine or glycine at five different concentrations: (0.1, 0.25, 0.5, 0.75, 1.0 mM) with controls without additional amino acids, making a total of 26 treatments.

All culture media were solidified with 0.35% (w/v) phytagel and the pH was adjusted to 5.7 - 5.8 before autoclaving. All cultures were subcultured every 4 weeks, maintained initially for 45 days in the dark. Thereafter, cultures were incubated in the growth room at 25 ± 1°C under 14 / 10 h day/ night light (1000 lux) from a cool white fluorescent lamp, and the relative humidity of the growth room was maintained at 75%. All the experiments were repeated three times and data were averaged on number of shoots produced. A completely randomized design experiment was conducted with three replicates per treatment. Six to seven pieces (4 - 5 mm/100 mg) of forty five days old callus (ca 0.7 g) per petri dish (100 × 20 mm) were subjected to each treatment. All cultures were transferred to fresh medium every 25 - 28 days. After the second subculture, the percentage and number of shoots produced were calculated per callus dividing the total number of shoots produced by the number of calli (6 - 7 mm/200 mg) produced. Each callus was also individually analyzed to verify the results. After the 3rd subculture, regenerated shoots were transferred to a similar medium composition containing 19.6 µM IBA for further shoot and root development. For convenience, the experimental design consisted of two sequential experiments, In the first set, calli were cultured on all the five selected amino acids at five levels, the effect of the amino acids in shoot regeneration were analyzed. While in the second experiment, the amino acids that showed best performance in the previous experiment were evaluated for their effect on callus growth index, percentage of embryogenic calli and number of shoots per calli. Slide write software was used for graphical presentation of data and test of significance was carried out using analysis of variance. Treatment means were separated using the Duncan's Multiple Range Test (PROC GLM, SAS Institute, 1996).

RESULTS AND DISCUSSION

Amino acids have been found critical to induce somatic embryogenesis in plant tissue culture medium. In orchard grass, embryos formed on amino acid containing medium showed high percentage of conversion and considerably less incidence of precocious germination (Trigiano et al., 1992). The yield of alfalfa embryos was also considerably improved when amino acids were added to callus maintenance medium. Apical leaf rolls of sugarcane variety SP- 241 initially produced white callus which subsequently turned into yellow nodular callus after six weeks of culturing. In experiment 1 we observed that somatic embryogenesis and shoot regeneration was high in three of the five amino acid treatments. Somatic embryogenesis percentages were higher either in the presence of glycine, arginine or cysteine up to 95% as compared to control 40% (Figures 1 and 2). These observations led us to evaluate the best performing amino acids, glycine, cysteine and arginine to determine their concentration for

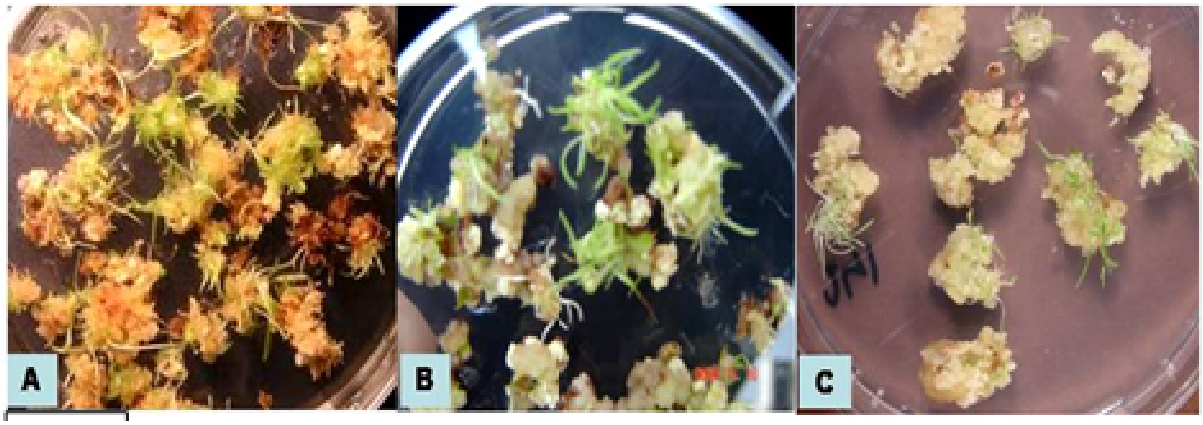

Figure 1. Regenerating callus on MSB supplemented with: (A) 0.75mM glycine (B) 0.50 mM arginine (C) 0.25 mM cysteine.

Table 1. Effect of cysteine, arginine and glycine on callus growth, embryogenesis and shoot regeneration.

Treatment	Callus weight (g)	Growth Index (G.I)	# of Embryogenic calli	Embryogenic calli (%)	# of Shoots/calli
So (Control)	8	11.4	30	36±4.0g	4±3.3d
So + Cysteine					
0.25 mM	5.8	8.28	49	69±2.88b	6±3.0b
0.5 mM	6.4	9.14	40	64±2.0c	5±2.9bc
0.75 mM	6	8.57	38	60±2.08d	4±3.6d
So + Arg.					
0.25 mM	7	10	36	51±3.0f	4±2.9
0.5 mM	6.2	8.8	40	66±1.6c	6±1.4b
0.75 mM	5.9	8.42	37	56±3.2e	5±2.0bc
So + Gly.					
0.25 mM	7.5	10.71	42	51±2.64f	4±2.2d
0.5 mM	6.9	9.8	48	72±2.5b	7±2.0b
0.75 mM	6.2	8.8	60	91.33±1.5a	14±1.5a

G. I = Final fresh weight /Initial fresh weight. S0 = MSB + 2, 4-D 6.8 µM+CH (0.5%) + CW (10%) + 3% glucose. Data is X ± S.D. Each treatment consists of 7 - 9 explants and 6 replication per treatment.
Means followed by same letters within a column do not differ significantly at 5% according to Duncan's Multiple Range Test.

optimal callus growth index, percentage of embryogenic calli, and number of shots per calli.

Effect of various amino acids on green shoot production was evaluated after 6 weeks using yellow nodular fresh calli as starting material. It was observed that type of amino acids and amount used in the medium had significant effect on the induction of green shoot meristems (Figure 1). Glycine promoted maximum shoots production among the tested amino acids at 0.75 mM (859, Figure 1B), while other amino acids and non-amino acid treatments induced fewer shoots (Gln. 200, Asn, 200, Arginine 560). Cysteine followed glycine and arginine producing shoots 280 at 0.25 mM (Figure 3). However, comparisons between individual best concentrations for each amino acid revealed that 0.75 mM glycine and 0.5 mM arginine followed by 0.25 mM (Figure 3) cysteine were optimum for rapid, continuous and efficient shoot production (Figure 1).

The experiment 2 was performed to study the concentration effect of optimized amino acids on callus. The results therefore showed that in the absence of amino acids, callus possessed high growth index but low percentage of embryogenic calli as well as less number of shoots per callus as compared to amino acid treatment (Table 1). Callus growth index was found high at low amino acid concentration which decreased with increased amino acid concentration but with high incidence of somatic embryogenesis and number of regenerated shoots per calli. Glycine was superior to other amino acids in percentage of embryogenic calli and number of shoots per calli at a concentration of 0.75 mM producing the highest embryogenesis and shoot regeneration per callus that is 14 ± 1.5 though it possessed low growth index (8.8) (Table 1). Although the embryogenic calli of

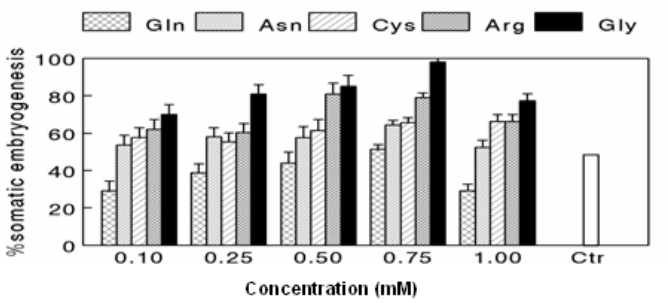

Figure 2. Effect of glutamine, asparagines, glycine, cysteine and arginine somatic embryogenesis in sugarcane.

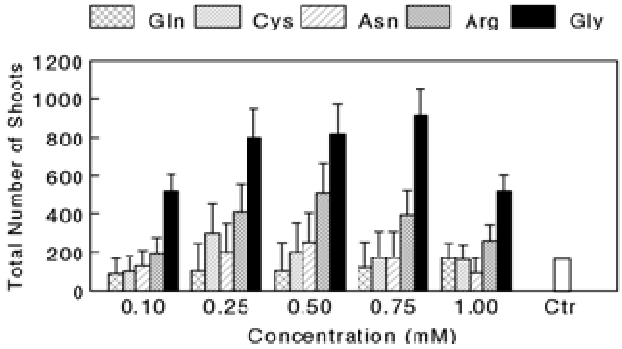

Figure 3. Effect of glutamine, asparagines, glycine, cysteine and arginine on total number of shoots produced from sugarcane from 9 week of culture.

sugarcane possess very good potential for plantlet regeneration, most of the time it exhibit precocious germination and shoot development with crowded nature and abnormal plant like albino, xantha were also observed (Fitch and Moore 1990). The precocious germination generally occurs in cultured embryos when the maturation has been short circuited and maturation is transitory, frequently indispensable stage between embryo development and embryo germination phases (Quatrano, 1987; Liu, 1993; Vasil, 1983). In the present study glycine surpassed all other amino acids in somatic embryogenesis and regeneration of shoots from sugarcane callus. It can be explained by its role as a source of organic nitrogen as well as its use in restructuring of dividing cells through synthesis of cell wall. So it allowed callus tissue to differentiate through restructuring the cells by synthesizing the cell wall. RNA levels of gene encoding cell wall specific glycine rich proteins (GRPs) have been found high in differentiating cells (Sato et al., 1995; Condit and Meagher, 1986).

From the present study, it is clear that amino acids (glycine, arginine or cystein) presence in the medium may promote the shoot production-through the differentiation of dividing cells that is the reason that it possess comparatively low growth potential because majority of dividing cells become differentiated rather undergoing faster cell proliferation. So it is advisable to include low concentrations of Auxin i.e. (6.8µM 2, 4-D) to support and maintain the momentum of cell division. In the future, the combined effects of evaluated amino acids on different sugarcane genotypes will be investigated as somatic embryogenesis and plant regeneration in sugarcane are strongly genotype dependent. To support this, work on endogenous free amino acid determination could be more explanatory to unravel the mechanism of promotion of shoot regeneration of sugarcane by amino acids (Sen et al., 2002).

Through this study, we were able to report a protocol using apical meristem as a source of explants culturing on MSB medium supplemented with 13.6 µM 2,4-D, 0.05% casein hydrolysate (w/v), 10% coconut water (v/v) and 3% sucrose (w/v) until yellow and nodular callus is formed. This callus could be used as a starting material on the MSB medium containing 6.8 µM 2,4-D supplemented with 0.75 mM glycine or 0.25 mM cysteine and 0.5 mM arginine. Upon elongation, shoots are transferred to MSB medium containing 19.7 µM IBA for rooting with subsequent transfer to the soil. This protocol could be applied in commercial propagation of elite cultivars, *in vitro* selection, production of disease free plants, and rapid, mass regeneration of transgenic plants from transformed sugarcane callus tissues as well as potential use of amino acids in promoting regeneration in other plant species.

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REFERENCES

Ahloowalia BS Maretzki A (1983). Plant regeneration via somatic embryogenesis in sugarcane. Plant Cell Rep. 2: 221-225.

Arencibia AD, Carmona R, Cornide MT, Menedez E, Molina P (2000). Transgenic sugarcane (*Saccharum* Species). In Bajaj YPS (eds), Biotechnology in Agriculture and Forestry 46. Transgenic crops Springer Verlag, Heidelberg. 1: 188-206.

Benson EE (2000). *In vitro* plant recalcitrance, an introduction. In Vitro Cell. Dev. Biol. Plant 36: 141-148.

Claparols I, Santosa MA, Torne MJ (1993). Influence of some exogenous amino acids on the production of maize embryogenic callus and on endogenous amino acid content. Plant Cell Tissue Organ Cult. 34: 1-11.

Chengalrayam K, GalloMeagher M (2001) Effect of various growth regulators on shoot regeneration of sugarcane. In Vtro Cell. Dev. Biol. 37: 434-439.

Condit CM, Meagher PB (1986). A gene encoding novel glycine-rich structural proteins of Petunia. Nature 323: 178-181.

Fitch MMM, Moore PH (1990). Composition of 2,4-D and picloram for selection of long term totipotent green callus cultures of sugarcane. Plant Cell Tissue Organ Cult. 20: 157-163.

Gamborg OL, Miller RA, Ojima K (1968). Nutrient requirements of suspension culture of soyabean root cells. Exp. Cell Res. 50: 150-158.
Grewel D, Gill R, Gosal S (2006). Role of cysteine in enhancing androgenesis and regeneration of indica rice (*Oryza sativa* L.). Plant Growth Reg. 49: 43-47.
Hamasaki RM, Purgatto E, Mercier H (2005). Glutamine enhances competence for organogenesis in pine apple leaves cultivated *in vitro* Braz. J Plant Physiol. 17(4): 383-389.
Helen B (1939). Distribution of Sugar Cane Production in Cuba. Economic Geography 15(3): 311.
Irvine JE, Benda GTA (1987). Transmission of Sugarcane diseases in plant derived by rapid regeneration from diseased leaf tissue. Sugarcane 6 (Nov/Dec): 14-16.
Irvine JE, Benda GTA, Legendre BL, Machodo GR (1991). The frequency of marker changes in sugarcane plants regenerated from callus culture. II. Evidence for vegetative and genetic transmission, epigenetic effects and chimeral disruption. Plant Cell Tissue Organ Cult. 26: 115-125.
Kim YW, Moon HK (2007). Enhancement of somatic embryogenesis and plant regeneration in Japanese Larch (Lerix leptolepis). Plant Cell Tissue Organ Cult. 88: 241-245.
Liu MC (1993). Factors affecting induction, Somatic embryogenesis and Plant regeneration of calli from culture immature inflourescence of Sugarcane. J Plant Physiol. 141: 714-720.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-479.
Quatrano RS (1987). The role of hormones during seed development In Devies PJ (eds) Plant hormones and their role in plant growth and development. drdrecht: Martinus Nijhoff, The Netherlands, pp. 495-514.
Rao AM, Sree KP, Kishor PBK (1995). Enhanced plant regeneration in grain and sweet sorghum by asparagine, praline and cefotaxime. Plant Cell Rep. 15: 72-75.
SAS Institute (1996). SAS/STAT guide for personal computers, version 6.12 ed. Cary, NC.
Sen J, Kali S, Mukherjee GS (2002). Level of endogenous free amino acids during various stages of culture of *Vigna mungo* (L.) Hepper-somatic embryogenesis, organogenesis and plant regeneration. Curr. Sci. 82(4): 429-433.
Skokut TA, Manchester J, Schaefer J (1985). Regeneration in alalfa tissue culture. Plant Physiol. 79: 579-583.
Sato S, Toya T, Kawahara R, Whitter FR, Fukuda H, Komamine A (1995). Isolation of a carrot gene expressed specifically during early-Sugarcane ENCARTA encyclopedia, 1994.
Trigiano RN, May RN, Conger BV (1992). Reduced nitrogen influences somatic embryo quality and plant regeneration from suspension cultures of orchardgrass. In Vitro Cell Dev. Biol. 28: 187-191.
Vasil IK (1983). Regeneration of plants from single cells of cereals and grasses. In: Lurquin P & Kleinhops A (eds) Genetic Engineering of Eukaryotes Plenum, New York, pp. 233-252.