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Improving sweet leaf (*Stevia rebaudiana* var. Bertoni) resistance to bialaphos herbicide via *bar* gene transfer

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Abstract

The main objective of the present study was to improve the productivity of sweet leaf (*Stevia rebaudiana* var. Bertoni) through *in vitro* propagation and transformation methods. Production of transgenic resistance to herbicide sweet leaf was achieved using *bar* gene. This was gained by biolistic bombardment gene delivery system. Histochemical assay and PCR analysis were used for detection the transformed tissue. The results of the study showed that 66.7% of transformed *S. rebaudiana* survived at 3 mg l^{-1} of bialaphos. However, only 4% of un-transformed explants were survived. Transgenic sweet leaf plants exhibited functional expression of the *bar* gene *in vitro* for bialaphos herbicide resistance. More than 3 mg l^{-1} of the herbicide resulted in non-survival of the un-transformed (wild type) sweet leaf explants. Transformed explants; however, could survive up to more than 5 mg l^{-1} of herbicide. Further filed evaluations are currently being carried out to evaluate the transgenic plantlets.

Keywords: Genetic transformation; herbicide resistance; micropropagation; sweetpotato; tissue culture; transgenic plant.
Abbreviations: NAA_naphthalene acetic acid; BA_6-benzyl adenine; GA: gibberellic acid; PPT_phosphinothricin; 2,4-D_2,4 Dichlorophenoxyacetic acid; IAA_indole-3-acetic acid; IBA_indole butyric Acid; X-gluc_5-bromo-4-chloro-3-indoly; GUS_*β*-glucordinase.

Introduction

Stevia rebaudiana Bertoni is a small shrub of the Asteraceae (Composite) family. Stevia seeds show a very low germination percentage (Toffler and Orio, 1981; Latha and Usha, 2003). It is a drought sensitive herb which needs continuously moist soil, not saturated (Kinghorn, 2002; Gupta et al., 2013). Vegetative propagation is also limited by the low number of individuals obtained from a single plant. In order to overcome these obstacles, micropropagation or *in vitro* culture technique can play a vital role for mass propagation and the production of genetically identical plants of *S. rebaudiana*. In tissue culture technique, the combination of NAA and BA was found to induce shoot formation in Stevia explants (Giridhar et al., 2010). Furthermore, addition of GA to callus and suspension cultures resulted in a significant increase in their fresh weight (Bondarev et al., 1998). Although, growth regulators have been shown to depress the content of steviol glycosides, the total glycosides remained the same (Bondarev et al., 2003).

Bialaphos is used in agriculture as a non-selective herbicide. It is a tripeptide which is composed of two L-alanine residues and an analogue of glutamic acid known as phosphinothricin (PPT) (Ogawa et al., 1973). *Bar* gene is widely used for producing herbicide-resistant plants in many crop species (Aasim et al., 2013). Acetyltransferase is encoded by the *bar* gene and inactivates phosphinothricin (PPT). When the active ingredient of herbicides such as Basta and Buster acetylate their free ammonium group, it becomes non-toxic (De Block et al., 1987; Strauch et al., 1988). Many methods have been described to introduce foreign DNA into plant cells such electrophoretic transfection, electroporation of intact plant cells and tissues, bioactive-bead-mediated gene transfer, microinjection, pollen-tube pathway, and silica carbide-mediated (Khan, 2010; Narusaka et al., 2012). The biolistic method has been used as an excellent approach for transforming monocots and recalcitrant agronomic crops. Herbicide resistant plants expressing the *bar* gene in sweetpotato embryogenic calluses were obtained using different methods such as *Agrobacterium tumefaciens*-mediated transformation or particle bombardment (Otani et al., 2003; Yi et al., 2007; Choi et al., 2007). *Gus* gene, as a reporter gene, has been reported to be excellent for many dicotyledonous plants (Jefferson et al., 1987). Plant genetic transformation has become a versatile tool for cultivar improvement as well as to studying gene function in plants. Transformation using the biolistic bombardment gene delivery system succeeded with *Vicia faba* (Jelenic et al.,

2000) soybean (Sato et al., 1993) peanut (Brar et al., 1994) and *Phaseolus vulgaries* (Aragão et al., 1996). This work aimed to optimize efficient protocol for *Stevia rebaudiana* (Sweet leaf) micropropagation through tissue culture technique. The study was also undertaken to produce transgenic stevia plants carrying *bar* gene as a selectable marker for bialaphos herbicide and *gus* gene as a reporter marker, using biolistic gene gun protocol.

Results

In order to maximize plant propagation of *S. rebaudiana*, grown *in vitro*, the effect of growth regulators as well as plant materials were studied. The callus productivity of *S. rebaudiana* was increased with increasing 2,4-D concentration (Table 1). Friable callus induction was noted from 3 to 5 mg l^{-1}, but condensed callus was obtained by 1 and 2 mg l^{-1}. The greatest callus fresh weight and viability were observed at 5 mgl^{-1} of 2,4-D.

Effect of different BA concentrations on S. rebaudiana development

The shoot and node numbers were increased by increasing BA concentration (Table 2). A significant reduction in shoot length, leave number and length was also obtained by elevating BA concentration compared with the control. When 1.5 mg l^{-1} was applied, a significant decrease in shoot length and leave number was observed and length reduced by 34%, 40% and 43%, respectively. The greatest segment length was obtained by 0.5 mg l^{-1} of BA, 34% higher than the control.

Effect of different IBA concentrations on S. rebaudiana development

A significant increase in the root and shoot number was observed when IBA concentration was increased from 0.5 to 1.5 mg l^{-1}. The increase in root number was 366% higher than the control value, in which 1.5 mg l^{-1} of IBA used (Table 3). On contrary, the shoot number was reduced 29% as compared with the control when these concentrations of IBA were used. Increasing IBA concentration significantly reduced the root length, compared to control. Both shoot length and node number varied insignificantly compared to control, except for IBA concentration of 1 mg l^{-1}. About 22% reduction in leave number was observed in this concentration compared to control (Table 3).

Gus and bar gene detection

Our results indicated that *gus* activity in the transformed Stevia explants could be detected histochemically for *gus* expression after two week as an evidence of transformation. Tissue of *S. rebaudiana* callus turned to deep blue color in culture media containing x-gluc substance (Fig 2).
Also, polymerase chain reaction (PCR) of the total genomic DNA of transformed and non-transformed Stevia callus confirmed the integration of *gus* and *bar* genes into the transformed callus. The amplified product *gus* and bar genes were observed at 750 and 484 bp, respectively (Fig 3).

Bialaphos herbicide sensitivity

When the bialaphos herbicide was applied (0-5 mg l^{-1}), the greatest amount of callus cultures obtained by the transformed *S. rebaudiana* cells (Fig. 4). However, the highly significant reduction in shoot number and survival percentage was observed at 3 mg l^{-1} in transformed explants (Table 4) with 94.1% and 94.4% reduction, respectively, compared to control. The lethal dose of bialaphos was obtained at 4 m gl^{-1} with the untransformed explants. In contrast, both shoot number and survival percentage of transformed explants were not significantly varied, when 1 to 3 mg l^{-1} bialaphos were used. In addition, transformed explants completed their growth at 4 and 5 mgl^{-1} bialaphos, while 40% reduction in shoot number and survival percentage was obtained at 5 mg l^{-1}.

Discussion

Our study reported the micropropagation of *S. rebaudiana* through shoot tip culture. The results confirmed that supplementation of culture medium with 2,4-D induced callus formation and viability of *S. rebaudiana in vitro*. The results are in agreement with those of Mohammed et al. (2006) who found that the greatest amount of *S. rebaudiana* callus was formed in MS medium inoculated with 3 mg l^{-1} of 2,4-D. Similarly, Ahmad et al. (2011) indicated that combination of BA and 2,4-D induced callus formation in *S. rebaudiana.* In addition, Das et al. (2011) reported the induction of IAA and BA for micropropagation of *S. rebaudiana*. Recently, Aman et al. (2013) reported that 2,4-D and BA along with different agar concentrations significantly enhanced callus formation of *S. rebaudiana*. Generally, auxins play an important role in morphogenesis of culture systems. It induces many processes like apical dominance, cell elongation in roots and shoots, permeability changes of the plasma lemma, formation of ethylene, induction of adventitious root formation, enhancement of the respiration rate, induction of disorganized growth at higher concentrations, inhibition of embryo formation in cell suspension cultures, formation of parthynocarpic fruits in some species, and mitotic regulation in long term tissue cultures (Chaturvedi et al., 1978; Sharma et al., 1981; Ammiraton, 1983). Higher concentrations of auxins increased root, shoot and leaves number, but inhibited their length. Our results are consistent with the findings of Kollmeier et al. (2000), who reported that root elongation phase was very sensitive to high auxin concentration. Moreover, Taiz and Zeiger, (2002) indicated that roots may require a less concentration of auxin to grow and strongly inhibit high auxin concentration, which induce ethylene production. Baker and Wetzstein, (2004) reported that higher concentration of auxin induces high level of degradative metabolites, which may block the regeneration process. Cell elongation involves sequential changes in levels and/or activity of certain enzymes (Cosgrove, 1999), which are triggered by auxin. Wada et al. (1998) reported that IBA promotes root elongation by influencing the synthesis of enzymes concerned to cell enlargement. As the *stevia* cultivation was successful in *in vitro* condition, an attempt to produce transgenic *stevia* plants carrying *bar* gene, as a selectable marker for herbicide bialaphos resistance; and *gus* gene, as a reporter marker was conducted using biolistic gene gun protocol. Histochemical staining technique and PCR analysis were used in this study to detect the presence of the *gus* and *bar* genes in the putatively transformed tissues. Using of x-gluc as a substrate for the GUS enzyme provided an effective method for monitoring the expression of *gus* DNA introduced into the plant cells. X-gluc is the best substrate currently available for histochemical localization of GUS activity in tissue and cells. Our results indicated that GUS activity in transformed stevia explants was detected histochemically after two weeks, as the first evidence of

Table 1. Effect of the different concentrations of 2,4-D on *S. rebaudiana* callus development.

Concentration mg l^{-1}	Characterization of callus			
	Fresh weight	Color	Type	Viability
0	0.00[f]	-	-	-
1	0.23[e]	Yellow	Compact	+
2	0.40[d]	Yellow	Compact	++
3	0.53[c]	White	Friable	+++
4	0.65[b]	White	Friable	++++
5	0.86[a]	White	Friable	++++++

++++++, excellent; ++++, very good; +++, good; ++, poor; +, very poor; -, no growth.
Different letters on the same column mean significant difference at p≤0.05.

Table 2. Effect of different concentrations of BA on *S. rebaudiana* development *in vitro*.

Concentration (mg l^{-1})	Shoot		Leaf		Node	
	Number	Length	Number	Length	Number	Segment length
0 (control)	25.5[d]	4.4[a]	6.0[a]	4.6[b]	24.0[c]	3.5[b]
0.5	30.9[c]	4.4[a]	6.4[a]	6.5[a]	26.2[c]	4.7[a]
1.0	48.4[b]	3.4[b]	4.9[b]	3.2[c]	35.3[b]	2.6[c]
1.5	55.2[a]	2.9[b]	3.6[c]	2.6[d]	44.2[a]	3.4[b]

Different letters on the same column mean significant difference at p≤0.05.

Table 3. Effect of different concentrations of IBA on *S. rebaudiana* development *in vitro*.

Concentration (mg l^{-1})	Root		Shoot		Node number	Leaf number
	Number	Length (cm)	Number	Length (cm)		
0 (control)	3.3[c]	6.1[a]	24.7[a]	4.0[a]	5.0[a]	9.0[ab]
0.5	10.2[b]	6.5[a]	9.7[c]	5.0[a]	5.0[a]	11.0[a]
1.0	12.3[ab]	4.7[b]	8.3[c]	4.0[a]	4.3[a]	7.0[b]
1.5	15.4[a]	3.4[c]	17.5[b]	6.0[a]	5.0[a]	11.3[a]

Different letters on the same column mean significant difference at p≤0.05.

Table 4. Effect of different concentrations of bialaphos on the shoot number and survival percentage of non-transformed and transformed *S. rebaudiana*.

Concentration of bialaphos (mgl^{-1})	Non-transformed plant		Transformed plant	
	Shoot number	Survival (%)	Shoot number	Survival (%)
0 (control)	10.2[a]	71.3[a]	11.2[a]	74.7[a]
1.0	8.2[b]	56.7[b]	10.2[a]	69.3[a]
2.0	4.8[c]	32.0[c]	11.0[a]	73.3[a]
3.0	0.6[d]	4.0[d]	9.4[a]	66.7[a]
4.0	0.0[d]	0.0[d]	7.8[b]	52.0[b]
5.0	0.0[d]	0.0[d]	6.6[b]	44.0[b]

Different letters on the same column mean significant difference at p≤0.05.

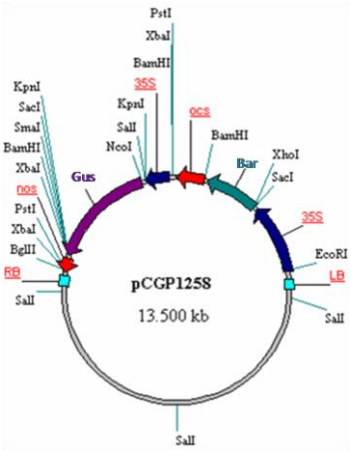

Fig 1. pCGP1258 binary vector containing the *bar* and *gus* genes.

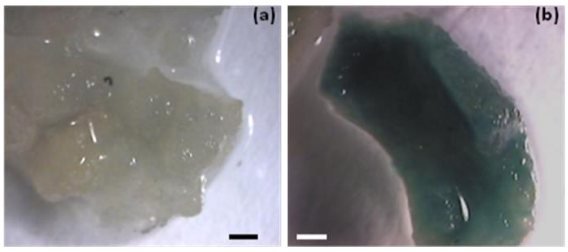

Fig 2. Detection of *gus* gene in callus explants of *stevia* by x-gluc after two weeks from transformation process. (a) untransformed explants; (b) transformed explants. (bar 50 µm).

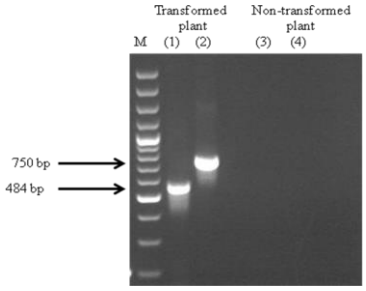

Fig 3. Amplification of partial sequences of *bar* (484 bp) and *gus* (750 bp) genes from *Stevia rebaudiana transformed* plants. Putative transformed plants showed successful amplification of both *bar* and *gus* sequences (lanes 1, 2 respectively), while non-transformed plants showed no amplification of either gene (lanes 3,4 respectively). (M) marker.

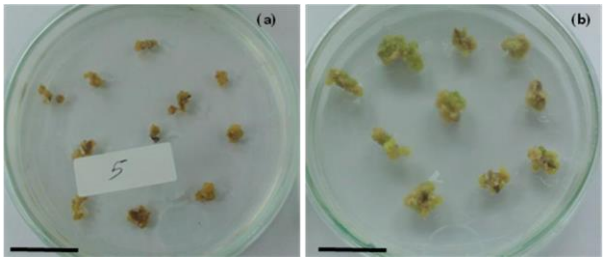

Fig 4. Effect of bialaphos (2 mg l^{-1}) on *Stevia rebaudiana* callus tissue after 4 weeks of cultivation. (a) untransformed and (b) transformed explants. (bar 20 mm).

transformation. No morphological variations were observed in transformed explants, compared with non-transformed ones. Stable integration of the *bar* and *gus* genes into the genome of *S. rebaudiana* plants was detected. Gonzalez et al. (2003) reported a stable integration of the *npt* II and *gus* genes into the genome of plum plants transformed with plasmid pBISNI, pGA482GG or pGA428GGi, using PCR analysis. The present work demonstrated that the transformed *S. rebaudiana* explants were more resistant than non-transformed one. The results are in agreement with those of Zang et al. (2009) who recorded 100% survival of 173 PCR-positive transgenic plants of sweet potato [*Ipomoea batatas* (L.) Lam.] transplanted into the soil. Moreover, the authors indicated a positive response of transformed plants to herbicide (Basta) applied directly to the leaves. It is important to mention that nematode resistance of transgenic sweetpotato (*cv.* Lizixiang) was developed by Shang et al. (2011) using *Oryzacystatin*-I (*OC*I) gene with *Agrobacterium tumefaciens*-mediated transformation. The *A. tumefaciens* strain EHA105 carried the binary vector pCAMBIA1301 with *OC*I, *gusA* and *hpt*II genes. GUS assay and PCR analyses of the putative transgenic sweetpotato plants showed that 90.54% of these plants were transgenic, which exhibited enhanced resistance to stem nematodes relatively to the non-transformed controls.

Materials and Methods

Plant materials

Seedlings of sweet leaf (*Stevia rebaudiana* var. Bertoni) were obtained from Sugar Crops Research Institute (SCRI), Ministry of Agriculture, Giza, Egypt. The seedlings were grown in a greenhouse at December 2010 under greenhouse conditions. Actively growing shoots were used as the explants during February to March, 2011. The terminal shoots were collected from the growing plants (2-3 months age) and were cut into 0.5-1.5 cm pieces.

Explants sterilization

Shoot tip explants, 5 to 10 mm in length, were excised and washed under running tap water. Explants were surface sterilized by immersing in an aseptic solution of Sodium hypochlorite (3.61 %) solution for 10 minutes, followed by Mercuric chloride ($1.5gl^{-1}$) for one minute and finally rinsed with distilled water several times.

Media preparation

The basal medium MS which described by Murashige and Skoog (1962) was used to cultivate *S. rebaudiana* var. Bertoni *in vitro*. The medium was solidified with phytagel ($2.5\ gl^{-1}$), and pH of the medium was adjusted to 5.7. Cultures were incubated in room chamber at 25±2ºC with 3000 lux and 16 h photoperiod.

Coating of DNA Plasmid with gold particles

Preparation of gold particles

A mixture of gold particles (30 mg) and ethanol (0.5 ml) were prepared in a micro tube and then vortexed for 1-2 min. The mixture was spin down at 10,000 rpm for 10 sec for 3 times. The supernatant was removed and 0.5 ml of sterile double distilled water was added.

DNA coating

50 µl aliquot of gold particles was added to 5 µl of DNA (1 µg/µl) of the plasmid pCG1258 (ICARDA), which contained *bar* and *gus*-intron genes (Fig. 1). It is then mixed with 50 µl of 2.5 M $CaCl_2$, 20 µl of 0.1 M spermidine. The mixture was vortexed for 3 min and spun down for 10 sec. The precipitate was rinsed by adding 250 µl of absolute ethanol, vortexed, spinned down, and the supernatant was removed. DNA gold particles were re-suspended in 70 µl of absolute ethanol. Aliquot of 10 µl was used for transformation.

Bombardment conditions

The small calli were placed in the center of Petri dish (5 cm diameter) and bombarded under a vacuum of 25 in Hg Vac with 1.0 µm golden micro carriers (163-283 Standard Pressure kit, with 1.0~L Gold + 1100psi disks Bio-Rad) coated with pCGP1258 plasmid using the Helium driven 1.0p Bio-Rad Biolistic Delivery system (Model PDS-1000/ He, Bio-Rad, USA). Target mature calli were bombarded at one distance (6 cm) from stopping screen with rapture disc strength of 900 and 1100 psi. After bombardment, callus was remained for additional 16 h on the same osmotic treatment. Then, callus was transferred to regeneration medium without mannitol.

Histochemical GUS assay

Transformation was confirmed by the *gus* assay in different stages of the experiment according to Jefferson et al. (1987). X-gluc produces the final insoluble blue precipitate dichloro-dibromoindigo (CIBr-indigo). Number of transformed explants and control were soaked in 500µl *gus* buffer (Daniell et al., 1990) containing 50µl X-gluc (5-bromo-4-chloro-3-indoly) glucuronide and rapped with aluminum foil (Jefferson et al., 1987). A blue staining was often visible in 24 h or less, even though tissues were incubated in the substrate solution overnight at 37˚C. After this incubation period, the tissues were cleared with chlorine solution (5%) for easily visualization of the blue staining. Blue color was detected under microscope.

PCR analysis

Total DNA was isolated from transformed and non-transformed Stevia tissues according to Delaborta et al. (1983). PCR amplification of *gus* and *bar* genes was carried out using the following primers *gus*-1: 5'-CCTGTAGAAACCCCAACCCG-3' *gus*-2: 5'-TGGCTGTGACGCACAGTTCA-3' and *bar*-1: 5'-TGC CAC CGA GGG GAC ATG CCG GC-3' and *bar*-2: 5' CCT GAA GTG GAG GCC ATG GGG 3', respectively. The target DNA sequences (*bar* and *gus* genes) were amplified, using the step cycle program as follows: the DNA was denaturated at 95ºC for 3min. Annealing was done at 60ºC for 1.5 min. The newly synthesized DNA was heated at 72ºC for 2 min for a total of 35 cycles. PCR products were analyzed by agarose gel electrophoresis. A 6 µl of each PCR reaction mixture was electrophoresed after mixing with 3µl loading buffer on a 1.3 % agarose gel at 105 Volt for 20 min.

Statistical analysis

The data of five replicates per treatment were statistically analyzed using Complete Randomized Design (CRD) by SPSS computer program V.10 (1999). The means were compared by Duncan's multiple range test (DMRT) (Duncan, 1955) at the 5% level.

Conclusion

In this study, we successfully optimized *in vitro* propagation of *S. rebaudiana* and then produced transformed plant with the *bar* gene. The results indicated the feasibility of transformation method for *S. rebaudiana*. This efficient transformation system was used to transfer the *bar* gene, as a selectable marker, to improve *S. rebaudiana* resistance to herbicides. Resistance to bialaphos herbicide was also clearly evidenced in transformed plants *in vitro*.

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Reference

Aasim M, Khawar K, Özcan S (2013) Production of herbicide-resistant cowpea (*Vigna unguiculata* L.) transformed with the bar gene. Turk J Biol. 37: 472-478.

Ahmad N, Fazal H, Zamir R, Khalil SA, Abbasi, BH (2011) Callogenesis and shoot organogenesis from flowers of *Stevia rebaudiana* (Bert.). Sugar Tech. 13, 174–177.

Aman N, Hadi F, Khalil SA, Zamir R, Ahmad N (2013) Efficient regeneration for enhanced steviol glycosides production in *Stevia rebaudiana* (Bertoni). C R Biol. 336: 486–492.

Ammiraton PV (1983) Emberyogensis In: Evans DA, Sharp WP, Ammirato PV, Mada Y (eds) Handbook of Plant Cell Culture Techniques for Propagation and Breeding. Macmillan Publishing Co., New York. London. 82-123.

Aragão FL, Barros LG, Brasileiro AM, Ribeiro SG, Smith FD, Sanford JC, Faria JC, Rech EL (1996) Inheritance of foreign genes in transgenic bean (*Phaseolus vulgaris* L.) co-transformed *via* particle bombardment. Theor Appl Genet. 93: 142–150.

Baker CM, Wetzstein HY (2004) Influence of auxin type and concentration on peanut somatic embryogenesis. Pl Cell Tissue Organ Cult. 36(3):361-368.

Bondarev NA, Nosov AM, Kornienko AV (1998) Effects of exogenous growth regulators on callusogenesis and growth of cultured cells of *Stevia rebaudiana* Bertoni. Rus J Pl Physiol. 45(6):770-774.

Bondarev NI, Reshtnyak O, Nosov A (2003) Effect of nutrient composition on development of *Stevia rebaudiana* shoots cultivated in the roller bioreactor and their production of steviol glycosides. Plant Sci. 165:845–850.

Brar GS, Barry CA, Carole VL, Johnson W (1994) Recovery of transgenic peanut (*Arachis hypogaea* L.) plants from elite cultivars utilizing ACCL technology. Plant J. 5:745-753.

Chaturvedi HC, Sharma AK, Prasad RN (1978) Shoot apex culture of *Bougainvillea glabra* 'Magnifica'. Hort Sci. 13:36.

Choi HJ, Chandrasekhar T, Lee HY, Kim KM (2007) Production of herbicideresistant transgenic sweetpotato plants through *Agrobacterium tumefaciens* method. Pl Cell Tissue Organ Cult. 91:235–242.

Cosgrove DJ (1999) Enzymes and other agents that enhance cell wall extensibility. Crit Rev Pl Physiol Pl Mol Biol. 50:391-417.

Daniell H, Vivekanada J, Nielson B, Tewari KK, Sanford JC (1990) Transient foreign gene expression in chloroplasts of cultured tobacoo cells following biolistic delivery of chloroplast vectors. Proc Natl Acad Sci. 87:88-92.

Das A, Gantait S, Mandal N (2011) Micropropagation of an elite medicinal plant: *Stevia rebaudiana* Bert. Int J Agric Res. 6:40-48.

De Block M, Botterman J, Vandewiele M, Dockx J, Thoem C, Gossele V, Roamovva N, Thompson G, Vanmontagu M, Leemans J (1987) Engineering herbicide resistance in plants by expression of aoxifin enzyme. EMBO J. 6:2513-2518.

Delaborta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Pl Mol Biol Rep. 4:19-21.

Duncan BD (1955) Multiple range and multiple F test. Biometrics 11:1- 42.

Giridhar P, Sowmya K, Ramakrishna A, Ravishankar G (2010) Rapid clonal propagation and stevioside profiles of *Stevia rebaudiana* Bertoni. International J Pl Develop Biol. 4(1):47-52.

Gonzalez IM, Padilla K, Scorza R (2003) Early antibiotic Selection and efficient rooting and acclimatization improve the production of transgenic plants (*Prunus domestica* L.). Pl Cell Rep. 22:38–45.

Gupta E, Purwar S, Sundaram S, Rai GK (2013) Nutritional and therapeutic values of *Stevia rebaudiana*. J Med Plant Res. 7(46) 3343-3353.

Jefferson RA, Kavanagh TA, Bevan M (1987) GUS fusion: beta glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901–3907.

Jelenic S, Mit, PT, Papes D, Jelaska S (2000) *Agrobacterium*-mediated transfor-mation of broad bean *Vicia faba* L. Food Tech. 38:167-172.

Khan KH (2010) Gene transfer technologies and their applications: Roles in human diseases. Asian J Biol Sci. 1 (1): 208-218.

Kinghorn DA (2002) Overview. In: Kinghorn, AD (Ed), Stevia the Genus Stevia (Medicinal and Aromatic Plants–Industrial Profiles). Taylor & Francis/CRC Press, New York/London, UK. 1-17.

Kollmeier M, Felle HH, Horst WJ (2000) Is basipetal auxin flow involved in inhibition of root elongation. Pl Physiol. 122:945-956.

Latha S, Usha M (2003) *In vitro* culture studies on *Stevia rebaudiana*. *In vitro* Cell Dev Biol Plant. 39(5):520-523.

Mohammed SU, Mohammad SH, Muoztaba MM, Mohammad BU, Romel A, Azizul MB (2006) *In vitro* propagation of *Stevia rebaudiana* Bert. in Bangladesh. Afr J Biotech. 5(13):1238-1240.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 15:473–497.

Narusaka Y, Narusaka M, Yamasaki S, Iwabuchi M (2012) Methods to Transfer Foreign Genes to Plants. In: Çiftçi YÖ (ed) Agricultural and Biological Sciences "Transgenic Plants - Advances and Limitations". In Tech Publishing. 173-188.

Ogawa Y, Tsuruoka T, Inouye S, Niida T (1973) Studies on a new antibiotic SF-1293. II Chemical Structure of Antibiotic, SF-1293, Sci. Rep. Meiji Seika. 13, 42-48.

Otani M, Wakita Y, Shimada T (2003) Production of herbicide-resistant sweetpotato (*Ipomoea batatas* (L.) Lam.) plants by *Agrobacterium tumefaciens* mediated transformation. Breeding Sci. 53:145–148.

Sato T, Newell C, Kolaez K, Tredo L, Finer J, Hinchee M (1993) Stable transformation *via* particle bombardment in two different Soybean regeneration systems. Plant Cell Rep. 12:408-413.

Shang GO, Bo YU, Hong Z, Shao-zhen HE, Qing-chang LU (2011) Enhanced stem nematode resistance of transgenic sweetpotato plants expressing *Oryzacystatin*-I gene. Agric Sci China. 10(4):519-525.

Sharma AK, Prasad RN, Chaturvedi HC (1981) Clonal propagation of *Bougainvillea glabra* 'Magnifica' through shoot apex culture. Plant Cell Tissue Organ Cult. 1:33-38.

Strauch E, Wohlleben W, Pühler A (1988) Cloning of a phosphinothricin Nacetyltransferase gene from Streptomyces viridochromogenes TU494 and its expression in *Streptomyces lividans* and *Escherichia coli*. Gene. 63:65–74.

Taiz L, Zeiger E (2002) Mineral Nutrition: *Plant Physiology*. 2nd ed. Sinaver Associates Inc Pub. 67-86.

Toffler F, Orio A (1981) Acceni sulla piñata tropicale 'Kaa-he-e' ou "erba dolce". Rev Soc Ita Sci Aliment. 4: 225-230.

Wada S, Tanimoto E, Masuda Y (1998) Cell elongation and metabolic turnover of the cell wall as affected by auxin and cell wall degrading enzymes. Pl Cell Physiol. 9(2):369-376.

Yi G, Shin YM, Choe G, Shin B, Kim YS, Kim KM (2007) Production of herbicide-resistant sweetpotato plants transformed with the bar gene. Biotechnol Lett. 29:669–675.

Zang N, Zhai H, Gao S, Chen W, He S, Liu Q (2009) Efficient production of transgenic plants using the bar gene for herbicide resistance in sweetpotato. Sci Hort. 122:649–653.