

Agrobacterium Mediated Transformation Optimizations for Sugarcane (*Saccharum Officinarum* L.) Cultivar SPF-234 with Direct Organogenesis

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AGROBACTERIUM MEDIATED TRANSFORMATION OPTIMIZATIONS FOR SUGARCANE (*SACCHARUM OFFICINARUM* L.) CULTIVAR SPF-234 WITH DIRECT ORGANOGENESIS

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ABSTRACT

Sugarcane (*Saccharum officinarum* L.) is the most important food and energy crop worldwide. In the present study, an efficient *Agrobacterium* mediated transformation and regeneration system for sugarcane cultivar SPF-234 was established. *Agrobacterium tumefaciens* strains EHA101 and LBA4404 using vector pIG121 Hm, having *GUS*, *HPTII* and *NPTII* genes were used. Polymerase chain reaction (PCR) and histochemical assays confirmed the *GUS* gene expression. A 620 bp fragment from *GUS* positive plants was amplified. The *GUS* expressing putative transformants were 35% of the total plants formed under 30 minute immersion time and 72 hr of incubation period. The co-cultivation media having 60 µM acetosyringone produced 66% *GUS* expressing plants for LBA4404 and 58% for EHA101. The maximum average number of directly produced shoot (59.5%) from leaf explant was in M6 media having 1.00 mg/l 6-Benzylaminopurine (BAP) and 2.5 mg/l Naphthaleneacetic acid (NAA). A significant decrease (17%) was observed when auxin (NAA) concentration was increased to 4.0 mg/l. The best response of shoot elongation was observed in SE4 media having equal concentration (2.00 mg/l) of both kinetin and BAP. Increased concentrations of kinetin significantly decreased shoot elongation of the subject cultivar. *Agrobacterium* strain LBA4404 performed better for genetic transformation of the said sugarcane cultivar. This quick and less expensive transformation and direct regeneration system could be exploited for sugarcane on commercial scale in general, and for this elite cultivar in particular.

Keywords: Genetic Engineering, Quick regeneration, Sugarcane, *Agrobacterium*.

INTRODUCTION

Genetic transformation in sugarcane (*Saccharum officinarum* L.) is of vital importance for induction of desired agronomic traits. It is vegetatively propagated in this area of world and every new variety is genetically modified individually for desired agronomic traits. The SPF-234 is a cultivated variety of sugar cane in the southern region of Punjab, Pakistan. Complex genome, low fertility, narrow genetic diversity and high ploidy levels are the bottle necks in genetic

improvement of sugarcane. In Pakistan, complicated flowering behavior of sugarcane adds more to this difficulty due to unfavorable climatic conditions. The sugarcane breeding in the country is based on imported fuzz and selection of exotic lines for better agronomic traits. Micro-propagation has been considered as method of choice for commercial scale production of sugarcane (Nawaz et al., 2013). For every event of genetic modification in sugarcane, transformation and regeneration system must be

optimized (Uzma et al., 2012) as every cultivar responds differentially in regeneration system (Popelka et al., 2003). Traits of agronomic importance are being introduced in many crops through genetic transformation.

There are many different methods available for genetic transformation of different crops. *Agrobacterium tumefaciens* had been used for genetic transformation of different major crops in past including wheat, maize, rice and sugarcane (Jones et al., 2005; Ahmadabadi et al., 2007). Sugarcane transformation is reported by many authors using biolistic method (Taparia et al., 2012; Olhoft et al., 2004; Fang et al., 2002). *Agrobacterium* mediated genetic transformation has been preferred in many cases due to its unique features (Singh et al., 2011; Malhotra et al., 2017).

Embryogenic callus is used as explant in most of the plant transformation events. Callus induction and regeneration is a time-consuming lengthy process involving repeated culturing activity and is also variety dependent thus limiting the transformation efficiency at the end of process (Danilova, 2007). It is necessary to expand the target tissue window for improvement of genetic transformation efficiencies in many plant species. Shoot tip has been reported as choice of explant in many studies due to its unavoidable features in terms of economics and reproducibility (Badoni and Chauhan, 2009; Bairu et al., 2010; Atak and Özge 2009).

Desired traits of commercial importance are being incorporated in crops including sugarcane through genetic transformation. It is believed that genetically modified crops with better traits are the promising sources to enhance world food production. Callus induction and sufficient regeneration through somatic embryogenesis is highly desired for genetic manipulation of different crops. Many studies including (Rahman et al., 2010) support this notion. In previous

experiments, we have developed an efficient, cost effective and reliable callus induction and regeneration system for non-transformed elite commercial cultivars (Nawaz et al., 2013) and are interested in improving drought tolerance of the elite cultivars through genetic engineering. Successful sugar cane genetic transformation events using *agrobacterium* and callus tissue have been reported in previous studies (Eldessoky et al., 2011). The present study was aimed at to establish a simple, efficient and reproducible *Agrobacterium* mediated transformation and direct regeneration system for sugarcane elite cultivar SPF-234 using inner leaf roll as explant.

MATERIALS AND METHODS

Plant Material

The vegetative parts of sugarcane cultivar SPF-234 were obtained from Ayub Agricultural Research Institute (AARI), Faisalabad Pakistan. The inner most spindle leaves were used as experimental material. Upper parts of the plants were cut having 10 cm spindle leaves. The inner most whorls of these spindles were used as explant for further studies.

The collected samples of the plants to be used as explants were washed twice in running water. The upper leaf sheaths of these disease-free spindles were removed. The excised tissues were soaked in antioxidant solution (150 mg/l Ascorbic acid) for 02 hours. The explants were surface sterilized with mercuric chloride (HgCl₂) solution (0.2% W/V) for two minutes and rinsed thrice with double distilled de-ionized water.

Cocultivation/Transformation

Fresh culture of *Agrobacterium* strains was prepared one day before use. Single colonies of EHA101 and LBA 4404 having pIG121 Hm were picked from plates in sterile environment of flow hood cabinet and cultured in a 250 ml flask

having 20 ml of liquid LB medium separately. Kanamycin (50 mg/l) and rifampicin (50 mg/l) antibiotics were added in the culture and incubated at 28 °C at constant shaking (150-200 g) for 48 hours. After adjusting the optical density to 0.6 units at 600 nm (OD₆₀₀ nm), the culture was used for transformation. The

T-DNA construct was having β -glucuronidase (*GUS*), neomycin phosphotransferase (*NPTII*) Hygromycin phosphotransferase (*HPTIII*) genes (Figure 1). The sterilised and double distilled deionised water washed leaf explant of SPF-234 were immersed in 30 ml bacterial suspension for 30 minutes.

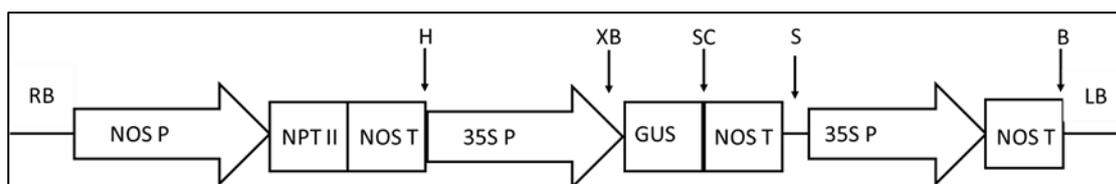


Figure 1: T-DNA Part of construct. RB: right border; LB: left border; GUS: β -glucuronidase, NPTII: neomycin phosphotransferase, NOS P: nopaline synthase promoter, NOS T: 3' signal of nopaline synthase, 35S P: 35S promoter, XB: XbaI, B: BamHI, H: HindIII, E: EcoRI, S: SmaI, SC: SacI.

The explants were blotted dry on autoclaved filter paper after removal from bacterial suspension. The treated explants were subjected to direct shoot formation media.

Direct Organogenesis and Shoot Multiplication

After co-cultivation the treated explants were subjected to direct organogenesis media having MS salt supplemented with different concentrations of BAP and NAA for 72 hr in the dark at 28±1 °C. The pH of all the media was set at 5.8. Direct organogenesis media was also augmented with antioxidant (ascorbic acid 2.5 mg/l). The experiment was conducted in three replicates each having 120 leaf disc explants.

The shoots formed were subjected to regeneration and elongation media having varying composition of plant growth hormones (Nawaz et al., 2013). After three days the explants were shifted to new media having same composition along with different concentrations of hygromycin (50 mg/l) and cefotaxime (1000 mg/l) for selection of putative transformant shoots and removal of excess

bacteria respectively. The developed shoots were excised and subjected to root development media and results were recorded in terms of % plantlets formed. The young plants were acclimatised in green house.

GUS Assay

The GUS assay of putatively transformed plants was carried out following previously described (Jefferson, 1987) method. The leaves of putatively transformed plants were soaked in GUS solution overnight at 37 °C. The samples were incubated at 37 °C in a rotatory shaker at 100 rpm and were examined under light microscope to observe the blue colour, indicating GUS gene expression. Results were expressed in order of percentage of plants expressing GUS.

PCR Analysis

GUS gene expression was confirmed through polymerase chain reaction (PCR). Leaf tissues of transformed plants were used for DNA extraction using Cetyl trimethylammonium bromide (CTAB) method (Nawaz et al., 2009). The sequence of the primers used for amplification of 620 bp fragment from *GUS* gene were as under.

FP 5' ACACCGATAACCATCAGCGAT 3'
RP 5' TCACCGAAGTTCATGCCAGT 3'.

PCR analysis was carried out as described by Nawaz et al. (2009) with little modifications, a reaction volume of 20 µl having 2.00 µl of 10X buffer {50 mM of Tris (pH8.3), 500 mM of KCl}, 1.5 mM of MgCl₂, 0.2 mM of dNTPs, 0.3 mM Primer (each reverse and forward), one unit of *Taq* DNA polymerase and 25 ng genomic DNA. The PCR profile was set at denaturation of 05 minutes at 94 °C, followed by 30 cycles of 94 °C for 30 Seconds, 55 °C for 30 seconds, 72 °C for 30 seconds followed by final extension of 05 minutes at 72 °C. The PCR products were analysed at 1% agarose gel.

Statistical Analysis

Complete randomised design (CRD) with three replicates of each treatment/experiment was applied. The data thus obtained were subjected to Analysis of Variance (ANOVA) using CoStat statistical software.

RESULTS AND DISCUSSION

Genetic engineering is considered as one of the best approaches for improvement of crops. The success story of sugarcane genetic transformation is built on the regeneration potential of the cultivar under study. Sugarcane regeneration can be achieved directly or indirectly. For indirect somatic embryogenesis callus induction and proliferation is the basic step. It is time consuming and laborious method involving a lot of culturing practices. In the present study inner most leaf discs were used as explant for direct organogenesis.

Direct Shoot Organogenesis

Direct shoot organogenesis has been reported in previous studies (Khan et al., 2009) and explant response for direct shoot formation has been related to type and size

of explant (Eldessoky et al., 2011). Direct regeneration of various monocots bypassing the callus phase has been reported in past (Sata et al., 2000; Vikrant and Rashid, 2001). Different problems related to regeneration of plantlets, maturity and embryo formation have been linked to callus culture in previous studies (Cho et al., 1998; Thorpe, 1994). Leaf explants of sugarcane cultivar SPF234 were cultured on MS media supplemented with different concentrations of BAP and NAA (Table 1).

The explants taken from younger leaves with 3-5 mm thickness exhibited best response towards shoot organogenesis (Figure 2). The mean number of shoots directly produced from explants ranged from 5.00-59.5 under different media concentrations. Before shoot formation a puffy appearance was observed at the cut ends of the explants after two weeks of culture.

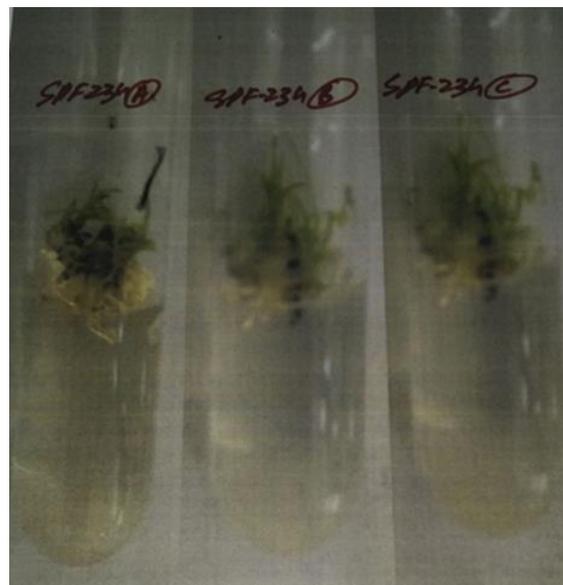


Figure 2: Direct regeneration of sugarcane cultivar SPF-234 from inner leaf roll explant.

Shoot initiation appeared on surface without callus formation during six weeks dark incubation period. Badawy et al. (2008) has reported that dark incubation increases direct shoot regeneration from explants as light negatively affects

metabolism and sugar uptake thus lowering the regeneration potential of cells.

The maximum mean number of shoots induced per explant was 59.5 in the MS salt medium containing 1mg/l BAP and 2.5mg/l NAA. Number of shoots per explant was significantly reduced in the media having only NAA and it went on increasing on addition of BAP in the same media. Reverse effect on shoot formation was observed with increasing

concentration of BAP and NAA beyond optimal dose. This also indicates a relationship between hormone dose level and physiological response of plant.

Regeneration is an essential feature of transformation events (Dey et al., 2012). The slight variation in media concentrations for maximum response may be due to genetic variations among the cultivars.

Table 1: Effect of BAP and NAA on direct organogenesis from inner most leaf roll of sugarcane cultivar SPF-234.

Media	BAP+NAA (mg/l)	Explants used	% Explants producing Shoots	Shoot organogenesis mean/Explant		
				1-15	16-25	>25
M1	MS only	120	0.000 ^e	00	00	00
M2	1+0.5	120	2.314 ^e	5	11	15
M3	1+1.0	120	4.410 ^e	8	23	26
M4	1+1.5	120	8.812 ^d	8	22.5	34
M5	1+2.0	120	10.734 ^{cd}	12	25	59
M6	1+2.5	120	20.312 ^c	11	25	59.5
M7	1+3.0	120	48.344 ^b	6	16	51.5
M8	1+3.5	120	70.121 ^a	5.8	14	50
M9	1+4.0	120	51.312 ^b	5.8	24.5	49.5

**P*<0.05

Table 2: Phytohormones effect on shoot regeneration and elongation produced from leaf explant of sugarcane cultivar SPF-234.

Media	Composition MS+Kin+BAP (mg/l)	Shoot producing explant %	Average Shoot Formed per explant	Elongated Shoots % Per Explant
SE1	MS+0.0+0.0	10.77 ^d	24.45 ^d	19.20 ^d
SE2	MS+1.0+0.0	47.65 ^c	55.00 ^{bc}	27.54 ^c
SE3	MS+1.5+0.2	58.90 ^c	76.00 ^b	40.00 ^b
SE4	MS+2.0+0.2	70.45 ^b	97.45 ^a	62.65 ^a
SE5	MS+2.5+0.2	85.55 ^a	61.25 ^b	45.00 ^b
SE6	MS+3.0+0.2	54.44 ^c	48.00 ^c	39.75 ^b
SE7	MS+0.0+0.2	15.21 ^d	32.00 ^d	21.32 ^d

Means with the same letter are statistically non-significant (*p*=0.05) Duncan's multiple range test (Duncan, 1955)

In the previous studies it has been reported the regeneration responses of different plant species/cultivars varies due to genetic distinctions among them (Ikeuchi et al., 2016).

Shoot Elongation and Root Formation

Seven different media were tested for regeneration and elongation of shoots produced previously on leaf explants. The spindles were shifted to MS medium having different concentrations of Kinetin and BAP to study the effect of cytokinin combination on shoot elongation and regeneration. The best response of shoot regeneration and elongation was observed in the MS media having 02 mg/l Kin and 02 mg/l BAP (Table 2).

In previous studies multiple shoots have been developed from sugarcane (*Saccharum officinarum* L.) using BAP and Kinetin (Baksha et al., 2002; Ali and Afghan, 2001). Cytokinins are thought to be involved in light mediated responses in higher plants. They induce many photomorphogenic responses in plants including chloroplast maturation (Stetler and Laetsch 1965), light regulated genes expression (Chory et al. 1994). The media used in the present study was simple in terms of chemical composition. Zeatin, thidiazuron, Coconut milk and casein hydrolysate was not included in media composition. This simple and cheap media produced best shoot elongation response in the sugarcane cultivar under study.

The regenerated shoots with 10 cm height were shifted to MS media with different concentrations of NAA for root initiation. MS medium with out NAA was used as control. The best response of root initiation was observed in RF5 medium (MS+2.0 mg/l NAA) among all the tested media (Figure 4). A 45.67% decline in plantlets formation response was observed in rooting media having 3.0 mg/l NAA. This may due to hormone dose saturation

response in physiological metabolism of plant cells.

The regenerated plants with well developed roots were shifted to pots having peat moss, clay and sand in 1:2:1 ratio. These plants were kept in shade for two weeks. In the first week, they were covered with polythene bags to maintain humidity. After two weeks hardening the established plants were transferred to earthen pots and put in field.

Transformation

Establishment of an efficient and reproducible transformation system is one of the prerequisites for improvement of agronomic characters in sugarcane (Mahmood et al., 2007; Weng et al., 2011). Success of transformation method is linked to many different factors including culture conditions, methodology used and explant regeneration. Transformation can be done directly or indirectly. The indirect transformation method using *Agrobacterium* has advantages over direct methods with respect to issues of copy number and gene instability (Weng et al., 2011).

In the present study two *Agrobacterium tumefaciens* strains, EHA101 and LBA 4404 were employed for genetic transformation of sugarcane cultivar SPF 234. The T-DNA insert was *GUS*, *NPTII* and *HPTII* genes. The transformation efficiency in terms of GUS expressing plant % was very low in control while maximum level of GUS expression (66% for LBA 4404 and 58% for EHA101) was observed at 60 μ M acetosyringone concentration in media. The maximum and minimum acetosyringone concentrations produced statistically non-significant results (Figure 5 B). We found that transformation efficiency increased with the increase in acetosyringone concentration in the co cultivation media for the sugarcane cultivar under study.

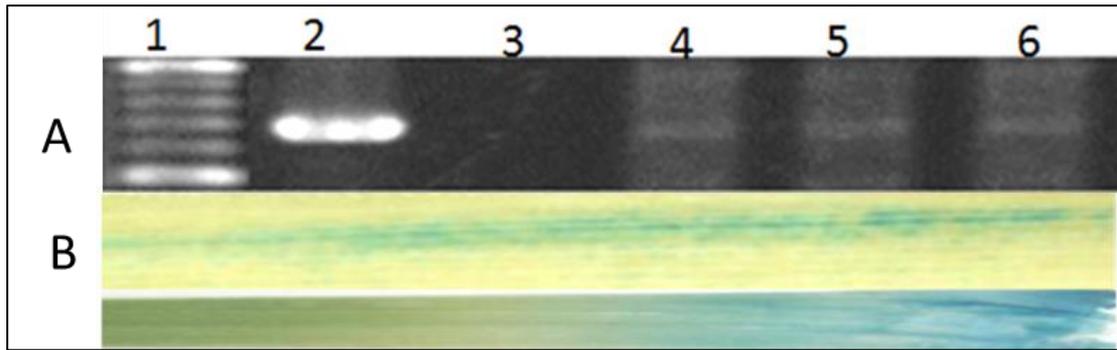


Figure 3: A. PCR amplification of *GUS* gene. Lane 1: 100 bp plus DNA ladder (Fermentas USA), Lane 2: Positive control of *GUS*, Lane 3: Negative control untransformed plant DNA, Lane 4-6: three accessions of SPF-234 transformed plants. B. GUS assay of transformed leaves

In the previous studies high concentration of acetosyringone has been reported for better results (Matsuoka et al., 2001; Manickavasagam et al., 2004). It has been known that acetosyringone interacts for *vir* gene expression in *Agrobacterium tumefaciens* during co-cultivation (He et al., 2010).

Effect of immersion time was also studied. It was observed that immersion time of 30 minutes produced maximum GUS expressing plant %age. A 62.86% decrease was observed in GUS expressing plants when immersion time was increased to 45 minutes. Greater immersion time increased *Agrobacterium* contamination and decreased plantlets regeneration from explant under study (Figure 5 A). This is probably due to damage of explant cells caused by overgrowth of bacteria. The same has been noticed by Ombori et al., (2012) for genetic transformation of maize hybrid lines.

***GUS* Expression Study**

The regenerated plants were subjected to histochemical assay for GUS expression study. The GUS expression was observed maximum in leaf as compared to all other parts of plants (Figure 3 B). The absence of blue colour indicated that gene transfer is unsuccessful by any means i.e. transformation failure, became silent or not expressing to make biological active product. Generally, GUS assay is considered as 1st step towards

transgene confirmation. In previous studies of sugarcane transformation, the GUS expression has been observed as initial indicator of successful gene transfer (Kharte et al., 2016; Prabu and Prasad, 2012).

PCR Analysis of Transgenic Plants

Screening of putative transformants in genetic engineering experiments had been important. PCR analysis is very vital for the selection of putative transformants to avoid any escapees during antibiotic selection phase (Arruda, 2012). Three GUS positive plants produced from different events were subjected to PCR analysis. Genomic DNA of these plants along with one positive and one negative plant, was isolated and used in PCR as template. Primers were designed from the construct in GUS region to amplify 620 bp fragment (Figure 3 A).

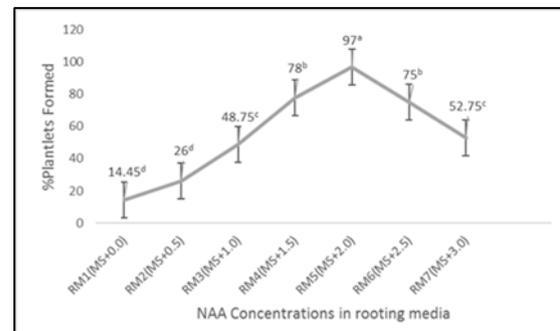


Figure 4: Effect of NAA in rooting media for young plantlets formation.

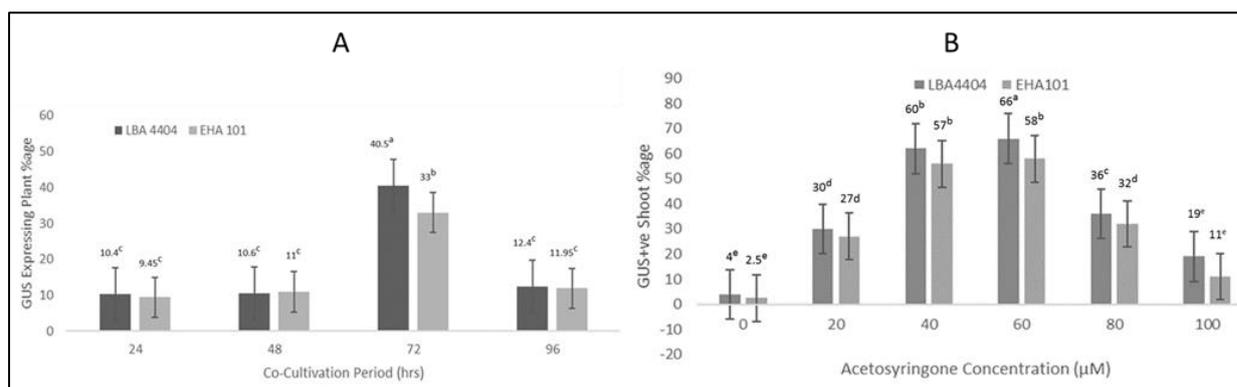


Figure 5: (A) Effect of cocultivation on GUS expression in plantlets formed using two *Agrobacterium* strains (B). Effect of acetosyringone on GUS expression in plantlets formed using two *Agrobacterium* strains.

In the present study many different factors of transformation including co-cultivation period, acetosyringone concentration, antibiotic concentration and direct regeneration of plantlets were studied.

All transformation factors studied have been found to affect the process significantly. In the present study 60 µM acetosyringone concentration and *Agrobacterium* strain LBA4404 was found most effective for transformation of the said cultivar. In this piece of research work, we established an efficient, cheap transformation and direct regeneration system for sugarcane cultivar SPF-234. This established protocol may be applied with reproducibility for desired agronomic traits in sugarcane.

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REFERENCES

Ahmadabadi M, Rauf S, Bock R (2007). A leaf-based regeneration and transformation system for maize (*Zea mays* L.). *Transgenic Res.*, 16: 437–448.

Ali K, Afghan S (2001). Rapid multiplication of sugarcane through

micropropagation technique. *Pak Sugar J.*, 16(6): 11-14.

Arruda P (2012). Genetically modified sugarcane for bioenergy generation. *Curr Opin Biotechnol.*, 23: 315–322.

Atak C, Celik Ö (2009). Micropropagation on *Anthurium andraeanum* from leaf explants. *Pak J Bot.*, 41: 1155-1161.

Badawy OM, Nasr MI, Alhendawi RA (2008). Response of sugarcane (*Saccharum* species hybrid) genotypes to embryogenic callus induction and in vitro salt stress. *Sugar Tech.*, 10: 243-47.

Badoni A, Chauhan J.S (2009). Effect of growth regulators on meristem-tip development and in-vitro multiplication of potato cultivar ‘Kufri Hilmalini’. *Nat Sci.*, 7: 31-34.

Bairu MW, Aremu AO, Staden JV (2010). Somaclonal variation in plants: Causes and detection methods. *Plant Growth Regul.*, 63: 147-173.

Baksha R, Alam R, Karim MZ, Paul SK, Hossain MA, Miah MAS, Rahman ABMM (2002). In vitro shoot tip culture of sugarcane (*Saccharum officinarum*) variety LSD28. *Biotechnol.*, 1(2-4): 67-72.

Cho MJ, Jiang W, Lemaux P (1998). Transformation of recalcitrant barley cultivars through improvement of regenerability and

- decreased albinism. *Plant Sci.*, 138: 229-244.
- Chory J, Cook RK, Dixon R, Elich T, Li H M, Lopez E, Mochizuki N, Nagpal P, Pepper A, Poole D, Reed J (1995). Signal-Transduction Pathways Controlling Light-Regulated Development in *Arabidopsis*. *Philosophical Transactions: Biological Scie.*, 350(1331): 59-65.
- Danilova SA (2007). The technologies for genetic transformation of cereals. *Russ. J Plant Physiol.*, 54: 569-581.
- Dey M, Bakshi S, Galiba G, Sahoo L, Panda SK (2012). Development of a genotype independent and transformation amenable regeneration system from shoot apex in rice (*Oryza sativa* spp. *indica*) using TDZ. *3 Biotech.*, 2: 233-240.
- Eldessoky DS, Ismail RM, Abdel-Hadi AH A, Abdallah NA (2011). Establishment of regeneration and transformation system of sugarcane cultivar GT54-9 (C9). *GM Crops*. 2(2): 126-134.
- Fang YD, Akula C, Altpeter F (2002). *Agrobacterium*-mediated barley (*Hordeum vulgare* L.) transformation using green fluorescent protein as a visual marker and sequence analysis of the T-DNA barley genomic DNA junctions. *J Plant Physiol.*, 159: 1131-1138.
- He Y, Jones HD, Chen S, Chen XM, Wang DW, Li KX, Wang DS, Xia LQ (2010). *Agrobacterium*-mediated transformation of durum wheat (*Triticum turgidum* L. var. durum cv Stewart) with improved efficiency. *J Exp Bot.*, 61:1567-1581.
- Ikeuchi M, Ogawa Y, Iwase A, Sugimoto K (2016). Plant regeneration: cellular origins and molecular mechanisms. *Development*. 143: 1442-1451 doi:10.1242/dev.134668
- Jahangir GZ, Nasir IA, Sial RA, Javid MA, Hussain T (2010). Various hormonal supplementations activate sugarcane regeneration in vitro. *J Agri Sci.*, 2(4):23-237.
- Jones HD, Doherty A, Wu H (2005). Review of methodologies and a protocol for the *Agrobacterium*-mediated transformation of wheat. *Plant Methods.*, 1: 5-6.
- Khalil MS (2002). Regeneration via somatic embryogenesis and microprojectile mediated co-transformation of sugarcane. *Arab J Biotech.*, 5:19-32.
- Khan IA, Dahot MU, Seema N, Yasmeen S, Bibi S, Raza G, Khatri A, Naqvi MH (2009). Direct regeneration of sugarcane plantlets: A tool to unravel genetic heterogeneity. *Pak J Bot.*, 41(2): 797-814.
- Kharte SB, Watharkar AS, Shingote PR, Sharanya C, Kawar PG, Pagariya MC, Govindwar SP (2016). Functional characterization and expression study of sugarcane MYB transcription factor gene PEaMYBAS1 promoter from *Erianthus arundinaceus* that confers abiotic stress tolerance in tobacco. *RSC adv.*, 6: 19576-19586.
- Mahmood A, Ishfaq M, Iqbal J, Nazir MS (2007). Agronomic performance and juice quality of autumn planted sugarcane (*Saccharum officinarum* L.) as affected by flat, ditch and pit planting under different spatial arrangements. *Int. J Agric Biol.*, 9: 167-169.
- Malhotra PK, Mittal A, Chopra R, Kaur M Thind KS (2017). Genetic Augmentation of Sugarcane with Stress-Inducible DREB1A Transcription Factor Using Biolistic Approach. *Sugar Tech.*, 19(05):469-475.
- Mandal SM, Chakraborty D, Dey S, (2010). Phenolic acids act as signaling molecules in plant

- microbe symbioses. *Plant Signal Behav.*, 5: 359-368.
- Manickavasagam M, Ganapathi A, Anbazhagan VR, Sudhakar B, Selvaraj N, Vasudevan A, Kasthuriengan S (2004). Agrobacterium-mediated genetic transformation and development of herbicide-resistant sugarcane (*Saccharum* species hybrids) using axillary buds. *Plant Cell Rep.*, 23: 134-143.
- Matsuoka M, Ideta O, Tanio M, Hayakawa A, Miwa H (2001). Agrobacterium tumefaciens-mediated transformation of sugarcane using cell suspension culture with a novel method. *International Society of sugarcane Technology. Proc. the xxiv Congress Brisbane Austr.*, 2: 660-662.
- Miki B, McHugh S (2004). Selectable marker genes in transgenic plants: applications, alternatives and biosafety *J Biotechnol.*, 107: 193-232.
- Mustafa G, Khan MS (2012). Prospecting the utility of antibiotics as lethal selection agents for chloroplast transformation in sugarcane. *Int. J Agric. Biol.*, 14: 307-310.
- Nawaz M, Ullah I, Iqbal N, Iqbal MZ, Javed MA (2013). Improving in vitro leaf disk regeneration system of sugarcane (*Saccharum officinarum* L.) with concurrent shoot/root induction from somatic embryos. *Turk J Biol.*, 37: 726-732.
- Nawaz M, Hussain SA, Ullah I, Younus M, Iqbal MZ, Rana SM (2009). Estimation of genetic diversity in wheat using DNA markers, *Am Eurasian J Sustain Agric.*, 3(3): 507-511.
- Olhoft PM, Flagel LE, Somers DA (2004). T-DNA locus structure in a large population of soybean plants transformed using the Agrobacterium-mediated cotyledonary-node method. *Plant Biotechnol J*, 2: 289-300.
- Ombori O, Muoma JVO, Machuka J (2012). Agrobacterium mediated genetic transformation of selected tropical inbred and hybrid maize (*Zea mays* L.) lines. *Plant Cell Tissue Organ Cult.*, 113: 11-23.
- Popelka JC, Altpeter F (2003). Evaluation of rye (*Secale cereale* L.) inbred lines and their crosses for tissue culture response and stable genetic transformation of homozygous rye inbred line L22 by biolistic gene transfer. *Theor Appl Genet.*, 107: 583-590.
- Prabu G, Prasad DT, (2012). Functional characterization of sugarcane MYB transcription factor gene promoter (PScMYBAS1) in response to abiotic stresses and hormones. *Plant Cell Rep.*, 31: 661-669.
- Rahman ZAS, Roowi W, Zaliha WS, Subramaniam S (2010). Regeneration of Malaysian indica rice (*Oryza sativa*) variety mr232 via optimised somatic embryogenesis system. *J Phytol.*, 2(3): 30-38.
- Sata SJ, Bagatharia SB, Thaker VS (2000). Induction of direct embryogenesis in garlic (*Allium sativum*). *Methods Cell Sci.*, 22: 299-304.
- Singh RK, Khan MS, Yadav S, Singh MR, Madhok HL, Srivastava S, Singh J (2011). Agrobacterium-mediated genetic transformation of subtropical sugarcane variety with Cry1Ab gene. *Nat Academy Sci Letters-Ind.*, 34: 103-111.
- Stetler DA, Laetsch WM (1965). Kinetin-induced chloroplast maturation in cultures of tobacco tissue. *Sci.*, 149: 1387-1388.
- Taparia Y, Fouad WM, Gallo M, Altpeter F (2012). Rapid production of transgenic sugarcane with the introduction of simple loci following biolistic transfer of a minimal expression cassette and

- direct embryogenesis. *In Vitro Cell Dev Biol Plant.*, 48: 15–22.
- Thorpe TA (1994). *In vitro* Embryogenesis in Plants. *Current Plant Science and Biotechnology in Agriculture*, Kluwer, Boston MA, 1995; vol. 20.
- Uzma MR, Khan A, Muhammad I, Hussain SH, Shah T, Kumar S, Inam M, Zubair HU, Rehman A, Sher N, Rehman S, Ahmad Ali GM (2012). Rapid *in vitro* multiplication of sugarcane elite genotypes and detection of sugarcane mosaic virus through two steps RT-PCR. *Int J Agric Biol.*, 14: 870–878.
- Vikrant A, Rashid A (2001). Direct as well as indirect somatic embryogenesis from immature (unemerged) inflorescence of a minor millet *Paspalum scrobiculatum* L. *Euphytica*, 120: 167-173.
- Weng LX, Deng HH, Xu JL, Li Q, Zhang YQ, Jiang ZD, Li QW, Chen JW, Zhang LH (2011). Transgenic sugarcane plants expressing high levels of modified cry1Ac provide effective control against stem borers in field trials. *Transgenic Res.* 20: 759–772.