Science, Technology and Development 37 (2): 93-100, 2018
ISSN 0254-6418 / DOI: 10.3923/std.2018.93.100

Effects of Growth Regulators on Photosynthetic Pigments and Some Biochemical Attributes in Shoot Cultures of *in vitro* Developed Virus Free Sugarcane Genotypes

[1]Shahid Nazir, [2]Iqbal Hussain, [2]Rubaika Khurshid, [1]Muhammad Aslam Javed, [1]Muhammad Zaffar Iqbal and [1]Saleem Akhtar

[1]Agricultural Biotechnology Research Institute, Ayub Agricultural Research Institute, Faisalabad, Pakistan
[2]Department of Botany, Government College University, Faisalabad, Pakistan

Abstract: Current study evaluates the impact of various plant growth regulators on sugarcane shoot proliferation, photosynthetic pigments and production of primary metabolites. Two sugarcane genotypes were *in vitro* maintained on MS medium having various amounts of 2,4-D for callogenesis, BAP and kinetin for shoot development and NAA for rooting. Among cytokinins, BAP was the most competent for shoot induction with maximum fresh weight and shoot length observed at 1.5 mgL^{-1} in S2007-AUS285 sugarcane genotype. Lower amounts of 2,4-D produced more callus in S2006-SP93 and higher fresh weight in S2007-AUS285. Genotype S2006-SP93 was highly susceptible to sugarcane mosaic virus and this disease incidence was reduced during *in vitro* propagation work. Disease free plants were exposed to different levels of hormones with various combinations to investigate the accumulation of primary metabolites/biochemicals and photosynthetic performance. Significantly higher photosynthetic pigments were recorded when BAP was used at 1.0 and 1.5 mgL^{-1} and kinetin was used at 1.0 and 0.5 mgL^{-1}. Moreover, some potential biochemical attributes like free proline, total soluble proteins and total free amino acids, were also tested in both sugarcane genotypes. S2006-SP93 showed increased levels at 1.0 mgL^{-1} of BAP and kinetin, while S2007-AUS285 depicted enhanced levels of biochemical at 0.5 and 1.0 mgL^{-1} of BAP. The study dissected out physiological impacts of plant growth regulators on photosynthetic pigments and other biochemical attributes of sugarcane genotypes under *in vitro* conditions.

Key words: Amino acid, 6-benzylaminopurine, free proline, kinetin, photosynthetic pigments, sugarcane shoots.

INTRODUCTION

Sugarcane is a versatile crop and a chief source of sugar production all over the world, including Pakistan. It drives large sugar industry and has significant importance in national economy. It also offers raw materials for numerous industries, including sugar, chipboard, paper and ethanol. Sugarcane was cultivated on 1217 thousand hectares with total production of 73.6 million tons (Anonymous, 2016-17). In Pakistan, normal yield of sugarcane is very poor in comparison to world average yield, which is 18,000-20,000 kg per acre (FAOSTAT, 2012). Pakistan is ranked 5th in cane production, but 15th in production of refined sugar. About 30% of the sugarcane is crushed in mills for sugar and other products, including Jaggery and gur, etc. Various byproducts, like alcohol for pharmacological industry, ethanol as fuel, bagasse in paper making, chipboard preparation and press mud, used as a main source of organic matter/nutrients, which add to soil as productivity enhancer (Hussain *et al.,* 2004).

In spite of the growth in sugarcane area and sugar industry, per acre yield and sugar recovery, the production is very low as compared to the actual potential due to various ecological restrictions. Biotechnology offers possible solutions to solve such issues and can provide a great assistance to the growers. Using micro propagation tools of biotechnology, one can regenerate the plants, develop new genotypes and produce novel crop in less time (Gill *et al.,* 2006). Micro-propagation is a process of *in vitro* culture, used to develop new replicas of plants. Growth regulators are utilized to produce new roots and shoots. Top genotypes, disease free production and development can be obtained via

Corresponding Author: Shahid Nazir, Agricultural Biotechnology Research Institute, Ayub Agricultural Research Institute, Faisalabad, Pakistan
E-mail: shahidnazir97@yahoo.com

different *in vitro* culturing protocols (Cruz-Cruz *et al.,* 2013) as it enables the viable propagation of plants cells. Vegetative plant proliferation like haploid and diploid embryos is cultured without gamete fusion (Emons, 1994). Callus culture develops infection free plantlets and is a useful, reproducible and cost-effective system. It is a known fact that regeneration efficiency of micro-propagated crops depends upon the cultivar used (Abe and Futsuhara, 1986).

Conventional techniques normally take around 10 years for the development of a better genotype. While, *in vitro* culture could be a decent option for genetic enhancement of asexually proliferated crops like sugarcane because it needs minimum time in contrast to traditional approaches. Callogenesis has been well recognized by different plant parts like shoot tips, fresh leaf and inflorescence on artificial medium having 2,4-D. Developed plantlets can display many changes for various traits like cane yield, sugar percentage, infection free and different stress tolerance capabilities (Lakshmanan *et al.,* 2006).

The variations produced in results of *in vitro* culture are known as soma-clonal variations. The developed soma-clones sometime are very valuable, particularly in growing a disease-free plant with superior characteristics. The occurrence of disease in soma-clones can be confirmed using different molecular biology tools like Enzymes Linked Immuno Sorbent Assay (ELISA). The theme of current study was the development of good sugarcane soma-clones having improved characters and also identification of best hormonal combinations for maximum shoot growth. Biochemical and morphological examination was also carried out for *in vitro* developed sugarcane plants.

MATERIALS AND METHODS

Ex-plant collection and preparation: Fresh leaf samples of two sugarcane genotypes (S2006-SP93 and S2007-AUS285) were selected from field area of Sugarcane Research Institute, Faisalabad. Ex-plant was the innermost leaf portion of around five leaves with 2-3 mm in length, washed under tap water for 10-15 minutes, rinsed with sterile water and washed with 70% alcohol for 1.0 min before transfer to a laminar air flow cabinet. All these activities were executed under hygienic conditions.

Preparation of culture medium and sterilization: The medium used was MS (Murashige and Skoog, 1962), having 4.33 gL^{-1} MS salts, 1.75 gL^{-1} phytagel, 30 gL^{-1} sucrose and different levels of growth regulators. The medium was sterilized at 15 lbs of pressure for 25 min at 121°C in an autoclave. All working tools, like forceps, scalpels and blades, etc., were decontaminated to maintain a germ-free environment.

Callogenesis: Four dissimilar levels of 2,4-D (1.0, 3.0, 5.0, 7.0 mgL^{-1}) were tried in MS medium. Disinfected explants were placed on callus induction medium for 2 weeks in incubation room in dark at 25 ± 2 °C and then transferred to continuous fluorescent light of 2000 to 2500 lux. On visual evaluation, poor to excellent (++++) callus data was noted after every seven days interval.

***In vitro* regeneration and organogenesis:** Organo-genesis was carried out at various levels of BAP and kinetin (Kn). Four levels of both Kn (0.5, 1.0, 1.5, 2.0 mgL^{-1}) and BAP (0.5, 1.0, 1.5, 2.0 mgL^{-1}) were used separately in MS medium. *In vitro* developed shoots were aseptically multiplied and shifted to the half strength of MS supplemented with 0.5, 1.0, 1.5, 2.0 mgL^{-1} of NAA for root initiation.

Acclimatization of *in vitro* propagated sugarcane clones: Vigorous and well rooted sugarcane clones were shifted into earthen pots, having sterilized sand, clay and well decomposed farmyard manure. Pots were cased with translucent polyethylene bags to maintain the humidity. Required inputs were regularly provided until the plantlets were shifted into soil under normal climatic situations.

Screening of virus free plants using ELISA technique: Mosaic virus disease in sugarcane was tested by ELISA on artificially inoculated *in vitro* developed plants. Soft leaves about 2-3 cm were ground in 0.5 M phosphate buffer. According to the method of Kemeny and Challacombe (1989), double antibody sandwich ELISA was performed to identify the disease free plants.

Biochemical analysis:

Amino acids' determination: Total free amino acids were measured by ninhydrin method as described by Hamilton and Van (1973). 1.0 gram of sugarcane leaf material was ground in phosphate buffer (pH 7.0), 1.0 ml of extract was poured in 25 mL tube and mixed with 1.0 mL of 10% pyridine and 1.0 mL of 2% ninhydrin mixture/solution. Water bath was used to heat the samples for 30 min and volume was made up to 50 mL using sterile water. OD was calculated at 570 nm with the help of UV-VIS spectrophotometer (Hitachi U-2910 Tokyo Japan). Free amino acids were measured from standard curve of Lucine expressed as mg g^{-1}.

Total protein determination: Fresh sugarcane plant material was used for the isolation of total proteins using phosphate buffer saline (PBS, pH 7.2). Bradford assay was used to measure the quantity of total proteins as described by Bradford (1976), and absorbance was calculated at 595 nm. BSA (Bovine serum albumin) was used as internal control. Total proteins were measured in mg mL^{-1}.

Measurements of free proline content: Free proline content in leaves was calculated with protocol of Bates *et al.* (1973). Fresh plant material of 0.1 g was thoroughly mixed in 5.0 mL of 3% aqueous sulphosalicylic acid. 1.0 mL remainder was mixed in 1.0 mL of ninhydrin and glacial acetic acid. The

mixed solution was warmed at 100 ºC for 10 min and chilled on ice. 4.0 mL of toluene was used and mixed using vortexer for 20 sec and cooled. The absorbance was calculated at 520 nm. Free proline quantity was determined using standard curve and measured in μmol g^{-1} of fresh weight.

Measurement of photosynthetic pigments: Photosynthetic pigments, i.e. chlorophylls (Chl) a, b, and carotenoids (Car), were measured using 0.1 g of top fresh leaves and thoroughly mixed in 1.0 mL of 80% acetone using pestle and mortar and sieved using filters. The absorbance was calculated at 480 nm for Car, 663 nm and 645 nm for Chl.a and Chl.b respectively using spectrophotometer. Chl.a Chl.b and their whole Chl contents were measured as described by Yoshida *et al.* (1976), and carotenoids were determined using the method of Davies (1976).

Statistical analysis:

The data for callus induction and regeneration, photosynthetic pigment and biochemical attributes was recorded and examined statistically using a completely randomized design (Steel *et al.* 1997).

RESULTS AND DISCUSSION

Response of sugarcane genotypes to callus induction medium: Sterilized tissues from both sugarcane genotypes were aseptically placed on artificial medium containing varying levels of 2,4-D for callogenesis. Significant differences were observed in callus formation to each level of 2,4-D, whereas the difference between genotypes was non-significant. The genotype S2006-SP93 showed maximum response to callus formation when 2,4-D concentration was 3 mgL^{-1}. At this level, callus formation frequency was 33%, whereas for S2007-AUS285, best callus formation was recorded at 3 mgL^{-1} of 2,4-D (22.22%). It was also recorded that at low level and very high doses of 2,4-D, milky types of callus were formed. Statistically significant differences were also observed in the fresh weight of callus on all tested levels of 2,4-D. Callus fresh weight was documented after 15 days of transferring in light. The maximum weight of genotype S2006-SP93 (0.57±0.01 g) was observed at 3 mgL^{-1} of 2,4-D, while S2007-AUS285 gained maximum weight of 0.66±0.01 g when 2,4-D level was 5 mgL^{-1} (Table 1).

Table 1: The effect of 2,4-D on callogenesis in sugarcane genotypes.

Genotypes	2,4-D (mgL^{-1})	Response towards callus formation	Callus formation % age	Callus weight (g)
S2006-SP93	0.0	Poor	0.00	0.05 ± 0.01
	1.0	++++	11.0	0.32 ± 0.01
	3.0	++++	33.0	0.57 ± 0.01
	5.0	++++	22.0	0.42 ± 0.02
	7.0	++++	22.0	0.37 ± 0.01
S2007-AUS285	0.0	Poor	0.00	0.05 ± 0.01
	1.0	++++	11.0	0.05 ± 0.01
	3.0	++++	22.22	0.26 ± 0.01
	5.0	++++	11.0	0.66 ± 0.01
	7.0	++++	11.0	0.45 ± 0.01

++++: excellent; 2,4-D: 2,4-dichlorophenoxyacetic acid

Regeneration and rooting response of sugarcane genotypes: Five weeks old calli were transferred onto regeneration medium for the development of *in vitro* shoots and roots. Various levels of Kn were practiced for initiation of shoot regeneration. Statistical analysis showed that difference was non-significant between these genotypes, whereas, the interactive effect of genotypes and treatments was highly significant. Genotypes S2006-SP93 gave maximum regeneration response at 1.0 mgL^{-1} (3.07 ± 0.35), whereas, S2007-AUS285 showed maximum shoot initiation (3.07 ± 0.35) when Kn dose was 1.5 mgL^{-1}. Shoot elongation and multiplication was carried out at different levels of BAP. Maximum shoots of sugarcane genotype S2006-SP93 (23.66 ± 0.33) per ex-plant was noted at 1.0 mgL^{-1} of BAP as compared to other variety (S2007-AUS285) develop maximum shoots (30.66 ± 0.33) at 1.5 mgL^{-1} of BAP levels. Overall, addition of BAP at 1.0 to 1.5 mgL^{-1} levels was the best in both genotypes. For initiation of roots, various levels of NAA were used in combination with half MS medium. When the shoot length reached 2-3 inches, it was exposed to half MS medium with four concentrations of NAA (0.5 to 2.0 mgL^{-1}). Genotype S2006-SP93 showed maximum number of roots (3.07 ± 0.34 and 3.10 ± 0.20) at 0.5 and 1.0 mgL^{-1} of NAA respectively. The maximum number of roots (3.06 ± 0.34 and 3.1 ± 0.20) was noted at 1.0 and 1.5 mgL^{-1} of NAA in S2007-AUS285 sugarcane genotype respectively (Table 2).

Table 2: The effect of Kn, BAP and NAA on *in vitro* regeneration and rooting of sugarcane genotypes.

Genotypes	Hormone level (mgL^{-1})	Shoot multiplication (MS + Kn)	No. of shoot/explants (MS + BAP)	Rooting (1/2 MS + NAA)
S2006-SP93	0.0	2.33 ± 0.33	7.66 ± 0.33	2.30 ± 0.33
	0.5	3.07 ± 0.35	15.00 ± 0.57	3.07 ± 0.34
	1.0	3.10 ± 0.21	23.66 ± 0.33	3.10 ± 0.20
	1.5	2.80 ± 0.15	18.00 ± 0.57	2.80 ± 0.15
	2.0	2.70 ± 0.35	2.33 ± 0.33	2.70 ± 0.35
S2007-AUS285	0.0	2.03 ± 0.32	17.33 ± 0.33	2.03 ± 0.31
	0.5	2.33 ± 0.33	22.33 ± 0.33	2.33 ± 0.33
	1.0	3.07 ± 0.35	29.33 ± 0.33	3.06 ± 0.34
	1.5	3.10 ± 0.21	30.66 ± 0.33	3.10 ± 0.20
	2.0	2.80 ± 0.15	14.66 ± 0.33	2.80 ± 0.15

MS: Murashige and Skoog; Kn: Kinetin; BAP: 6-benzylaminopurine; NAA:α-Naphthaleneacetic acid

Screening of sugarcane mosaic virus free plants: Well rooted and healthy *in vitro* plants were shifted into soil for further development of roots and proliferation of shoots. Plants were exposed to the natural environment under field conditions. Thirty six plants were selected after one month of exposure for ELISA based screening of sugarcane mosaic virus free plants. Leaf samples were ground in phosphate buffer and ELISA was performed for the investigation of transmission of SCMV disease. All the samples and controls were poured in duplicate fashion in 96 well plates. Negative and positive control samples were also run in ELISA to verify the testing procedure. Results were according to expectations because only 15 plants displayed positive and 21 gave negative reactions. Hence, *in vitro* developed sugarcane genotypes showed 68% disease free plants.

Young sugarcane leaves are a good source of ex-plant for callogenesis (Brisibe *et al.,* 1994; Chengalrayan and Gallo-Meagher, 2001) and 2,4-D has been added to encourage calli from different sugarcane parts (Oropeza and Garcia, 1996; Gallo-Meagher *et al.,* 2000). So, young leaves of elite sugarcane genotypes such as S2006-SP93 and S2007-AUS285 were used. The present study indicated maximum root/shoot length, number of leaves and number of shoots in MS medium containing 3.0 mgL^{-1} of 2,4-D. The genotype S2006-SP93 showed maximum response to callus formation at 3 mgL^{-1} of 2,4-D. The callus formation frequency at this concentration was 33%. The genotype S2007-AUS285 showed the best callus initiation percentage (22.22%) when the medium was containing 3 mgL^{-1} of 2,4-D (Table 1). The hormonal effects on growth, physical and biochemical qualities after one month of culture were examined. Different levels of BAP (1.0, 1.5 mgL^{-1}) for multiplication and the Kn (0.5, 1.0 mgL^{-1}) for regeneration process was used. By increasing the level of BAP (1.0, 1.5 mgL^{-1}) and Kn (0.5, 1.0 mgL^{-1}) in MS also decreased shoot/root length of plants *in vitro* in both genotypes. The shoot length, number of leaves and number of shoots were improved at concentration of 1.0 and 1.5 mgL^{-1} of BAP and 1.0 mgL^{-1} of Kn (Table 1). These findings are in accordance to some previous results of Capelle *et al.* (1983); Murthy *et al.* (1995); Hutchinson and Sexena (1996).

Photosynthetic pigment analysis of *in vitro* developed disease free sugar cane plants: The growth regulator (BAP) differently controlled the photosynthetic pigments contents in *in vitro* developed disease free both sugarcane genotypes. For Chl.a, the data indicated a significant ($P<0.001$) difference in genotypes and treatments with significant ($P<0.001$) interaction between genotypes and BAP treatments. Levels of photosynthetic pigment were more in S2006-SP93 sugarcane genotype as compared to S2007-AUS285 on all tested levels of hormones (Fig. 1). For S2006-SP93, greater Chl.a content (7.26 ± 0.62 mg) produced at 1.0 mgL^{-1} levels of BAP, while quite similar results, i.e. 0.57 ± 0.01 mg and 0.63 ± 0.057 mg in genotype S2007-AUS285, were obtained at 1.0 mgL^{-1} and 1.5 mgL^{-1} of BAP respectively. For Chl.b, data showed a non-significant difference in the genotypes ($P>0.05$) and treatments ($P<0.001$), and a significant ($P<0.01$) interaction between the treatments and genotypes. Both *in vitro* developed sugarcane genotypes showed the highest Chl.b at the control level of BAP, as compared to other levels. Greater Chl.b content produced in S2006-SP93 (8.19 ± 0.45 mg) than S2007-AUS285 (2.17 ± 0.19 mg) at a control level. For total Chl. and Car., the data showed a significant difference ($P<0.001$) in the genotypes and treatments (BAP). A greater total Chl. content (14.01 ± 0.23 mg) was produced in S2006-SP93 than in S2007-AUS285 (2.31 ± 0.01 mg) at 0 mgL^{-1} of BAP. Total Car. contents increased in both genotypes S2006-SP93 (2.75 ± 0.11 mg) and S2007-AUS285 (1.13 ± 0.05 mg) at 1.0 mgL^{-1} of BAP (Fig. 1).

The growth regulator (Kn) differently modified the photosynthetic pigments in both sugarcane disease free genotypes. For Chl.a content, the result revealed significant (P<0.001) difference in genotypes, treatments (Kn) (P<0.001) with significant interaction between these aspects. The response of both genotypes of sugarcane was not the same with respect to Chl.a. However, a reduction in Chl.a was observed at all levels of Kn except in control (7.29 ± 0.26 mg) in S2006-SP93 genotype, while S2007-AUS285 accumulated (0.58 ± 0.02 mg) more Chl.a at 1.5 mgL^{-1} of Kn. For Chl.b, data indicated non-significant (P>0.05) difference in genotypes and significant (P<0.001) in treatments (Kn). The highest Chl.b contents (2.75 ± 0.11 mg) were documented in genotype S2006-SP93 at 1.0 mgL^{-1} of Kn, while S2007-AUS285 accumulated more Chl.b contents (1.031 ± 0.37 mg) at 1.5 mgL^{-1} of Kn. For total Chl. and Car., the data showed significant (P<0.001) variations in genotypes, and treatments (Kn) by significant interaction between these factors. Genotype S2006-SP93 presented nearly same findings at all levels of Kn, while S2007-AUS285 displayed maximum response (2.13 ± 0.12) at 1.5 mgL^{-1} of Kn. Maximum carotenoid accumulation was found at 0.5 mgL^{-1} (2.94 ± 0.23) and 1.0 mgL^{-1} (2.95 ± 0.23) in S2006-SP93 and 0.87 ± 0.013 in S2007-AUS285 genotype at 1.5 mgL^{-1} of Kn (Fig. 1).

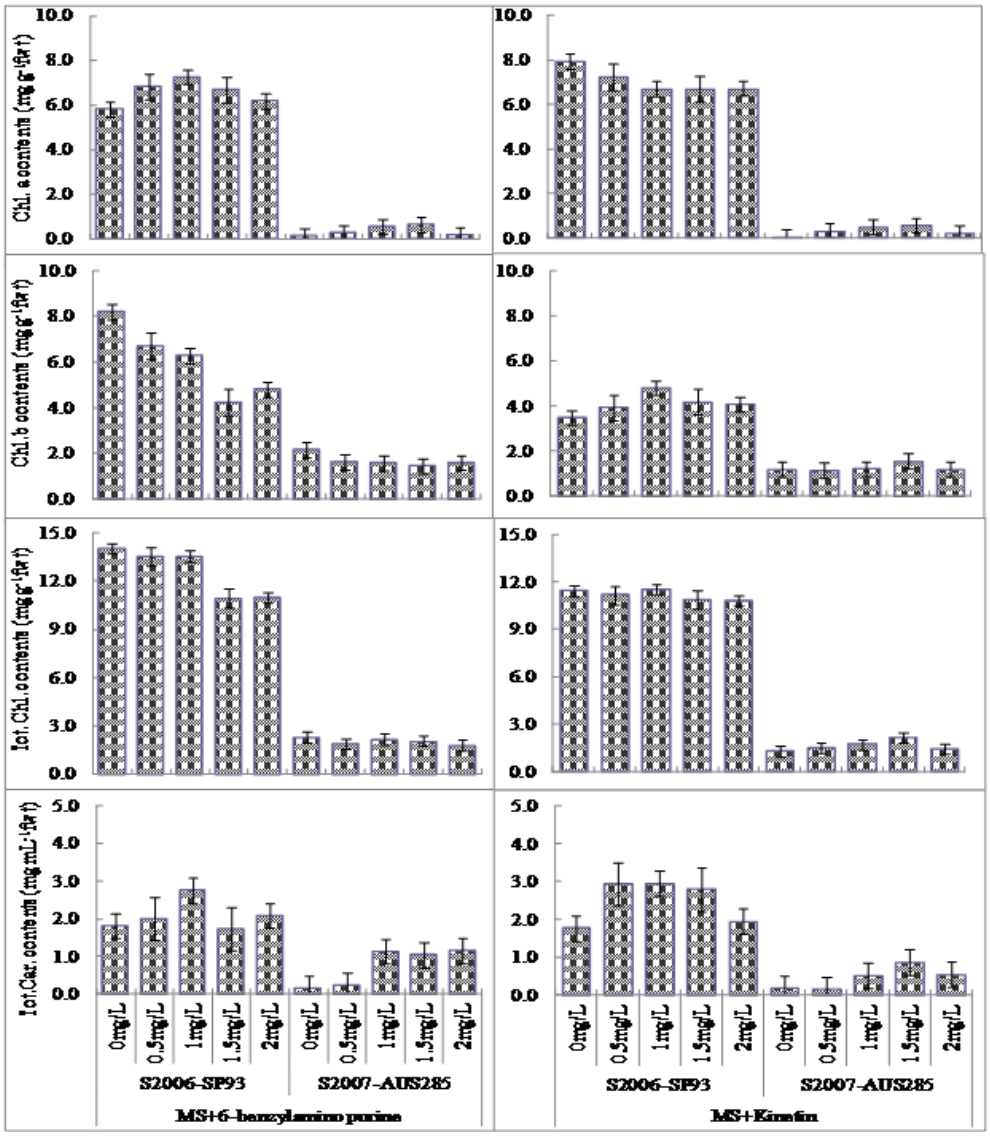

Fig. 1: The effects of some growth regulators on photosynthetic pigments in shoot cultures of *in vitro* developed virus free sugarcane genotypes. The data presented are mean values (n = 3 ± SE).

Photosynthetic pigments are significant to plants, primarily for collecting light and manufacturing of reducing powers (Eux *et al.,* 2004). It was also stated that BAP and Kn application reduced the degeneration in chlorophyll content and increased cell division, elongation, increased chlorophyll biosynthesis and delayed the leaf senescence (Liu and Huang, 2002). This may be due to reduced enzymatic activities intricate in chlorophyll catabolism, like chlorophyllase, Mg-dechelatase and peroxide linked with chlorophyll bleaching in broccoli (Costa *et al.,* 2005). The current study indicated that among two chlorophyll types, Chl.b is more prone to degradation by BAP in S2007-AUS285 genotypes than S2006-

SP93. From these changes in chlorophyll concentrations levels, it can be assumed that sensitivity of Chl.b to BAP is higher (Fig. 1).

Biochemical studies of *in vitro* developed disease free sugarcane plants: Total soluble proteins, amino acids and proline contents were recorded in *in vitro* developed disease free sugarcane genotypes under different levels of BAP. The reaction of sugarcane genotypes was different with respect to these attributes. For total soluble protein contents, the data revealed non-significant (P>0.05) variations in genotypes, while different treatments (BAP) varied significantly (P<0.001). S2006-SP93 genotype showed maximum total soluble protein contents at 1.0 mgL^{-1} (7.90 ± 0.90) of BAP, while S2007-AUS285 showed the highest production of protein at 0.5 mgL^{-1} (7.13 ± 0.34) of BAP. For total free amino acids, data showed a significant (P<0.1) difference in the genotypes, and treatments (P<0.001) with significant (P<0.001) interactions between these factors. S2006-SP93 genotype showed a maximum accumulation of free amino acids (4.66 ± 0.40) at 1.0 mgL^{-1} of BAP, while S2007-AUS285 also showed the highest production of amino acids (9.01 ± 0.43) at 1.0 mgL^{-1} of BAP. In leaf free proline contents, data showed significant (P<0.001) variations in genotypes, and BAP with non-significant (P>0.05) interaction between these features. The response of both genotypes was not the same with respect to this attribute. Maximum free proline accumulation (4.06 ± 0.01) was recorded in S2006-SP93 at the 1.0 mgL^{-1} level of BAP, while it reduced gradually at all levels except in control (2.70 ± 0.08) in S2007-AUS285 (Fig. 2).

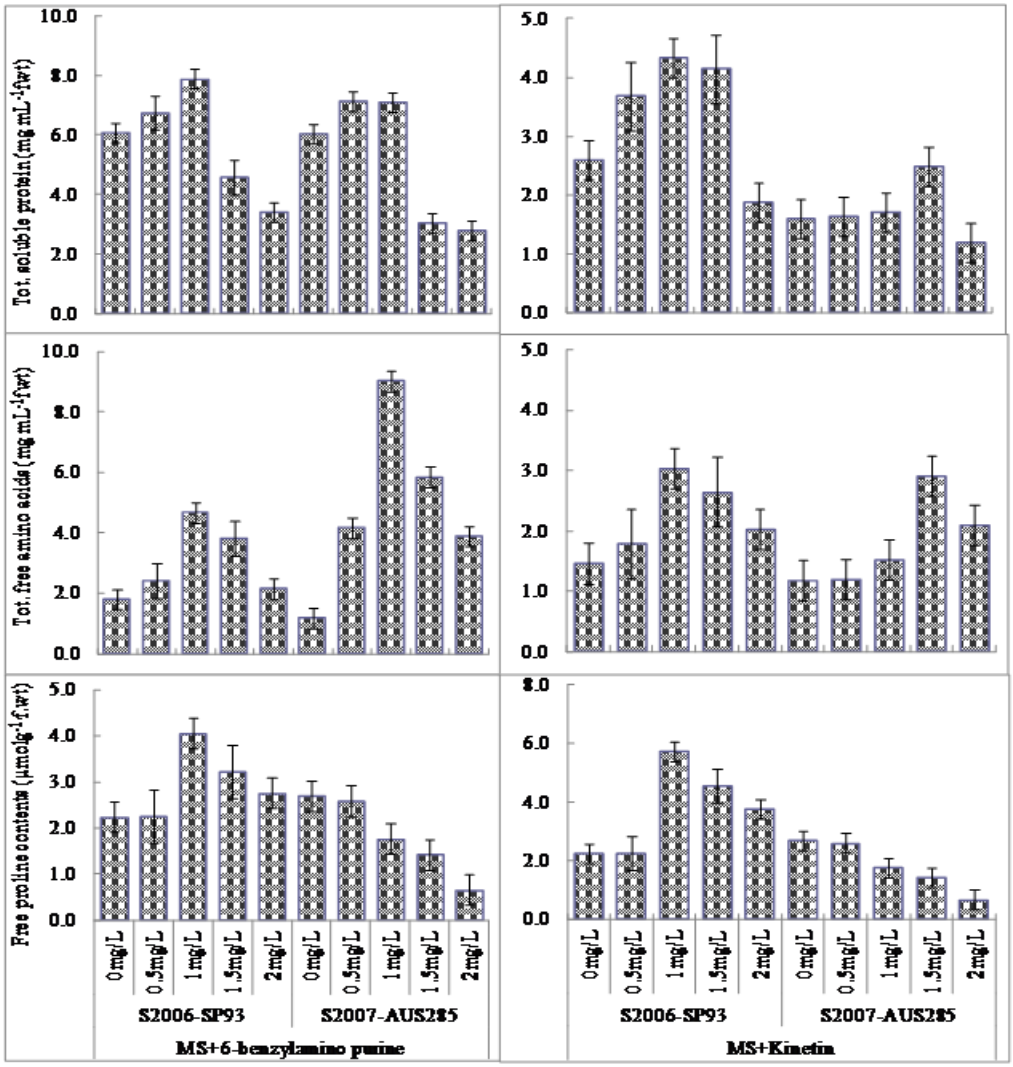

Fig. 2: The effects of growth regulators on some biochemical attributes in shoot cultures of in vitro developed virus free sugarcane genotypes. The data presented are mean values (n = 3 ± SE).

Free amino acids, free proline and total soluble protein contents were also estimated in disease free plants of sugarcane genotypes under different levels of Kn. For total soluble protein contents leaf, the results showed non-significant (P>0.05) difference in genotypes, and significant (P<0.001) in treatments (Kn) with significant (P<0.001) interaction between these factors (P<0.001). Leaf protein contents increased in both the genotypes (Fig. 2). The higher amount of protein (4.333 ± 0.731) accumulated in

S2006-SP93 at 1.0 mgL^{-1} of Kn, while S2007-AUS285 showed greater accumulation of protein at 1.5 mgL^{-1} of Kn. For amino acid contents, a significant ($P<0.001$) difference in the genotypes and treatments (Kn) along with significant interaction between these factors was noted. The level of free amino acid accumulation was noted in both genotypes. The genotype S2006-SP93 showed optimum accumulation of amino acid (1.19 ± 0.05) at 1.0 mgL^{-1}, while S2007-AUS285 showed the highest accumulation at 1.5 mgL^{-1} (2.91 ± 0.11) of Kn. For leaf free proline contents, the data showed significant difference in the genotypes ($P<0.001$) and treatments (Kn) with non-significant ($P>0.05$) interaction between these factors. The free proline contents improved in both the genotypes. Leaf free proline increased (5.73 ± 0.34) in S2006-SP93 genotype at 1.0 mgL^{-1}, while the production of free proline contents decreased gradually at all levels in S2007-AUS285 and showed maximum (2.70 ± 0.08) accumulation at 0 mgL^{-1} of Kn. Overall, S2006-SP93 exhibited higher quantity of total free proline contents under varying Kn levels than S2007-AUS285 (Fig. 2). A higher accumulation of proline is normally linked to tolerance against various types of abiotic stresses like chilling, drought and heat in sugarcane (Rasheed *et al.,* 2010).

Biotechnological techniques are very important tool for the improvement of stress tolerance. Protein has been proposed as a significant molecular marker (Pareek *et al.,* 1997). The effect of various hormonal levels on protein contents in two sugarcane genotypes was also predictable in current study. BAP and Kn slowly increased in medium, but decreased in protein contents of both tested varieties (S2006-SP93 and S2007-AUS285) (Fig. 2). These protein contents were also reported in sugarcane plants for maintaining the osmotic imbalance and synthesis of induced proteins (Rahnama and Ebrahimzadeh, 2004).

CONCLUSION

Minor doses of 2,4-D produced maximum callus in S2006-SP93 and higher fresh weight of callus in S2007-AUS285. Both genotypes S2006-SP93 and S2007-AUS285 displayed similar findings at 1.0 mgL^{-1} and 1.5 mgL^{-1} Kn. The highest fresh mass and shoot length was observed in S2007-AUS285 at 1.5 mgL^{-1} of BAP. Various levels of BAP (1.0 and 1.5 mgL^{-1}) and Kn (1.0 and 0.5 mgL^{-1}) have markedly increased the photosynthetic pigments. Moreover, various biochemical attributes, like free proline, total soluble proteins and total free amino acids qualities, improved at 1.0 mgL^{-1} of BAP and Kn in S2006-SP93, while values of these attributes increased at 0 mgL^{-1}, 0.5 mgL^{-1} and 1.0 mgL^{-1} of BAP and Kn in S2007-AUS285 respectively. An ELISA test revealed that sugarcane accession S2006-SP93 is highly susceptible to SCMV and the micro propagation of this genotype reduced the SCMV incidence.

REFERENCES

Abe, T. and Y. Futsuhara, 1986. Genotypic variability for callus formation and plant regeneration in rice. Theor. Appl. Genet., 72: 3-10.

Anonymous, 2016-17. Economic Survey of Pakistan (2016-17). Government of Pakistan. Finance division. Economic Advisory Wing, Islamabad, pp. 20.

Bates, L.S., R.P. Waldren and I.D. Teare, 1973. Rapid determination of free proline for water stress studies. Plant Soil, 39: 205-207.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Brisibe, E.A., H. Miyake, T. Taniguchi and E. Maeda, 1994. Regulation of somatic embryogenesis in long term callus cultures of sugarcane (*Saccharum officinarum* L.). New Phytol., 126: 301-307.

Cruz-Cruz, C.A., M.T. González-Arnao and F. Engelmann, 2013. Biotechnology and conservation of plant biodiversity. Resources, 2(2): 73-95.

Capelle, S.C., D.W.S. Mok, S.C. Kirchner and M.C. Mok, 1983. Effects of TDZ on cytokinin autonomy and the metabolism of N6-(Δ2-isopentenyl)[8-14C] adenosine in callus tissues of *Phaseolus lunatus* L. Plant Physiol., 73: 796–802.

Chengalrayan, K. and M. Gallo-Meagher, 2001. Effect of various growth regulators on shoot regeneration of sugarcane. In Vitro Cell Dev. Biol. Plant, 7: 434-439.

Costa, M.L., P.M. Civello, A.R. Chaves and G.A. Martinez, 2005. Effect of ethephon and 6-benzylaminopurine on chlorophyll degrading enzymes and a peroxidase linked chlorophyll bleaching during post-harvest senescence of broccoli (*Brassica oleracea* L.) at 20°C. Postharvest Biol. Tech., 35: 191-199.

Davies, B.H. 1976. Carotenoids. In: T.W. Goodwin (ed), Chemistry and Biochemistry of Plant Pigments, Academic Press London, UK, pp 138-165.

Emons, A.M.C., 1994. Somatic embryogenesis cell biological aspects. Acta Bot. Neerl., 43: 1-14.

Eux, A.R., K.V. Chaitanya and M. Vivekanandan, 2004. Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. J. Plant Physiol., 161: 1189–1202.

FAOSTAT, 2012. Crop production. Food and Agriculture Organization of the United Nations.

Gallo-Meagher, M., R.G. English and A. Abouzid, 2000. Thidiazuron stimulates shoot regeneration of sugarcane embryogenic callus. In Vitro Cell. Dev. Biol. Plant, 36: 37-40.

Gill, R., P.K. Malhotra and S.S. Gosal, 2006. Direct plant regeneration from cultured young leaf segments of sugarcane. Plant Cell Tissue Organ Cult., 84: 227-231.

Hamilton, P.B. and S.D.D. Van, 1973. Amino acid determination with ninhydrin. J. Biol. Chem., 150: 231-233.

Hussain, A., Z.I. Khan, M.Y. Ghafoor and M. Ashraf, 2004. Review sugarcane, sugar metabolism and some abiotic stresses. Int. J. Agric. Biol., 6(4): 732–742.

Hutchinson, M.J. and P.K. Saxena, 1996. Role of purine metabolism in TDZ-induced somatic embryogenesis of geranium (Pelargonium × horturum) hypocotyl cultures. Physiol. Plant, 98: 517–522.

Kemeny, D.M. and S.J. Challacombe, 1989. ELISA and other solid phase immunoassays. John Wiley and Sons, USA. pp. 1–16.

Liu, X. and B. Huang, 2002. Cytokinin effects on creeping bentgrass response to heat stress leaf senescence and antioxidant metabolism. Crop Sci., 42: 466–472.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant, 15(3): 473-497.

Murthy, B.N.S., S.J. Murch and P.K. Saxena, 1995. Thidiazuron-induced somatic embryogenesis in intact seedlings of peanut (*Arachis hypogaea* L.) endogenous growth regulator levels and significance of cotyledons. Physiol. Plant, 94: 268–276.

Lakshmanan, P., R.J. Geijskes, L. Wang, A. Elliott, C.P. Grof, N. Berding and G.R. Smith, 2006. Developmental and hormonal regulation of direct shoot organogenesis and somatic embryogenesis in sugarcane (*Saccharum* spp. interspecific hybrids) leaf culture. Plant Cell Rep., 25(10): 1007-1015.

Oropeza, M. and E. Garcia, 1996. Somaclonal variants resistant to sugarcane mosaic virus and their agronomic characterization. In Vitro Cell Dev. Biol. Plant, 32: 26-30.

Pareek, A., S.L. Singla and A. Grover, 1997. Salt responsive proteins: genes in crop plants. In: P.K. Jaiswal, R.P. Singh, A. Gulati (Eds.), 1997. Strategies for improving salt tolerance in higher plants. Oxford and IBH Publishing, New Delhi, pp. 365–391.

Rahnama, H. and E. Ebrahimzadeh, 2004. The effect of NaCl on proline accumulation in potato seedlings and calli. Acta Physiol. Plant, 26: 263-270.

Rasheed, R., A. Wahid, M.Y. Ashraf and S.M.A. Basra, 2010. Role of proline and glycinebetaine in improving chilling stress tolerance in sugarcane buds at sprouting. Int. J. Agric. Biol., 12: 1560-8530.

Steel, R.G.D., J.H. Torrie and D.A. Dicky, 1997. Principles and procedures of statistics: a biological approach, 3rd edition, McGraw Hill Book Co. Inc., New York, USA, pp: 352–358.

Yoshida, S.D.A., J.H. Forno and K.A. Cock Gomez, 1976. Laboratory manual for physiological studies of rice. IRRI, Los Banos Philippines.