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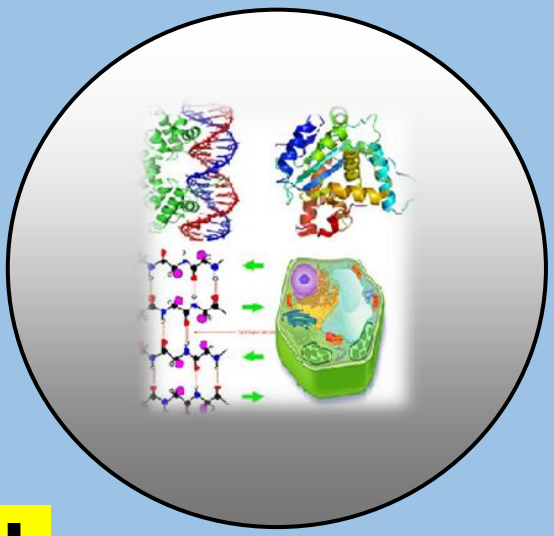
ISSN 0970-4973 Print
ISSN 2319-3077 Online/Electronic

Global Impact factor of Journal: 0.756
Scientific Journals Impact Factor: 3.285
Index Copernicus International Value
IC Value of Journal 6.01 Poland, Europe

J. Biol. Chem. Research
Volume 32 (1) 2015 Pages No. 231-241



Journal of Biological and Chemical Research

An International Journal of Life Sciences and Chemistry

Indexed Abstracted and Cited in about 25 different Scientific Databases around the World

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 32, No. 1: 231-241, 2015
(An International Journal of Life Sciences and Chemistry)

ISSN 0970-4973 (Print)
ISSN 2319-3077 (Online/Electronic)

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RESEARCH PAPER
Received: 15/01/2015 Revised: 09/02/2015 Accepted: 28/02/2015

Effect of Sodium Chloride on Plant Multiplication from Immature Meristematic Leaf-Tips of Sugarcane (*Saccharum officinarum* L.) under the Influence of Abscisic Acid

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ABSTRACT

In present work, aseptic plant initiation (organogenesis) and plant multiplication (micro-propagation) were assessed in salt tolerant (HSF-240) and salt sensitive (CPF-237) sugarcane (Saccharum officinarum L.) cultivars under sodium chloride (NaCl) and abscisic acid (ABA) stresses. Almost 3-5 mm meristematic leaf-tips were cultured onMS_1 (1.2 mg L^{-1} BAP, 0.8 mg L^{-1} NAA, 0.5 mg L^{-1} kinetin) organogenesis medium for 1-week.Low number of shoot initiation was observed on MS_{1a} (MS_1 + 50 mol m^{-3} NaCl) than MS_1 cultures, while on MS_{1b} (2.5 mg L^{-1} ABA) and MS_{1c} (50 mol m^{-3} NaCl + 2.5 mg L^{-1}ABA)shoot initiation was observed. For plant micro-propagation, 1-weeks old explants(after organogenesis on MS_1) were sub-cultured on ABA and NaCl stressed plant micro-propagation MS_2(0.8 mg L^{-1} BAP, 0.6 mg L^{-1} NAA) medium for 3-weeks. Increase in size of plants but almost no multiplication was observed under ABA stressed MS_{2b} (MS_2 + 2.5 mg L^{-1} ABA) cultures, while high number of plantlets [5.50±0.646 (HSF-240), 6.50±0.289 (CPF-237)] per explant were observed in MS_{2c} (MS_2 + 50 mol m^{-3} NaCl + 2.5 mg L^{-1} ABA) than MS_{2a} (MS_2 + 50 mol m^{-3} NaCl) but less than MS_2[10.5±1.555 (HSF-240), 11.75±0.629 (CPF-237)] control cultures ($p<0.001$). Stress reducing markers like as proline, reducing sugars, glycinebetaine and carotenoids were increased as MS_{2c}>MS_{2a} cultures ($p<0.001$). ABA was seems to perm role in elevation of NaCl stress in plant multiplying cultures. Trend of incline or decline was same in both cultivars but order was CPF-237>HSF-240. It means ABA (MS_{2c}) is plant saver under NaCl stressed conditions especially for salt sensitive cultivars, presence of ABA (MS_{2b}) in absence of NaCl causes inhibition of plant multiplication in both salt sensitive and tolerant cultivars of sugarcane.

Key words: Sugarcane, In-vitro, Salinity, Plant multiplication, Abscisic acid, Sodium chloride and Carotenoids.

INTRODUCTION

Today, salinization is an increasing threat for human nutrition and environmental resources. According to FAO, it has been affecting more than 1 billion hectares area of agriculture land all over the world (Vincent et al., 2006; FAO, 2011). Approximately, 5% of cultivated crops are affected due to salt (Munns et al., 1999). Among nutritional crops, sugarcane (*Saccharum officinarum* L.) is a very useful and major sugar producing crop with 60% world's sugar contribution, while salinity not only arrest plant growth also decreases sugar contents (Anderson et al., 1994). High salinity in soil account for decrease in yield of all crops (Tester and Davenport, 2003). It has been decreasing significant vegetative growth because of increase in osmotic potential of medium that causes inhibition for uptake of water and other biochemical (Dubey, 1994; Mohana et al., 2011). Salt stressed medium of sugarcane have decreased its productivity (Shrivastava et al.,1993).Like as in other crop, various studies has been showing that salinity reduces both plant initiation from bud or even germination from seed and its further growth (Lutts et al.,1995; Chowdhury et al., 2001).Data concerned to this study about *in-vitro* plant multiplication under aseptic salt stress is not much available. Few of such studies are available but performed in pot experiment, which does not reflect the inside actual phenomena (Kumar and Naidu, 1993; Chowdhury et al., 2001). Salt as well as dehydration stresses have shown a higher degree of similar effects on plant physiological, biochemical and genetical characters (Cushman et al., 1990).High salinityhas inverse relationship with stomatal conductance and net photosynthetic rate (Curtis and Lauchli, 1986; Lopez et al., 2002). Each of them lead to decrease in photo-assimilation and production of dry matter (Rozeff, 1995; Lingle and Weigand, 1997). Among salt tolerant plants, it has been observed that abscicic acid (ABA) plays very important roles to maintain various biological processes including seed development, dormancy, germination, vegetative growth under salt stressed conditions (Schwartz et al., 2003),while ABA avoid plant growth under non-stressful conditions. It mitigates the stress-damage tissues by the activation of stressed responsive genes that causes biosynthesis of various compatible stress tolerant osmolytes (Hasegawa et al., 2000; Mills et al., 2001; Bray, 2002; Finkelstein et al., 2002). Like as ABA has regulatory effects on proline metabolism pathways (Dallmier and Stewart, 1992; Savoure et al., 1997), other amino acid and carbohydrates that assure beneficial effects in osmotic adjustment in stressed tissues (Handa et al., 1986; Wang et al.,1999). Novel biological techniques are required to select the salt tolerant cultivars as well as to improve their yields in saline and dry area of agriculture soils (Wherheim and Martius, 2008; Egamberdieva and Lugtenberg, 2014). *In-vitro* propagation is an invaluable tool to study basic plant growth aspects and also to manipulate biological processes without interference of environmental factors since it is possible to conduct on plant bulks in a small space. No need to wait for specific season to plane an experiment. This system offer a remarkable dissection bio-molecular regulation involved in plant growth under stress phenomena. In recent years, a number of studies have been conducted for the selection of stress tolerant genotypes (Sancho-Carrascosa et al., 2000; Bhivare and Nimbalkar, 1984). By keeping in view of the above cited reports, aim of present study is to investigate the effect of NaCl and abscisic acid (ABA) on the organogenesis in explant, development of plantlets than their multiplication and biochemical analysis in two salt variant responsive sugarcane cultivars under aseptic conditions.

MATERIAL AND METHODS

For current proposed experiment two sugarcane (*Saccharum officinarum* L.), cultivars [i.e. relative salt tolerant (HSF-240)and salt sensitive (CPF-237)]were collected from the open air field like as by Sreenivasan and Jalaja, (1992) and Jiménez et al., (1995).Almost 70 innermost 3-5mm meristematic bases of leaf sheath whorls from top of plant were excised and washed with dH_2O. They were used as explant and sterilized from microbes by immersing in 70% ethanol for 1minute and stirred with 10% sodium hypochlorite solution for 20minutes.

After sterilization, explants were cultured on shoot initiation (organogenesis) MS_1[MS - Murashige and Skoog , (1962) basal salts with B5 vitamins (Gamborg et al., 1968) + 1.2 mg L^{-1} BAP + 0.8 mg L^{-1} NAA + 0.5 mg L^{-1} kinetin] for 1-week than sub-cultured on shoot multiplication (micro-propagation)MS_2 [MS_1 except kinetin] medium for 2-weeks. Both organogenesis and micro-propagation cultures were maintained under NaCl and ABA stresses. organogenesis cultures were represented as MS_1 (control), MS_{1a} (MS_1, 50 mol m^{-3} NaCl, MS_{1b} (MS_1, 2.5 mg L^{-1} ABA), MS_{1c} (MS_1, 50 mol m^{-3} NaCl, 2.5 mg L^{-1} ABA), while micro-propagation cultures were symbolized as MS_2 (control), MS_{2a} (MS_2, 50 mol m^{-3} NaCl), MS_{2b} (MS_2, 2.5 mg L^{-1} ABA) and MS_{2c} (MS_2, 50 mol m^{-3} NaCl, 2.5 mg L^{-1} ABA)as shown in Table1.

After 1-week of explants cultured for organogenesis on MS_1 medium were sub-cultured on NaCl and ABA stressed plant micro-propagation (MS_2 series) medium and incubated in growth room. After 21 days of culture, ex-plants were removed from jars, washed with water, dried on filter paper and subjected to take fresh weight, chlorophyll pigments (Arnon, 1949), carotenoids (Snell and Snell, 1937)and incubated at 70°C for dry mass.

Among the biochemical contents, total protein was determined through Lowery et al., (1951), total sugars as Montgomery, (1960) and reducing sugars with Miller's method (1959). Similarly other organics like as proline **(**Bates et al., 1973), glycine-betaine contents (Grieve and Gratter, 1983) and phenolics (Ozyigit et al., 2007) were analyzed, while inorganic contents like as nitrate contents were determined by manual spectrophotometric method as described by Morris and Riley, (1963).

The pH of each nutrient medium was adjusted between 5.7-5.8before its sterilization. Cultures were maintained in $\frac{16}{8}$ hrs day and light conditions (light intensity 15 µmol m^{-2} s^{-1}) at 25ºC±1.Data significance of each treatment was computed by using COSTAT Computer Package (*Co Hort Software, Berkeley, USA*).

RESULTS AND DISCUSSIONS

Aseptic plant micro-propagation has been used for development of surface as well as intra-cellular spaces growing pathogen free plants. Its optimization for a specific crop could be useful for the determination of plant growth decreasing effects of abiotic stresses by their external environmental factors. Among the stresses, salinity has always been growth decreasing abiotic stress in all biological systems. This experiment was conducted to know about the effects of relative growth retarder sodium chloride (NaCl) on the aseptic organogenesis in explants than micro-propagation of plantlets as well as how and at which stage of plant growth abscisic acid (ABA) is beneficial for plants growing under NaCl stressed conditions. A number of morpho-physiologicalattributes of *in-vitro* multiplying two sugarcane (*Saccharum officinarum* L.) cultivars under NaCl and ABA stresses has shown variation in micro-propagation rate as well as metabolic assimilations.

Among the organogenesis cultures less shoot initiation seems in NaCl stressed (MS_{1a}) cultures, while complete inhibitions was observed in ABA (MS_{1b}) and NaCl with ABA (MS_{1c}) cultures in comparison to control MS_1medium. Presence of ABA in these cultures causes inhibition of shoots or organogenesis. After organogenesis in MS_1 medium, explants were sub-cultured on NaCl and ABA supplied plant multiplication (MS_2) medium. Increase in plant numbers per explant by the addition of ABA in NaCl stressed (MS_{2c}) culture was observed, while in the cultures with ABA (MS_{2b}) less number of plantlets was measured even in comparison to NaCl stressed cultures (MS_{2a}) also. Same instable criteria in plant biomass were also observed in both cultivars (Fig 1, Table 1).A significant relationship between chlorophyll b and carotenoids under NaCl stress was observed. Elevation of NaCl stress in the presence of ABA is known when relative increase in proline contents, reducing sugars and glycine-betaine was observed in among the cultures of both HSF-420 and CPF-237. These changes in biochemical contents were observed comparatively very similar to an increase in number of plantlets as well as plant biomass also. These superficial facts could not be directly involved to the phenomena but their induction under stressed conditions in the presence of ABA is mainly because of specific gene activation for enzymes that are responsible salt tolerance. Similarly, salinity stress is also involved in the induction of ABA biosynthesis (Xiong et al., 2002; Chinnusamy et al., 2004, 2006). Accumulation of ABA either by addition in culture or by plant biosynthesis itself could be retained or degraded by plant cells on the basis of presence or absence of environmental stresses (Yamaguchi-Shinozaki et al., 1993; Shinozaki et al., 1997).

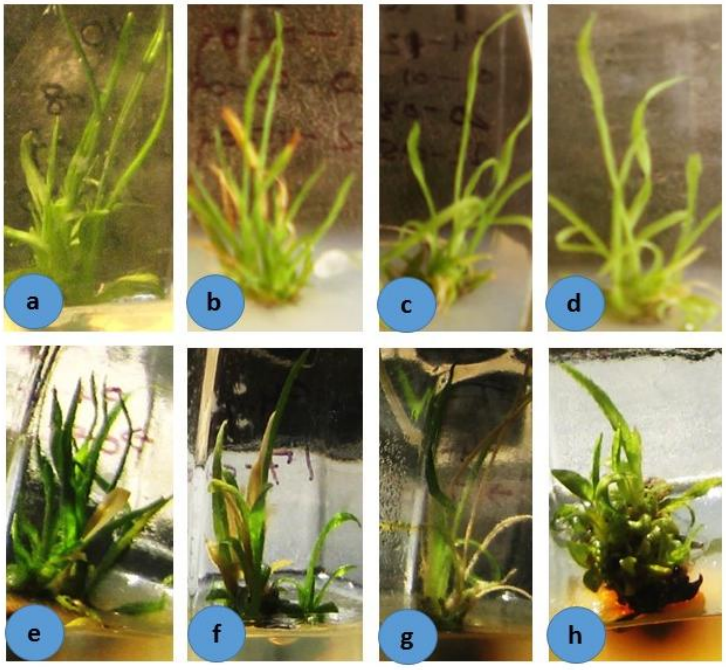

Figure 1. Effect of NaCl on plant multiplication from immature meristematic leaf-tips and their biochemical attributes intwo sugarcane (*Saccharum officinarum* L.) cultivars HSF-240 (a-d) and CPF-237 (e-h).a & e: Plantlets of HSF-240 and CPF-237 are multiplying on control MS_2 (0.8 mg L^{-1} BAP, 0.6 mg L^{-1} NAA) medium; **b & f**: Plantlets on MS_{2a} (MS_2 + 50 mol m^{-3} NaCl) medium; **c & g**: ABA stressed MS_{2b} (MS_2 + 2.5 mg L^{-1} ABA) cultures; **d & h**: Plantlets of both cultivars growing on MS_{2c} (MS_2 + 50 mol m^{-3} NaCl + 2.5 mg L^{-1} ABA) cultures.

Table 1. Effect of NaCl onplant multiplication from immature meristematic leaf-tips and their biochemical attributes in sugarcane (*Saccharumofficinarum* L.) cultivars

#s	Characters	MS_2	MS_{2a}	MS_{2b}	MS_{2c}	Treatment Significance
		HSF-240 *(3-weeks culture)*				
a.	# of plantlets explant^{-1}	10.5±1.555	4.50±0.289	2.00±0.408	5.50±0.646	***
b.	Plant height (cm)	4.50±0.108	2.20±0.071	5.025±0.239	4.05±0.155	***
c.	Dry weight (g)	4.14±0.117	1.81±0.025	0.85±0.011	1.598±0.027	***
d.	Fresh weight (g)	0.38±0.002	0.17±0.001	0.07±0.001	0.153±0.002	***
e.	Chlorophyll b (mg g^{-1})	0.27±0.002	0.24±0.001	0.23±0.001	0.27±0.002	***
f.	Carotenoids (mg g^{-1})	3.28±0.005	5.65±0.013	3.93±0.001	4.26±0.019	***
g.	Total protein (mg g^{-1})	2.33±0.004	1.62±0.004	1.45±0.004	1.66±0.003	***
h.	Total sugars (mg g^{-1})	2.49±0.007	1.28±0.008	1.68±0.010	1.54±0.010	***
i.	Reducing sugars (mg g^{-1})	0.99±0.003	1.02±0.005	0.89±0.005	1.02±0.006	***
j.	Proline contents (mg g^{-1})	2.36±0.004	2.79±0.003	2.10±0.003	2.67±0.003	***
k.	Glycinebetaine (mg g^{-1})	0.65±0.002	1.26±0.003	0.67±0.002	1.12±0.002	***
l.	Phenolics(mg g^{-1})	1.10±0.002	1.52±0.006	1.68±0.002	1.42±0.002	***
m.	Nitrate (mg g^{-1})	3.30±0.004	3.01±0.003	2.83±0.003	2.52±0.050	***
		CPF-237 *(3-weeks culture)*				
a.	# of plantlets explant^{-1}	11.75±0.629	2.75±0.479	2.00±0.408	6.50±0.289	***
b.	Plant height (cm)	4.63±0.086	1.65±0.065	4.70±0.108	3.68±0.075	***
c.	Dry weight (g)	5.22±0.054	0.95±0.011	0.82±0.007	1.86±0.027	***
d.	Fresh weight (g)	0.47±0.002	0.08±0.001	0.06±0.001	0.17±0.001	***
e.	Chlorophyll b (mg g^{-1})	0.028±0.001	0.25±0.001	0.24±0.001	0.27±0.001	***
f.	Carotenoids (mg g^{-1})	3.29±0.006	5.77±0.012	3.45±0.012	4.25±0.013	***
g.	Total protein (mg g^{-1})	2.34±0.004	1.35±0.003	1.42±0.003	1.56±0.002	***
h.	Total sugars (mg g^{-1})	2.43±0.006	1.49±0.026	1.26±0.004	1.59±0.012	***
i.	Reducing sugars (mg g^{-1})	0.97±0.003	1.01±0.003	0.87±0.002	0.980±0.003	***
j.	Proline contents (mg g^{-1})	2.23±0.002	2.74±0.047	1.99±0.003	2.56±0.002	***
k.	Glycinebetaine (mg g^{-1})	0.63±0.002	1.20±0.002	0.65±0.002	1.08±0.004	***
l.	Phenolics(mg g^{-1})	1.07±0.003	1.45±0.002	1.55±0.002	1.34±0.002	***
m.	Nitrate (mg g^{-1})	3.27±0.010	2.87±0.023	2.23±0.006	2.35±0.059	***

Accumulation of proline in the stressed cultures that could also be mediated by both ABA-dependent and ABA-independent signaling pathways (Knights et al., (1997; Sanan-Mishra et al., 2005; Mahajan et al., 2006). Meanwhile, plant morphology is a mutual result of internal cell physiology in relation to applied environment. Overall increase or decrease in plant height or biomass under NaCl and or ABA stress is a reflection of un-easiness of internal metabolism. Such reduction could be held in initial due to loss of relative water contents that indirectly reduce the cell elongation and cell division (Herandaz et al., 1995; Dinar et al., 1999;Chartzoulakis and Klapahi,2000).Plant growth is the result of integrated and regulated physiological processes. Limitation in plant growth for environmental stress is not assigned for a specific single physiological process, like as synthesis of amino acids depends a number of anabolic and catabolic processes. Among the photosynthetic pigments, Chl a, Chl b, total chlorophyll and carotenoids, are also playing important role for photochemical reaction (Taiz and Ziegar, 2006), except Chl b and carotenoids others decreases.

In adaptation of plantlets in saline stress cultures, primary step is metabolic adjustments to initiate and accumulate many organic solutes like sugars and certain free amino acids. These phenomena vary among the plant tissues when initially sub-cultured on saline stressed condition (Greenway and Munns, 1980; Ashraf and Foolad, 2007). Even NaCl stress reduces total sugars and proteins (Riazi et al., 1985), while reducing sugars increases (Table 1). These metabolites act as osmo-regulators in abiotic stressed plants (Banzel and Reuveni, 1999). Proline and glycine-betaineare considered as major osmo-protectant (Rontein et al., 2002).Role of glycine- betaine is conforming in proteins to stabilize them for osmotic adjustments under salinity stresses (Khan et al., 1998; Yeo et al., 1998). Surprisingly, these osmo-regulating solutes accumulate in high concentration in the cell without cell metabolism disturbance (Bohnert and Jensen, 1996). Phenolics are also synthesized under both biotic and abiotic stressed plant tissues. Its synthesis is dependent on *phenol oxidase* (POD) and *polyphenol oxidase* (PPO) activity (Cox, 1996; Laukkanen et al., 1999; Thomos and Ravindra, 1999). High phenolics syntheses were observed in NaCl stressed cultures with ABA. It means that phenols are also one among the other osmotic adjusters performing their role in abiotic stressed plant tissues. Meanwhile, Lorenzo et al., (2001) demonstrated that phenolic compounds excretion is linked with shoot multiplication, which mostly this phenomena appears during the establishment of plant tissues in aseptic environment because of explant injury or external stresses (Haq et al., 2011). With passage of time,phenolics decreases as reported by Cvikrová et al., (1996) in alfalfa and Legrand and Bouazza (1991) in *Cichoriumintybus*, while Preece and Compton, (1991) point out some positive correlations of phenolics with totipotency, when cathecol and phloroglucinol supplied in cultures for the initiation of shoot induction in blackberry. High concentration of shikimic acid (polyphenolic) induces high shoot formation and low level of this acid cause less number of shoots in *Pinussylvestris* even poor metabolic activity as well as senescence (Herman, 1991). In short, phenolics are being beneficial for the plant tissues either secreted by cell injury, cell stress or initiation of shoots. This work also suggests that inhibition of shoot initiation is caused by ABA in the sugarcane cultivars.

CONCLUSIONS

Successful and efficient *in-vitro* plant multiplication is a key source to analyze the growth reducing environmental stresses at plant tissue level. Salinity is being a major loser of agriculture including all biological systems. In present work, NaCl also retarded plant multiplication rate in cultured explants. It causes to reduce metabolic processes because of the increase in osmotic potential in medium that taking-off water for the cell and increases the level of osmo-regulators in the cell like as free amine acids i.e. proline, glycine betaine), reducing sugars and carotenoids also. Further increase in these stress markers by the supplementation of abscisic acid in saline stressed cultures, while not same in cultures with abscisic acid only. It means that presence of abscisic acid when plant tissue is multiplying under stressed conditions is helpful in the elevation of stresses by the synthesis of osmo-regulators. Almost same behavior of growth of plant tissue under sodium chloride and abscisic acid stresses is seen in both sugarcane cultivars but relatively abscisic acid performs best in CPF-237 salt sensitive cultivars. Adjustments of these incline or decline in certain plant growth attributes as well as biochemical can be helpful in the development of salt tolerance line of sugarcane.

In-vitro plant culture have ability to modify itself or may adopta specific trait against continues applied stress. In future, such study may be hopeful for developing salt tolerance by inducing stress adjusting mechanism in the plant of sugarcane or other crops because salinity resistance is need of time scenario.

ACKNOWLEDGEMENTS

Author is grateful to acknowledge Pakistan Agriculture Research Council (PARC) for financial support and thankful for all the technicians or staff members for their cooperation throughout the experimentation period.

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