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REGULAR ARTICLE

EFFECT OF SALINITY ON SOME PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS IN EXPLANTS OF TWO CULTIVARS OF SOYBEAN (*GLYICINE MAX* L.)

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SUMMARY

Effect of NaCl on fresh mass, contents of sugars, free proline, protein, activity of polyphenol oxidase (PPO) and peroxidase (POD) in explants of two cultivars (Hack and Zan) of soybean were investigated. Explants were grown for one month in medium with different NaCl concentrations (0, 50, 100, 150 and 200 mM). Fresh mass of explants in both cultivars decreased continuously with an increase in salinity. Proline, reducing sugars, soluble sugars and total sugars contents enhanced under salinity in both cultivars. Oligosaccharide content in both cultivars increased up to 50 mM and declined at higher salinities. Polysaccharide of Hack explants increased up to 100 mM and then diminished, but in Zan explants, it reduced at 50 mM and increased at higher salinities. Protein content in Hack improved by salinity but in Zan explants, protein content decreased up to 100 mM NaCl and then increased at higher salinities. PPO activity in two cultivars increased up to 150 mM NaCl and then decreased. POD activity in both explants increased up to 200 mM NaCl. Fatty acid composition was modified by salinity. Saturated fatty acids content in explants of both cultivars declined but unsaturated fatty acids content (linoleic and arachidonic acid) improved under salinity. These results showed that cv. Hack exhibit a better protection mechanism against salinity damage and more salt-tolerant by maintaining and/or increasing fresh mass, osmolyte accumulation and activity of antioxidant enzymes than cv. Zan.

Keywords: Salinity stress, Tissue culture, Osmolytes, Fatty acids, PPO and POD Activities.

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1. Introduction

Salinity is the major environmental factor limiting plant growth and productivity. The detrimental effects of high salinity on plants can be observed at the whole-plant level as the death of plants and/or decreases in productivity (Parida, and Das, 2005). The use of iv vitro cultures to study stress responses is based on the fact that in vitro cultured cells behave similarly to cells of intact plants subjected to water deficit and salinity stress condition (Al-Khayri and Al-Bahrany, 2004). Moreover, species differing in drought tolerance at the whole plant level also usually

exhibit differences in drought tolerance in cell cultures (Santos-Diaz and Ochoa-Alejo, 1994). Undifferentiated cells and callus cultures eliminate complications related to genetic and morphological variability inherent to different tissues of whole plant. Moreover, cell culture systems eliminate the responses related to water stress except those operative at the cellular level (Al-Khayri and Al-Bahrany, 2004).

Exposure of cells to salt stress causes a set of metabolic and developmental changes. The identifications of specific characteristics related to salt resistance such as compatible osmolytes (e.g. proline and sugars) will provide potential biological markers useful in the identification and genetic manipulation of salt resistant plant and plant cells (Niknam et al. 2004).

Several reports have suggested that lipids might be involved in the protection against salt stress (Huflejt et al. 1990; Khamutov et al. 1990; Ritter and Yopp, 1993). One common effect of salinity reported for salt sensitive and tolerant species is the increasing degree of saturation of membrane fatty acids and decreasing membrane fluidity (Mansour et al. 2002; Kerkeb et al. 2001), which was proposed to regulate Na^+ and Cl^- permeability under salt stress.

In order to look into osmotic stress induced biochemical changes and to elucidate adaptive mechanisms at cellular level, the status of growth, sugars, free proline, protein, fatty acids composition and activity of some enzymes in explants of two cultivars of soybean grown under salinity were investigated.

2. Materials and methods

Seeds of soybean (*Glycine max* L. cv. Hack and cv. Zan) were provided from Agricultural Research and Education Organization Seed and Plant Improvement Institute. Seeds of soybean were surface sterilized in 20% (v/v) sodium hypochlorate solution containing a few drops of tween 20 for 20 min, followed by 3 times washes with sterile distilled water. Calli were produced from hypocotyl explants on MS (Murashige and Skooge, 1962) medium containing 0, 50, 100, 150 and 200 mM NaCl and supplemented with 2 mg dm-3 2, 4-D (2, 4 Dichlorophenoxyacetic acid) and 0.5 mg dm-3 BA (Benzyladenine) under 16-h photoperiod (with fluorescent lamps: irradiance of 46 µmol m-1s-1) and day/night temperature of 25/20 ˚C. Explants were maintained in a growth chamber for 30 d under mentioned condition. Fresh mass of explants was recorded in 30-d-old explants. Three replicates containing 4 explants each were taken for measurements.

Free proline was determined according to Bates et al. (1973) using L-proline as a standard. High-speed centrifuge (Beckman J2-21M, USA) and UV-visible spectrophotometer (Shimadzu UV- 160 Japan) with 10 mm matched quartz cells were used for centrifugation of the extracts and determination of the absorbance, respectively.

For determination of sugar content, 0.5 g of powder was extracted using 10 cm^3 of ethanol-distilled water (8:2 v/v), and supernatants were collected after twice centrifugation at 1480 g. The residue from ethanol extraction was subsequently used for polysaccharide extraction by boiling water (Seyyednejad et al. 2001). Total Sugar contents were estimated by the method of Dubois et al. (1956). Reducing sugars were quantified according to Nelson (1944). Oligosaccharide content was obtained from difference between soluble and reducing sugars contents.

For determination of protein content, fresh explants (0.5 g) were homogenized in a chilled (4°C) mortar using a 500 mM Tris-HCl buffer,

pH 7.0. After centrifugation at 13000 g for 45 min at 4°C, the supernatant was filtered and then transferred to Eppendorf tubes and the samples kept on ice at 4°C. Enzyme extraction was stored at -70°C for enzyme activity assay. Total protein contents were measured by the spectrophotometric method of Lowry at al. (1951) using bovine serum albumin (BSA) as the standard.

Peroxidase (POD) activity was measured by the H_2O_2-dependent oxidation of benzidine at 530 nm, in a reaction mixture containing 2 ml of 0.2 M acetate buffer (pH 4.8), 0.2 ml of 3% H_2O_2, 0.2 ml of 0.04 M benzidine and 0.1 ml of extracted protein (Abeles and Biles, 1991).

Polyphenol oxidase (PPO) activity was measured by the increase in absorbance at 420 nm. The reaction mixture containing of 2.5 ml of 0.2 M phosphate buffer (pH 6.8), 0.2 ml of 0.02 M pyrogallol and 0.2 ml of protein extract at 25°C (Raymond et al. 1993).

Extraction of total lipid was performed following the methods of Folch et al. (1957) as modified by Bligh and Dyer (1959). The fatty acid methyl esters were prepared following the Carreau and Dubacq (1987) and analyzed by gas liquid chromatography. A Shimadzu GC-16A equipped with a flame ionization detector and 1.6 m × 3.2 mm i.d. glass column packet with OV-1 was used. Operation temperatures were 220°C for the oven and 230°C for both the injector and detector block. Nitrogen was used as carrier gas at a flow rate of 50 ml min-1. Flow rates of hydrogen and air were 55 and 400 ml min-1, respectively. The peaks were identified by comparison of the retention times with authentic standards.

The data determined in triplicate were analyzed by analysis of variance (ANOVA) using SPSS (version 9.05). The significance of differences was determined according to Duncan's multiple range test (DMRT). P values < 0.05 are considered to be significant.

3. Results

In both cultivars of Hack and Zan increasing of salinity caused a linear reduction in growth as expressed in explants fresh mass (Tab. 1). Moreover, biomass in explants of Hack was higher than that of Zan at all treatments.

In present study, steady increasing in free proline content in cv. Zan was observed in response to the increase in NaCl concentration. In contrast, in cv. Hack increase of salinity up to 50 mM increased free proline content significantly, and then further increase of salinity up to 200 mM NaCl decreased the proline content. In general, in explants of cv. Hack free proline content of explants under salinity was higher than that of control (Tab. 1). Moreover, proline content in explants of Hack was higher than that of Zan at all treatments.

Effect of NaCl on various sugar contents was presented in Tab.2. NaCl increased the contents of reducing sugars, soluble sugars and total sugar contents in both explants comparing to that of control. Oligosaccharide content in explants of both cultivars increased up to 50 mM and then diminished at higher salinities. Polysaccharide content of Hack explants increased up to 100 mM and then diminished at 150 and 200 mM NaCl. In contrast, polysaccharide content in Zan explants at 50 and 150 mM was lower than that of control but at 100 and 200 mM NaCl was higher than that of the control.

Protein content in Hack explants increased by salinity. In contrast, Protein content in Zan explants decreased up to 100 mM and then increased at higher salinities (Tab. 1). Moreover, protein content in explants of Hack was higher than

that of Zan at all treatments.

Salinity increased significantly total POD activity in explants of both cultivars comparing to that of control (Tab. 1). The maximum POD activity, in both explants was obtained at 150 and 200 mM NaCl, ca. 492 and 302% compared with controls in Hack and Zan, respectively. At all treatments, POD activity in Hack was higher than that of Zan.

PPO activity in Hack explants increased up to 150 mM NaCl and then decreased (Tab. 1). In Zan explants, PPO activity increased up to 50 mM and then declined with an increasing salinity. The maximum PPO activity, in both explants was obtained at 150 and 50 mM NaCl, ca. 390 and 113% compared with controls in Hack and Zan, respectively.

The results of fatty acid analysis based on calculations from the GLC data for eight major fatty acids present in soybean explants and seeds are summarized in Tab. 3. Palmitic acid ($C_{16:0}$) and myristic acid ($C_{14:0}$) were found to be the most abundant saturated fatty acids. Palmitic acid, myristic acid and margaric acid ($C_{17:0}$) contents in explants of both cultivars under saline conditions were lower than that of the control explants. Lauric acid ($C_{12:0}$), in cv. Zan declined with increasing NaCl conditions, whereas in cv. Hack, lauric acid improved by salinity. Linoleic acid ($C_{18:2}$) is the major unsaturated fatty acid in explants of both cultivars and increased with increasing of salinity. The other fatty acids such as arachidic acid (C_{20}), behenic acid (C_{22}) and arachidonic acid ($C_{20:4}$) contents were only slightly modified.

Table 1. Fresh mass [g], contents of free proline [%(d.m.)], protein [%(f.m.)] and activity of polyphenol oxidase (PPO) and peroxidase (POD) [unit g-1(f.m.)] in explants of *Glycine max* L. cvs. Hack and Zan under NaCl stress.

Cultivar	NaCl [mM]	Fresh mass [g]	Free Proline [%(d.m.)]	Protein [%(f.m.)]	PPO [unit g-1 (f.m.)]	POD [unit g-1 (f.m.)]
Zan	0	0.59 ± 0.16a	0.25 ± 0.08c	0.33 ± 0.03b	5.31 ± 0.11b	18.25 ± 0.22c
	50	0.53 ± 0.1ab	0.39 ± 0.09b	0.27 ± 0.02 b	6.02 ± 0.32a	21.88 ± 0.32c
	100	0.47 ±0.13bc	0.52 ± 0.14a	0.17 ± 0.05c	4.40 ± 0.21c	39.60 ± 0.16b
	150	0.43 ±0.07bc	0.33 ± 0.17b	0.20 ± 0.03c	5.54 ± 0.23b	33.24 ± 0.19b
	200	0.35 ± 0.07c	0.54 ± 0.07a	0.53 ± 0.04a	3.41 ± 0.15c	55.20 ± 0.33a
Hack	0	0.69 ± 0.30a	0.35 ± 0.16b	0.40 ± 0.01a	1.11 ± 0.09c	12.32 ± 0.12c
	50	0.59 ± 0.08b	1.07 ± 0.09a	0.41 ± 0.06b	2.05 ± 0.07b	60.27 ± 0.32a
	100	0.52 ± 0.08b	0.66 ± 0.08b	0.43 ± 0.03c	2.72 ± 0.06b	31.51 ± 0.22bc
	150	0.42 ± 0.10c	0.62 ± 0.07b	0.46 ± 0.02c	4.34 ± 0.16a	60.72 ± 0.25a
	200	0.34 ± 0.06c	0.45 ± 0.09ba	0.60 ± 0.03a	3.22 ± 0.11ab	40.52 ± 0.21b

Values are means ± SE of 4 determinations. Data were analyzed by Duncan's multiple range test and means followed by identical letters were not significantly different within the columns ($P < 0.05$)

Table 2. Reducing sugar (RS), oligosaccharide (OS), polysaccharide (PS), soluble sugar and total sugars contents [%(d.m.)] in explants of *Glycine max* L. cvs. Hack and Zan under NaCl stress.

Cultivar	NaCl [mM]	RS [%(d.m.)]	OS [%(d.m.)]	PS [%(d.m.)]	SS [%(d.m.)]	TS [%(d.m.)]
	0	0.43 ± 0.05c	1.37 ± 0.16b	1.02 ± 0.07b	1.80 ± 0.14b	2.82 ± 0.24b
	50	0.58 ± 0.15a	1.45 ± 0.23a	0.78 ± 0.19c	2.03 ± 0.28a	2.81 ± 0.37b
Zan	100	0.48 ± 0.13c	1.43 ± 0.62a	1.28 ± 0.16a	1.91 ± 0.21b	3.19 ± 0.17a
	150	0.56 ± 0.09b	1.27 ± 0.10c	0.97 ± 0.12b	1.82 ± 0.30b	2.80 ± 0.22b
	200	0.57 ± 0.17ab	1.34 ± 0.20b	1.28 ± 0.07a	1.91 ± 0.11b	3.19 ± 0.39a
	0	0.37 ± 0.08c	1.00 ± 0.39b	0.91 ± 0.17c	1.37 ± 0.18c	2.28 ± 0.35c
	50	0.49 ± 0.06c	1.45 ± 0.35a	1.14 ± 0.18bc	1.98 ± 0.22a	3.08 ± 0.28b
Hack	100	0.71 ± 0.10a	0.96 ± 0.3ab	2.73 ± 0.18a	1.67 ± 0.28b	4.40 ± 0.31a
	150	0.81 ± 0.09a	0.67 ± 0.02c	1.30 ± 0.10b	1.48 ± 0.19c	2.78 ± 0.22b
	200	0.57 ± 0.07b	1.19 ± 0.30b	1.47 ± 0.11b	1.76 ± 0.21ab	3.23 ± 0.19b

Values are means ± SE of 4 determinations. Data were analyzed by Duncan's multiple range test and means followed by identical letters were not significantly different within the columns ($P < 0.05$).

Table 3. Fatty acid composition (% total) in seeds and explants of *Glycine max* L. cvs. Hack and Zan under NaCl stress.

Cultivar	NaCl [mM]	C12: 0	C14: 0	C16: 0	C17: 0	C20: 0	C22: 0	C18: 2	C20: 4
	0	10.59	44.27	46.02	4.21	3.18	1.99	12.99	1.29
	50	2.64	36.54	45.38	2.42	2.92	1.48	13.54	1.43
Zan	100	4.48	43.62	20.62	3.04	2.35	1.59	14.63	1.89
	150	7.30	39.19	17.72	2.20	2.57	1.63	14.34	1.80
	200	5.89	39.85	32.31	3.26	1.47	1.70	15.60	1.75
	Seed	17.27	32.33	29.98	1.05	2.18	1.34	7.91	3.50
	0	5.95	32.20	55.25	5.79	1.73	2.31	5.60	2.70
	50	12.42	22.36	54.96	4.19	0.98	1.42	6.05	2.10
Hack	100	6.76	27.98	46.14	1.84	1.31	0.69	6.46	2.91
	150	12.66	30.79	37.53	1.78	2.31	1.26	7.27	1.52
	200	8.64	29.78	39.64	1.99	2.17	1.50	8.05	1.73
	Seed	8.89	26.96	27.79	9.17	1.60	0.78	15.89	3.50

4. Discussion

Salinity decreased fresh mass in both cultivars (Tab. 1). Legume species vary widely in response to salt stress, ranging from extremely sensitive to tolerant species. Low concentration of NaCl (10 mM) inhibits the growth of salt sensitive soybean genotypes (Lauchli and Wieneke, 1979). Grattan and Maas (1988) observed that high concentration of salinity (60, 80 and 120 mM NaCl) decreased the growth of soybean.

Free proline content in both explants promoted with increases in salinity comparing to that of control (Tab. 1). Accumulation of proline is frequently reported in many plants or tissues in response to salinity stress (Khatkar and Kuhad 2000, Jain et al. 2001, Jaleel et al. 2007a, Jaleel et al. 2008). According to our results, this accumulation is more prominent in cv. Hack than that cv. Zan. Many plants accumulate proline as an osmo-regulator (Delaurey and Verma, 1993) or osmo-protector (Csonka, 1989) under saline condition.

Sugar contents [reducing sugar, oligosaccharide (at low salinity), soluble sugar and total sugar] in both cultivars, except polysaccharide, promoted with an increase in salinity, comparing to that of control (Tab. 2). Carbohydrates (such as glucose, fructose, and

fructans) and starch accumulate under salt stress (Parida et al. 2002, Aghaleh, et al. 2009). The major functions of sugars are osmoprotection, osmotic adjustment, carbon storage, and radical scavenging (Parida and Das, 2005). Salt stress increased reducing sugars and sucrose in two wheat cultivars (Khatkar and Kuhad, 2000).

Protein content in Hack increased by salinity but in Zan explants, protein content decreased up to 100 mM NaCl and then increased at higher salinities. These results are in a good agreement with the results of workers who found that a higher content of soluble proteins has been observed in salt tolerant than in salt sensitive cultivars of sunflower (Ashraf and Tufail, 1995) and rice (Lutts and Kinet, 1996). According to our results, this increase is more prominent in cv. Hack than that cv. Zan.

Peroxidase catalyzes H_2O_2-depended oxidation of substrate. It seems that under salt stress conditions, the product of the SOD reaction, H_2O_2, was eliminated by higher constitutive and induced levels of POD activity with increasing salinity (Tab. 1). The enhancement of POD activity by salinity has also been observed in the rice leaves (Lee et al, 2001), callus cultures of Suaeda nudiflora (Cherian and Reddy, 2003), apple rootstock MM 106 (Molassiotis et al. 2006) and *Catharanthus roseus* (Jaleel et al. 2007) (.Gossett et al. (1994 and 1996) reported that the activity of antioxidative enzymes (catalase, ascorbate peroxidase and peroxidase) under salt stress increased in the callus tissue of the salt-tolerant cultivars of cotton and decreased or remained unchanged in non-tolerant cultivars. They believed that the increase in activity of the enzymes in callus of the salt-tolerant cultivar could be associated with its salt tolerance character (Gossett et al. 1994).

In cv. Hack, PPO activity increased with an increasing salinity, whereas in cv. Zan, it activity promoted at 50 mM and reduced at higher salinities (Tab. 1). These results are in a good agreement with the results of Niknam et al. (2006) who reported that salt-stress induced PPO activity in calli and seedlings of *Trigonella aphanoneura* and seedlings of *T. foenum-graecum*.

These results showed that treatment with salinity alters membrane fatty acid composition. In other hand, saturated fatty acids in the soybean explants reduced with increasing of salinity, whereas unsaturated fatty acid composition enhanced by salinity (Tab. 3). Elkahoui et al. (2004) reported that salt treatment decreased palmitic acid level and increased linolenic acid in Catharanthus roseus cell suspensions. However, these results are in discordance with those obtained with halophytes such as *Dunaliella salina* and Spartina patens (Peeler et al., 1989; Wu et al., 1998). An increase in unsaturation and length of fatty acid chain enhance membrane fluidity and thickness, respectively, which might affect membrane permeability to ions (Elkahoui et al. 2004).

In summary, explants growth was reduced under salinity. Osmotic adjustment through accumulation of proline, reducing sugar, oligosaccharide (at low salinity) and soluble sugar was positively related to an increasing of NaCl concentration. This study also demonstrated that salinity changes fatty acid composition and PPO and POD activities in two cultivars differently. In cv. Hack higher fresh mass, free proline, protein, reducing sugar, total sugars contents, and POD activity in control and salt treated explants were observed in comparison to that of cv. Zan. Therefore, cv. Hack exhibit a better protection mechanism against salinity damage and more salt-tolerant than that of cv. Zan.

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