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EFFECT OF SALT STRESS ON PROLINE ACCUMULATION, PHOTOSYNTHETIC ABILITY AND GROWTH CHARACTERS IN TWO MAIZE CULTIVARS

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Abstract

Salt stress strongly affects on plant growth and development, especially maize plant, which is reported as a salt sensitive species. The salt tolerant identification in the large genetic resources and breeding population is a profitable research topic for solving the salinity problem. Two maize cultivars, viz., sweet (*Zea mays* L. cv. Saccharata) and waxy (*Z. mays* L. cv. Ceratina) seedlings were treated with 0 (control), 100, 200, 300 or 400 mM NaCl. Osmotic potential (ψ_s) or water availability in the culture media was limited, relating to increase in the NaCl concentrations of the growth medium. The chlorophyll degradation in the salt stressed seedlings was positively related to ψ_s in the culture media. Chlorophyll a (Chl_a), chlorophyll b (Chl_b) and total chlorophyll (TC) concentrations in the salt stressed leaves significantly dropped, depending on salt treatments except total carotenoids (C_{x+c}) content which was decreased by the factors of salt concentrations, cultivars and their interaction. Proline in the salt stressed leaves accumulated to 600.9 μmol g^{-1}FW, especially in sweet maize treated with 400 mM NaCl. The chlorophyll degradation in both cultivars was progressively correlated with maximum quantum yield of PSII (F_v/F_m) as well as the photon yield of PSII (Φ_{PSII}) was related to net photosynthetic rate (P_n), leading to growth reduction. Chlorophyll a fluorescence parameters, F_v/F_m, Φ_{PSII} and photochemical quenching (qP), in the leaf tissues were reduced, while non-photochemical quenching (NPQ) was exhibited. The biochemical, physiological and morphological changes in salt stressed maize cultivars were subjected to K-Means Cluster in SPSS software and classified the two cultivars as waxy salt tolerant and sweet salt sensitive.

Introduction

Salinity is one of the most important abiotic stresses widely distributed in both irrigated and non-irrigated areas of the world. Soil contaminated salts (ECe > 4 dS m^{-1} or 40 mM NaCl or osmotic potential < 0.117 MPa) are defined as salinity land, which directly affects plant growth and development in vegetative growth prior to reproductive stage, especially crop species (Allakhverdiev *et al*., 2000; Sairam & Tyagi, 2004; Chinnusamy *et al.*, 2005; Ashraf *et al.*, 2008; Ashraf, 2009). Most of crop species *i.e.* bean, eggplant, onion, pepper, corn, sugarcane, potato and cabbage are sensitive to salinity (ECe 1.0-1.8 dS m^{-1}), which reduce crop productivity about 6-19%. In general, biochemical, physiological, morphological and anatomical characteristics of crop species directly affected by soil salinity are well established (Ashraf, 2004; Ashraf & Harris, 2004; Chinnusamy *et al.*, 2005; Parida & Das, 2005). There are many reports which show that salinity induces water deficit in many crop species such as corn, sunflower, potato and soybean (Katerji *et al*., 1996; Katerji *et al*., 1998; Katerji *et al*., 2004). A primary response in salt stressed plants is a decrease in plant water potential, resulting in decreased water use efficiency, leading to the overall toxic damages and yield reduction

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(Glenn & Brown, 1998; El-Hendawy *et al*., 2005; Mansour *et al*., 2005). The role of proline in cell osmotic adjustment, membrane stabilization and detoxification of injurious ions in plants exposed to salt stress is widely reported (Hare *et al*., 1999; Kavi Kishor *et al*., 2005; Ashraf & Foolad, 2007). There are several techniques to enhance the endogenous proline accumulation for salt defense mechanism such as exogenous application (Santos *et al*., 1996; Hoque *et al*., 2007; Kaya *et al*., 2007), biosynthesis gene(s) overexpression (Zhu *et al*., 1998; Han & Hwang, 2003) and degradation gene(s) knock-out (Nanjo *et al*., 1999). The endogenous proline accumulation in salt stressed plants has been utilized as effective indicator for salt tolerance. Moreover, multivariate biochemical and physiological parameters, growth performances and yield have been applied to classify salt tolerant cultivars in maize (Neto *et al*., 2004), wheat (El-Hendawy *et al*., 2005), rice (Zeng, 2005), cowpea (Murillo-Amador *et al*., 2006), tomato (Juan *et al*., 2005), seashore paspalum (Lee *et al*., 2008), and chickpea (Maliro *et al*., 2008).

Maize (*Zea mays* L.) belonging to Poaceae family of C_4 type is reported as salt susceptible (Katerji *et al*., 1996; Chinnusamy *et al*., 2005). In the recent study, salt tolerance trait is a major target of maize breeding program, especially in the CIMMYT organization (Bänziger *et al*., 2006). The aim of this investigation was to find-out the effective criteria in terms of biochemical, physiological and morphological changes taking place in maize cultivars differing in some qualitative traits.

Materials and Methods

Plant materials: Seeds of sweet corn (cv. Saccharata) and waxy-corn (cv. Ceratina) provided by Lion Seed Ltd., were surface disinfected using 5% Clorox® overnight, 30% Clorox® for 30 min., rinsed thrice by sterile-distilled water and then cultured on the MS media (Murashige & Skoog, 1962) containing 3% sucrose and 0.25% Phytagel®. Seedlings were cultured *In vitro* under condition of 25±2°C ambient temperature, 60±5% relative humidity (RH) and 60±5 μmol $m^{-2} s^{-1}$ photosynthetic proton flux (PPF) provided by fluorescent lamps with 16 h d^{-1} photoperiod for 2 weeks. Maize seedlings were transferred to MS sugar-free liquid media (photoautotrophic condition) using vermiculite as a supporting material for 1 week. The number of air-exchanges in the glass vessels was adjusted to 2.32 h^{-1} by punching a hole in the plastic cap (∅ 1 cm) and covering the hole with a microporous filter (0.20 μm of pore size). Sodium chloride concentration in the culture media was adjusted to 0, 100, 200, 300 or 400 mM for 5 days. Photosynthetic pigments, proline content, chlorophyll a fluorescence, net-photosynthetic rate and growth characters were measured.

Chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (TC) and total carotenoid (C_{x+c}) concentrations were determined following the methods of Shabala *et al*., (1998) and Lichtenthaler (1987), respectively. One hundred milligrams of leaf material were collected from the second and third nodes of the shoot tip. The leaf samples were placed in a 25 mL glass vial, added with 10 mL of 95.5% acetone, and blended with a homogenizer. The glass vials were sealed with parafilm to prevent evaporation and then stored at 4°C for 48 h. The Chl_a and Chl_b concentrations were measured using a UV-visible spectrophotometer at 662 nm and 644 nm wavelengths. The C_{x+c} concentration was measured spectrophotometrically at 470 nm. A solution of 95.5% acetone was used as a blank. Pigment degradation percentage was calculated using the following equation:

$$\text{Pigment degradation (\%)} = \left[1 - \frac{\text{Salt treatment}}{\text{Control}} \right] \text{X } 100$$

Proline content in the leaf tissues was extracted and analyzed according to the method of Bates *et al*., (1973). Fifty-milligram fresh leaf materials were ground in a motar with liquid nitrogen. The homogenate powder was mixed with 1 mL aqueous Sulfosalicylic acid (3 % w/v) and filtered through (Whatman #1) filter paper. The extracted solution was reacted with an equal volume of Glacial acetic acid and ninhydrin reagent (1.25 mg Ninhydrin in 30 mL of Glacial acetic acid and 20 ml 6 M H_3PO_4) and incubated at 95°C for 1 h. The reaction was terminated placing in an ice bath. The reaction mixture was vigorously mixed with 2 mL toluene. After warming at 25°C, the chromophore was measured at 520 nm. L-proline was used as a standard.

Chlorophyll *a* fluorescence emission from the third leaf was measured using a Fluorescence Monitoring System in the pulse amplitude modulation mode as described by Loggini *et al*., (1999) and Maxwell & Johnson (2000).

Carbon dioxide (CO_2) inside (C_{in}) and outside (C_{out}) the culture vessel containing seedlings was measured using a Gas Chromatograph and net photosynthetic rate (P_n) was calculated according to Fujiwara *et al*., (1987).

$$\mathbf{[P_n] = K \times E \times V (C_{out} - C_{in}) / Leaf\ area}$$

where, K is a conversion factor converting CO_2 amount from volume to mole (40.5 mol m^{-3} at 28°C).
E is a number of air exchanges per hour (2.32 h^{-1}).
V is an air volume of the vessel (0.0025 m^3).

Fresh and dry weights, shoot height, root length and leaf area of maize seedlings were measured as described by Cha-um *et al*., (2006). Maize seedlings were dried at 110°C in a hot-air oven for 2 days and then incubated in desiccators before measurement of the dry weight. Leaf area of maize seedlings was measured using a leaf area meter (DT-scan).

The experiment was arranged as 2×5 factorials in a completely randomized design (CRD) with six replicates and four plantlets per replicate. The mean values obtained were compared by Duncan's New Multiple Range Test (DMRT) and analyzed by the SPSS software. The correlations between physiological and biochemical parameters were calculated using Pearson's correlation coefficients.

Results and Discussion

Osmotic potential (ψ_s) in the culture media containing Sodium chloride (NaCl) was decreased, depending on salt concentrations. Decrease in ψ_s in the culture media was positively related to pigment degradation in both Saccharata (r^2 = 0.98) and Ceratina (r^2 = 0.99) (Fig. 1). Chlorophyll a (Chl_a), chlorophyll b (Chl_b) and total chalorophyll (TC) contents in salt-stressed seedlings of Saccharata and Ceratina were significantly dropped when exposed to salt stress. Total carotenoid (C_{x+c}) content was decreased, relating to genotype and salt stress factors (Table 1). On the other hand, proline content in the salt stressed seedlings reached to 600.9 (3.67 folds of control) and 339.2 μmol g^{-1} FW (2.86 folds of control) in Saccharata and Ceratina cultivars, respectively under 400 mM NaCl stress (Table 1). The proline content in the salt stressed tissues of cv. Saccharata was higher than that in Ceratina. The pigment degradation in the salt stressed leaves was positively correlated with low maximum quantum yield of PSII (F_v/F_m) in both Saccharata (r^2 = 0.85) and Ceratina (r^2 = 0.90) (Fig. 2). Chlorophyll a fluorescence

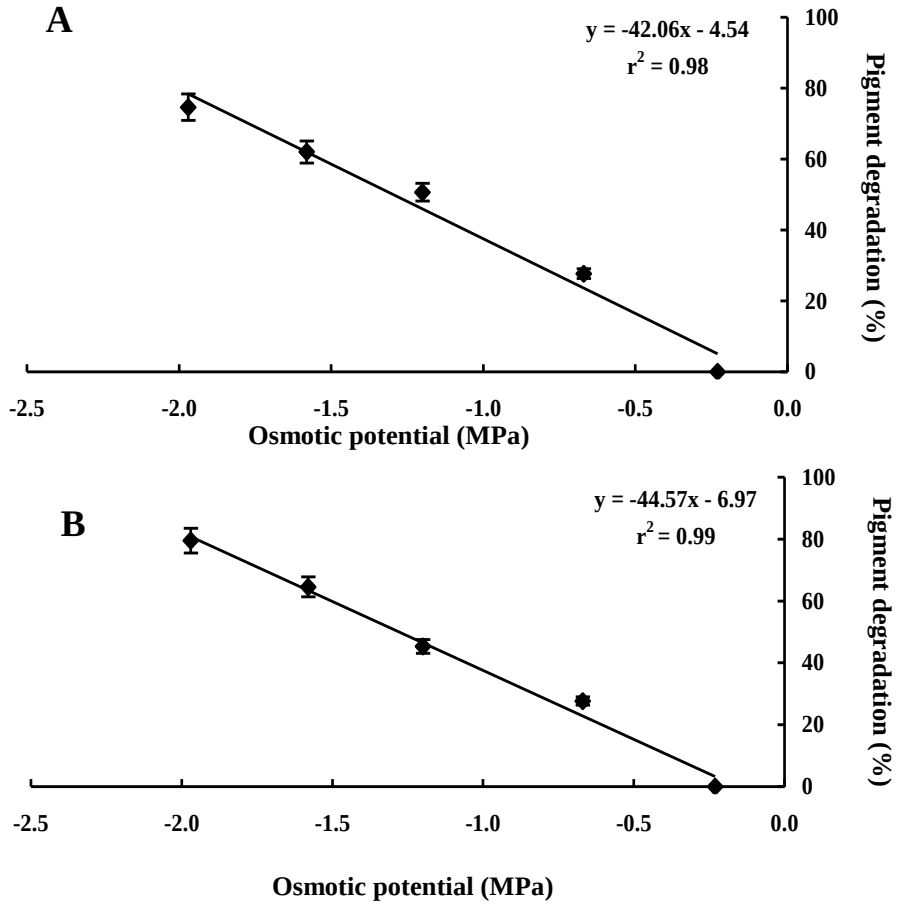

Fig. 1. Relationship between osmotic potential in the culture media and pigment degradation of Saccharata (A) and Ceratina (B) maize seedlings grown under salt stress for 5 days. Error bars represent ±SE.

parameters, including F_v/F_m, photon yield of PSII (Φ_{PSII}), and photochemical quenching (qP) in the salt stressed leaves were significantly diminished corresponding to cultivar, salt stress and their interactions, while non-photochemical quenching (NPQ) was increased (Table 2). The reduction in Φ_{PSII} in the salt stressed seedlings of maize was positively correlated to net photosynthetic rate (P_n) in both Saccharata (r^2 = 0.91) and Ceratina (r^2 = 0.96) (Fig. 3). The P_n in salt stressed seedlings was sharply dropped in both cultivars (Table 2), leading to considerable growth reduction (Fig. 4). In 400 mM NaCl treatment, the P_n was reduced to as low as 1.05 μmol m^{-2} s^{-1} in Saccharata (7.76 folds of control) and 0.99 μmol m^{-2} s^{-1} in Ceratina (5.27 folds of control) (Table 2). The relationship between biochemical and physiological parameters are presented in Table 3. The Chl_a, Chl_b, C_{x+c}, F_v/F_m, Φ_{PSII}, qP and P_n showed positive correlations, while proline and NPQ was negatively related. In addition, the fresh weight, dry weight and leaf area in both cultivars were reduced significantly due to salt stress (Table 4). The data for pigment degradation, photosynthetic ability and growth reduction in salt stressed seedlings were subjected to K-Means Cluster in SPSS software to classify the cultivars, Saccharata (sweet) was found to be salt susceptible and Ceratina (waxy) the salt tolerant.

Table 1. Chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (TC), total carotenoids (C_{x+c}) and proline contents of maize seedlings grown under salt stress for 5 days.

Cultivar (CV)	NaCl (mM)	Chl_a ($\mu g\ g^{-1}FW$)	Chl_b ($\mu g\ g^{-1}FW$)	TC ($\mu g\ g^{-1}FW$)	C_{x+c} ($\mu g\ g^{-1}FW$)	Proline ($\mu mol\ g^{-1}FW$)
Saccharata	0	200.4a	87.8a	288.2a	64.9a	163.6fg
	100	137.1b	71.3ab	208.4b	55.4ab	271.5d
	200	89.9cd	52.3bc	142.2c	52.3abc	294.9cd
	300	73.2de	36.3cd	109.5d	49.9abc	520.0b
	400	45.2ef	28.0cd	73.2e	38.3bcd	600.9a
Ceratina	0	200.1a	89.2a	289.3a	62.9a	118.5g
	100	152.5b	56.7bc	209.2b	50.3abc	188.5ef
	200	106.7c	51.5bcd	158.2c	46.1abc	234.8de
	300	64.7def	37.9cd	102.6d	32.9cd	273.8d
	400	38.7f	20.7d	59.4e	26.8d	339.2c
Significant level						
CV		NS	NS	NS	**	**
NaCl		**	**	**	**	**
CV×NaCl		NS	NS	NS	NS	**

Different letters in each column show significant difference at $p \leq 0.01$ (**) by Duncan's New Multiple Range Test (DMRT). Non significant difference represented by ^{NS}.

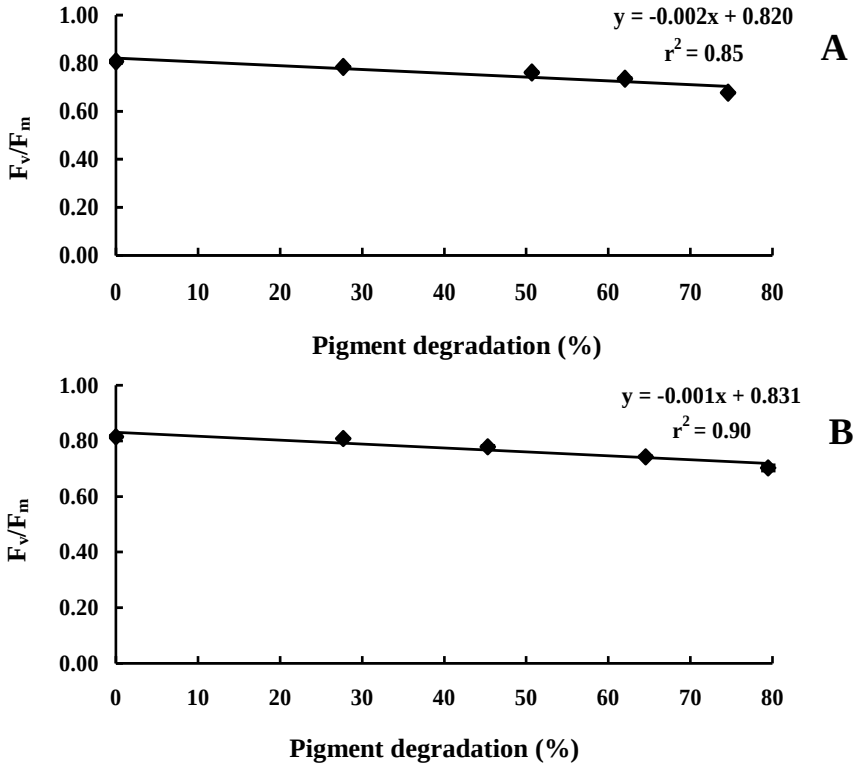

Fig. 2. Relationship between pigment degradation and maximum quantum yield of PSII (F_v/F_m) of Saccharata (A) and Ceratina (B) maize seedlings grown under salt stress for 5 days. Error bars represent ±SE.

Table 2. Maximum quantum yield of PSII (F_v/F_m), photon yield of PSII (Φ_{PSII}), photochemical quenching (qP), non-photochemical quenching (NPQ) and net-photosynthetic rate (P_n), of maize seedlings grown under salt stress for 5 days.

Cultivar (CV)	NaCl (mM)	F_v/F_m	Φ_{PSII}	qP	NPQ	P_n (μmol m^{-2} s^{-1})
Saccharata	0	0.807a	0.716a	0.914a	0.033c	8.15a
	100	0.785b	0.688ab	0.850a	0.051c	5.70b
	200	0.761c	0.603bc	0.731bc	0.110abc	4.73c
	300	0.736d	0.503cd	0.686cde	0.167abc	2.02ef
	400	0.677f	0.385e	0.461f	0.271a	1.05g
Ceratina	0	0.815a	0.657ab	0.826ab	0.031c	5.22bc
	100	0.808a	0.530cd	0.718bcd	0.044c	3.58d
	200	0.779b	0.495d	0.679cde	0.155abc	2.49e
	300	0.743d	0.458de	0.614de	0.161abc	1.54fg
	400	0.703e	0.439de	0.576e	0.211ab	0.99g
Significant level						
CV		**	**	**	NS	**
NaCl		**	**	**	**	**
CV×NaCl		NS	**	**	NS	**

Different letters in each column show significant difference at $p \leq 0.01$ (**) by Duncan's New Multiple Range Test (DMRT). Non-significant difference represented by NS.

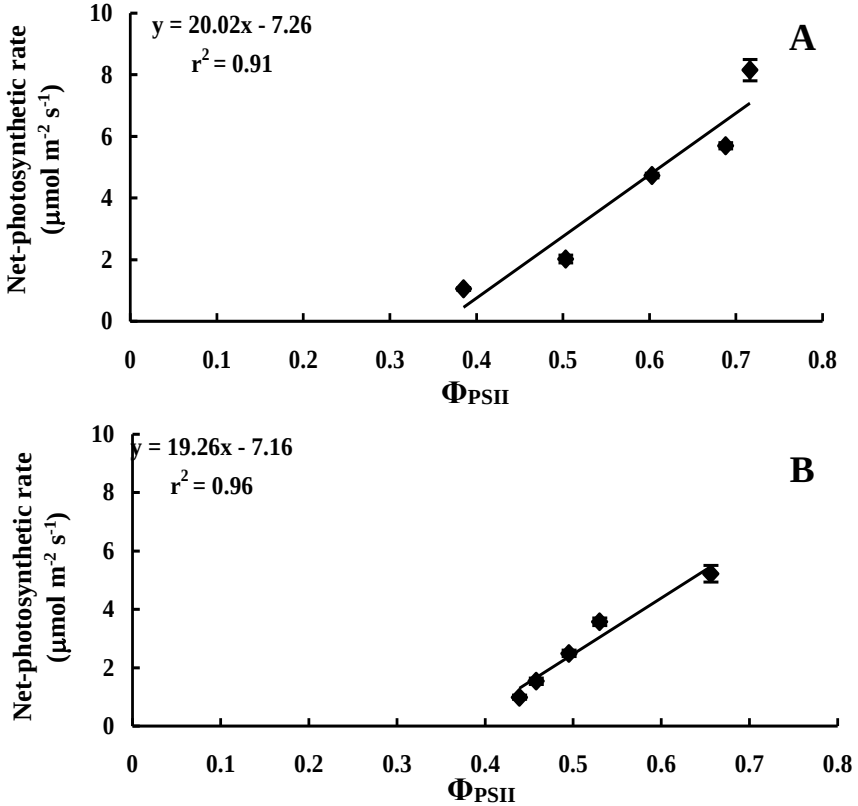

Fig. 3. Relationship between photon yield of PSII (Φ_{PSII}) and net-photosynthetic rate (P_n) of Saccharata (A) and Ceratina (B) maize seedlings grown under salt stress for 5 days. Error bars represent ±SE.

Table 3. Relationship between physiological and biochemical parameters of maize seedlings grown under salt stress for 5 days.

Parameters	Chl_a	Chl_b	C_{x+c}	PRO	F_v/F_m	Φ_{PSII}	qP	NPQ	P_n
Chl_a	-	-	-	-	-	-	-	-	-
Chl_b	0.734**	-	-	-	-	-	-	-	-
C_{x+c}	0.736**	0.612**	-	-	-	-	-	-	-
PRO	-0.720**	-0.570**	-0.338**	-	-	-	-	-	-
F_v/F_m	0.856**	0.750**	0.674**	-0.657**	-	-	-	-	-
Φ_{PSII}	0.737**	0.656**	0.607**	-0.560**	0.829**	-	-	-	-
qP	0.633**	0.595**	0.449**	-0.533**	0.661**	0.857**	-	-	-
NPQ	-0.614**	-0.526**	-0.419**	0.612**	-0.666**	-0.549**	-0.432**	-	-
P_n	0.810**	0.738**	0.641**	-0.619**	0.860**	0.840**	0.761**	-0.590**	-

Significant level at $p \leq 0.01$ is represented by ** using Pearson's correlation coefficients.

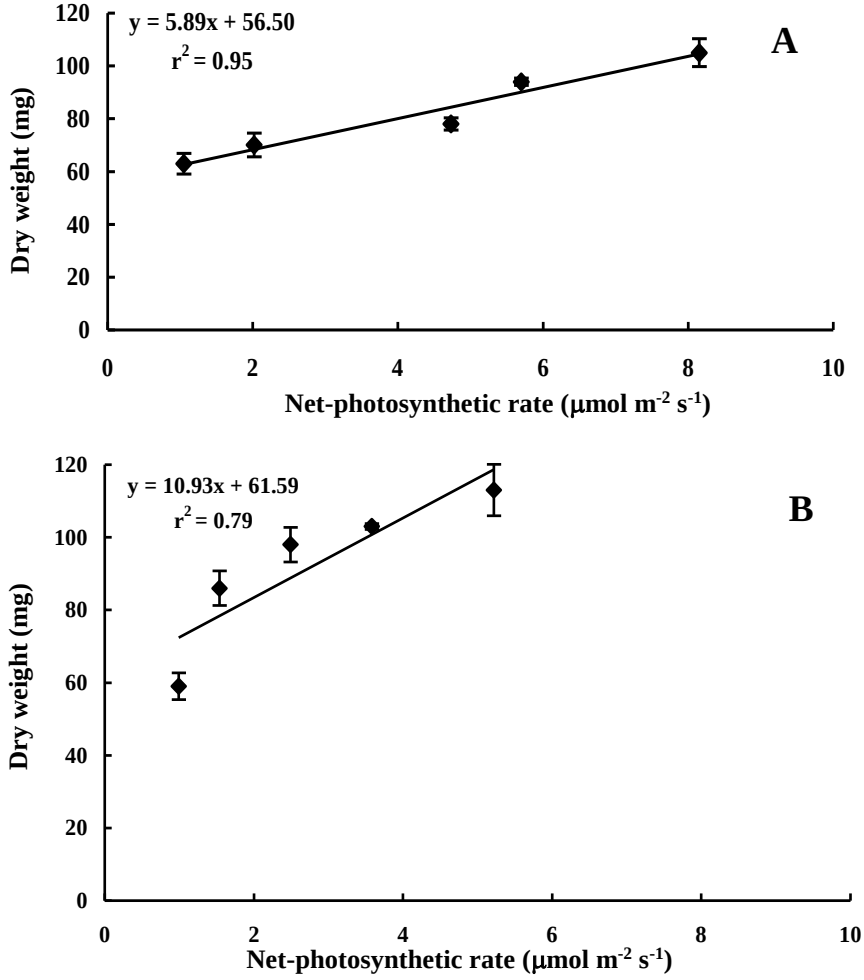

Fig. 4. Relationship between net-photosynthetic rate (P_n) and dry weight of Saccharata (A) and Ceratina (B) maize seedlings grown under salt stress for 5 days. Error bars represent ± SE.

Table 4. Growth characters, fresh weight, dry weight and leaf area of maize seedlings grown under salt stress for 5 days.

Cultivar (CV)	NaCl (mM)	Fresh weight (mg)	Dry weight (mg)	Leaf area (mm^2)
Saccharata	0	1238a	105ab	2207a
	100	855bc	94bcd	1522c
	200	668cd	78def	1056d
	300	562de	70fgh	658e
	400	453e	63gh	360f
Ceratina	0	1289a	113a	2079ab
	100	1099a	103abc	1872b
	200	903b	98abc	1470c
	300	852bc	86cde	1132d
	400	662cd	59h	899de
Significant level				
CV		**	**	**
NaCl		**	**	**
CV×NaCl		NS	NS	**

Different letters in each column show significant difference at $p \leq 0.01$ (**) by Duncan's New Multiple Range Test (DMRT). Non-significant difference represented by ^{NS}.

In the present study, the osmotic potential dramatically decreased when NaCl was supplied in the culture media. This led to water deficit in the maize seedlings. Osmotic stress and ionic toxicity resulted from salt stress in maize plants are well established in many reports (Fortmeier & Schubert, 1995; Katrrji *et al*., 1996; Katrrji *et al*., 2004; Mansour *et al*., 2005; Eker *et al*., 2006). Low osmotic potential in the media containing salts is one of the most factors, which directly affect water use efficiency in plants of maize (Neto *et al*., 2004; Mansour *et al*., 2005), wheat (El-Hendawe *et al*., 2005), barley (Chen *et al*., 2007) and soybean (Çiçek & Çakırlar, 2008).

Proline accumulation in salt stressed plants is a primary defense response to maintain the osmotic pressure in a cell, which is reported in salt tolerant and salt sensitive cultivars of many crops (de Lacerda *et al*., 2003; Kumar *et al*., 2003; de Lacerda, *et al*., 2005; Demiral & Türkan, 2005; Mansour *et al*., 2005; Misra & Gupta, 2005; Desingh & Kanagaraj, 2007; Koca *et al*., 2007; Veeranagamallaiah *et al*., 2007). In the present study, proline accumulation in the salt tolerant maize (cv. Ceratina) was significantly lower than that in the salt sensitive maize (cv. Sacharata). Similar results have been reported in rice [IR28 (salt susceptible) < Pokkari (salt tolerant)] and sorghum [CSF18 (salt susceptible) < CSF20 (salt tolerant)] grown under salt stress (de Lacerda, *et al*., 2003; de Lacerda, *et al*., 2005; Demiral & Türkan, 2005). Salt tolerant plant species may possibly survive in salt stress condition using other defense mechanisms such as ion homeostasis, antioxidation and hormonal systems (Zörb *et al*., 2005; Neto *et al*., 2006; Zhang *et al*., 2006). Due to this, evaluation of a number of parameter in salt stressed plant would result in the identification of some effective criteria to classify plants for salt tolerance.

The pigment degradation, chlorophyll a fluorescence weakness and P_n reduction in salt stressed maize cultivars were found to be the sensitive parameters to determine the pigment stability, photosystem II (PSII) efficiency and CO_2 assimilation rate in the leaf tissues. The chlorophyll content in 100 mM NaCl stressed maize (cv. Helix) for 8 days was significantly reduced to 8% when compared to control, causing low CO_2 assimilation

and transpiration rates (Lohaus *et al*., 2000). The chloroplast in the bundle sheath zones of 513 mM NaCl stressed maize (cv. Golden Bantam) is drastically damaged 2.26 folds of control, leading to low F_v/F_m and Φ_{PSII} (Hasan *et al*., 2006). The reduction in both light reaction and dark reaction of photosynthesis of salt stressed maize is related to growth reduction and low productivity (Fortmeier & Schubert, 1995; Lohaus *et al*., 2000; Katerji *et al*., 2004; Rodríguez *et al*., 2004). In addition, the salt tolerant cultivars, Pioneer 3769, Pioneer 3906, Giza 2, and salt sensitive cultivars, Pioneer 3751, Across 8023, Trihybrid 321, of maize have been categorized (Fortmeier & Schubert, 1995; Mühling & Läuchli, 2002; Mansour *et al*., 2005). In this study, cv. Ceratina was identified as salt tolerant, while Saccharata as salt susceptible using K-Means Cluster analysis. There are several reports, which show that salt tolerant cultivars of maize can be identified using biochemical, physiological and morphological changes as well as productivity criteria. From previous publications, maize cultivar, Maverik (hybrid), 2572 (sweet corn) and BR5033, were identified as salt tolerant, and 7993 (hybrid), Reliance and BR5011 as salt susceptible (Pasternak *et al*., 1995; Neto *et al*., 2004; Eker *et al*., 2006).

In conclusion, the photosynthetic parameters in both light and dark reactions in cv. Ceratina (waxy) and cv. Saccharata (sweet) cultivars were the sensitive parameters, which related to overall growth reduction under salt stress. There was a significant relationship between biochemical and physiological characters. Saccharata cultivar of maize was classified as salt susceptible, whereas cv. Ceratina as salt tolerant, based on various biochemical, physiological and growth parameters appraised in the present study.

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