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Physiological Changes in Pea (*Pisum sativum* L. cv. Green Arrow) under NaCl Salinity

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Abstract: The effects of salt-stress on physiological parameters in pea (*Pisum sativum* L. cv. Green Arrow) plants were investigated. Ten weeks old pea plants were treated with NaCl at 0, 50, 100 and 150 mM in nutrient solution. Plants were grown under controlled environment and harvested at each 3 days interval for measurement of physiological parameters. With increasing NaCl concentration in nutrient solution, photosynthetic rate, relative water content, stomatal conductance and chlorophylls contents decreased while CO_2 compensation concentration and free proline content increased. The effects of salinity were increased when higher NaCl concentrations were accompanied with increasing stress duration such that plants died in 150 mM NaCl after 15 days. Results indicated that Pea plants could tolerate 50 and 100 mM NaCl, however, were sensitive to 150 mM NaCl. In Plants treated with high concentrations of NaCl P_n, g_s and chlorophylls contents were strongly decreased at the initial stages of impose salinity.

Key words: Pea, *Pisum sativum* L., photosynthetic rate, relative water content, salt-stress, stomatal conductance

INTRODUCTION

Osmotic stress is the main result of drought, chilling, freezing, or excessive salt in water (Zhu *et al*., 1997). Osmotic stress induces specific cellular responses that includes change in the activity of solute transporters (Lucht and Bremer, 1994; Luyten *et al*., 1995), the expression of genes that encode enzymes required for osmolytes synthesis (Albertyn *et al*., 1994; Levin and Errde, 1995) and stress resistance (Vaidyanathan *et al*., 1999; Xu *et al*., 1996). Certain plants, marine algae, bacteria and other organisms accumulate organic solutes such as sugar alcohols, the amino acid proline, quaternary ammonium and/or tertiary sulphonium compounds in response to osmotic stress (Yancey *et al*., 1982). Salinity seems to affect two plant processes: water relations and ionic relations. During initial exposure to salinity, plants experience water stress, which in turn reduce leaf expansion. During long-term exposure to salinity, plants experience ionic stress, which can lead to premature senescence of adult leaves and thus a reduction in the photosynthetic rate available to support continued growth (Cramer and Nowak, 1992). Reduced photosynthesis with increasing salinity is attributed to either stomatal closure, leading to a reduction in intracellular CO_2 partial pressure, or non-stomatal factors (Bethke and Drew, 1992). There is increasing evidence that salinity changes photosynthetic parameters, including osmotic and leaf water potential, transpiration rate, leaf temperature and relative leaf water content. Salt also affects photosynthetic components such as enzymes, chlorophylls and carotenoids. Changes in these parameters depend on the severity and duration of stress (Lakshmi *et al*., 1996; Misra *et al*., 1997) and on plant species (Dubey, 1999).

In this research, physiological changes of pea plants in saline environment are evaluated. Results indicated that with decreasing relative water content of leaves, stomatal conductance and photosynthetic rate decreased. Additionally, with increasing NaCl salinity free proline content in plants was increased for osmotic adjustment.

MATERIALS AND METHODS

Pea seeds (*Pisum sativum* L. cv. Green Arrow) were surface-sterilized (1 min in ethanol 70% and 15 min in sodium hypochlorite 2%), germinated and grown in perlite with Hoagland solution. After 10 days, seedlings

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Table 1: Effects of NaCl on photosynthetic rate (P_n), CO_2 compensation concentration (Γ), stomatal conductance (g_s), relative water content (RWC) and proline content of salt-stressed pea plants during experimental period

NaCl (mM)	Time of sampling (day)	P_n(μmol CO_2 m^{-2} S^{-1})	Γ (μL L^{-1})	g_s (mm s^{-1})	RWC (%)	Free proline (μg g^{-1} fresh leaf)
0	3	3.6±0.60^{cde}	114±7.20^{d}	4.0±0.30^{cde}	91±3.40^{abc}	2.3±0.50^{g}
	6	3.6±0.30^{cde}	102±1.30^{d}	6.6±0.90^{b}	93±0.70^{ab}	2.0±0.80^{g}
	9	4.2±0.40^{c}	99±2.10^{d}	6.5±0.63^{b}	93±1.70^{ab}	1.5±0.90^{g}
	12	5.3±0.20^{b}	111±4.30^{d}	7.8±0.40^{a}	94±2.10^{ab}	2.5±0.30^{g}
	15	6.4±0.30^{a}	105±7.70^{d}	7.8±0.32^{a}	95±1.50^{a}	2.0±0.00^{g}
50	3	3.2±0.50^{cde}	142±10^{c}	3.7±0.37^{de}	95±0.60^{ab}	4.5±0.60^{g}
	6	3.0±0.10^{def}	146±6.40^{c}	4.4±0.16^{cd}	90±1.40^{abc}	16.5±1.30^{f}
	9	2.8±0.50^{ef}	146±1.50^{c}	5.0±0.13^{c}	90±0.20^{abc}	20.5±0.60^{f}
	12	2.0±0.20^{fg}	145±4.50^{c}	4.6±0.60^{cd}	82±0.60^{ef}	40.5±0.60^{e}
	15	0.4±0.05^{h}	175±2.20^{b}	0.4±0.07^{ij}	86±2.40^{cde}	51.5±1.30^{d}
100	3	4.0±.50^{cd}	169±18.0^{b}	3.5±0.40^{def}	92±1.20^{abc}	20.5±1.50^{f}
	6	3.2±0.00^{cde}	162±2.00^{bc}	3.1±0.40^{efg}	90±0.80^{abc}	38.7±1.30^{e}
	9	1.6±0.10^{g}	170±3.50^{b}	2.0±0.24^{gh}	90±1.30^{abc}	60.0±2.30^{d}
	12	1.6±0.20^{g}	182±2.30^{b}	2.0±0.40^{gh}	84±1.70^{def}	83.0±2.50^{c}
	15	0.5±0.03^{h}	222±20.0^{a}	0.1±0.01^{ij}	82±2.10^{ef}	92.0±1.00^{bc}
150	3	3.4±0.04^{cde}	205±4.00^{a}	2.4±0.20^{fgh}	88±3.40^{bcd}	26±2.00^{f}
	6	2.6±0.30^{efg}	173±2.80^{b}	1.3±0.10^{hi}	83±2.00^{def}	60±0.80^{d}
	9	2±0.60^{fg}	202±3.50^{a}	0.8±0.10^{ij}	80±3.00^{ef}	100±2.70^{b}
	12	0.5±0.04^{h}	220±7.00^{a}	0.7±0.10^{ij}	78±1.10^{f}	264±15.0^{a}
	15	0.0±0.00	000±0.00	0.0±0.00	00±0.00	000±0.00

(Means±SE) of four replicates. Numbers followed by the same letter are not significantly different ($p>0.05$)

were treated with NaCl at 0, 50, 100 and 150 mM in nutrient solution, with pH 6.5 and renewed every 5 days. The plants were grown under controlled environment (17 h light periods, 300 μmol quanta m^{-2} s^{-1} light intensity, day/night temperatures of 25/18°C) in a greenhouse. For per treatment four plants were harvested after 3, 6, 9, 12 and 15 days of growth for physiological analyses. Leaf Relative Water Content (RWC) was estimated by recording the Saturated Mass (SM) of 0.5 g fresh leaf (FM) samples by keeping them in water for 4h, followed by drying in a hot air oven until constant by mass (DM) was achieved according described by Weatherley (1950). Chlorophylls a and b contents were estimated as described by Arnon (1949). Free proline concentration was determined as described by Bates *et al.* (1973). Values were expressed as micrograms per gram fresh weight. Photosynthetic rate (P_n) and CO_2 compensation concentration (Γ) were determined in plants under salt-stress, applying an infrared gas (CO_2) analyser (225 MKS, Analytical Development Co., UK) as described by Khavari-Nejad (1980, 1986). Stomatal conductance (g_s) was measured on abaxial leaf surfaces using a Delta-T AP4 transit porometer (Delta-T Davices, Cambridge, UK).The study was conducted completely randomized design with four replications. Data were statistically analyzed using SAS software.

RESULTS

Because the photosynthetic rate (P_n) of leaves of both C_3 and C_4 plants decreases as their Relative Water Content (RWC) and water potential (ψ) decrease (Chaves, 1991; Cornic, 1994; Kramer and Boyer, 1995; Lawlor, 1995; Cornic and Massacci, 1996), both relative water content and photosynthetic rate were measured. In 50 and 100 mM NaCl, P_n was significantly decreased at the 9 days sampling, but in days of 3 and 6 was not significantly decreased. P_n was decreased further in 150 mM NaCl, reaching very low values at 12 days sampling (Table 1). Also at 6, 9 and 12 days sampling in 150 mM NaCl, RWC was decreased to 83, 80 and 78% , respectively, however, in 50 and 100 mM NaCl, RWC until the day of 9 sampling was 90% and did not has significantly changes. CO_2 compensation concentration (Γ) increases as RWC falls (Lawlor, 2002), present results indicated that Γ was significantly enhanced in all of treatments but increasing of Γ in plant treated with 150 mM NaCl was higher than that of plants in 50 and 100 mM NaCl (Table 1).

Stomatal conductance (g_s) drastically reduced in plants treated with NaCl; this might be attributed to the lower leaf water potential and a reduction in relative leaf water content, which resulted in loss of turgor (Sultana, *et al.*, 1999). Present results indicated that g_s was very low in the leaves of plants treated with 150 mM NaCl with respect to other treatments. In 50 and 100 mM NaCl, g_s was significantly decreased at 6 days sampling (Table 1).

Because in response to water stress and salinity proline accumulation was observed in many organisms, including bacteria, fungi, algae, invertebrates and plants (Csonka and Hanson, 1991; Delauney and Verma, 1993; Hanson and Hitz, 1982; Yoshiba *et al.*, 1995), we aimed at producing free proline in leaves of plants grown in the presence or absence of salinity. In all of NaCl concentrations free proline content of leaves was

Table 2: Effects of NaCl on chlorophyll a and chlorophyll b content, total chlorophyll and Chlorophyll a/b ratio of salt-stressed pea plants during experimental period

NaCl(mM)	Sampling time (day)	Chl.a (mg g^{-1} fresh matter)	Chl.b (mg g^{-1} fresh matter)	Chl.a+Chl.b (mg g^{-1} fresh matter)	Chlorophyll a/b ratio
0	3	1.47±0.21^{defg}	0.70±0.07^{cde}	2.20±0.27^{def}	2.09±0.14^{bcd}
	6	1.80±0.10^{cde}	0.81±0.03^{abc}	2.61±0.12^{cd}	2.33±0.14^{bcd}
	9	1.85±0.14^{cd}	0.89±0.07^{ab}	2.75±0.12^{c}	2.14±0.33^{bcd}
	12	2.30±0.24^{b}	0.91±0.04^{a}	3.20±0.25^{b}	2.50±0.24^{abc}
	15	2.80±0.06^{a}	0.91±0.01^{a}	3.76±0.05^{a}	3.12±0.10^{a}
50	3	1.25±0.03^{fgh}	0.65±0.02^{def}	1.90±0.07^{efgh}	1.93±0.22^{bcd}
	6	1.60±0.06^{cdef}	0.72±0.02^{cd}	2.36±0.07^{cde}	2.27±0.02^{bcd}
	9	1.66±0.26^{cdef}	0.69±0.04^{cde}	2.34±0.20^{cde}	2.41±0.40^{abcd}
	12	1.93±0.21^{bcd}	0.79±0.06^{abc}	2.72±0.20^{c}	2.50±0.38^{abcd}
	15	1.96±0.30^{bc}	0.80±0.07^{abc}	2.75±0.30^{c}	2.61±0.07^{ab}
100	3	1.07±0.09^{gh}	0.55±0.02^{f}	1.60±0.11^{gh}	1.93±0.10^{bcd}
	6	0.96±0.01^{h}	0.57±0.02^{ef}	1.54±0.02^{h}	1.67±0.05^{d}
	9	1.29±0.04^{fgh}	0.71±0.03^{cd}	2.00±0.03^{efgh}	1.81±0.10^{bcd}
	12	1.33±0.19^{fgh}	0.77±0.01^{bcd}	2.10±0.10^{efg}	1.72±0.27^{cd}
	15	1.06±0.05^{gh}	0.54±0.07^{f}	1.61±0.10^{gh}	2.05±0.28^{bcd}
150	3	1.00±0.05^{gh}	0.53±0.01^{f}	1.63±0.07^{gh}	2.00±0.07^{bcd}
	6	1.13±0.05^{gh}	0.57±0.01^{ef}	1.71±0.06^{fgh}	1.95±0.07^{bcd}
	9	1.19±0.02^{fgh}	0.65±0.04^{def}	1.85±0.06^{fgh}	1.83±0.08^{bcd}
	12	1.34±0.03^{efgh}	0.64±0.03^{def}	1.99±0.06^{efgh}	2.07±0.05^{bcd}
	15	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00

(Means±SE) of four replicates. Numbers followed by the same letter are not significantly different ($p>0.05$)

significantly increased and in 150 mM NaCl free proline accumulation was in highest content in respect of 50 and 100 mM NaCl (Table 1).

A decrease in chlorophyll concentration in salinized plants could be attributed to increased activity of the chlorophyll- degrading enzyme chlorophyllase (Reddy and Vora, 1986). In this research chlorophylls contents of leaves in 100 and 150 mM NaCl after 6 days sampling were significantly decreased in respect of control and 50 mM NaCl (Table 2). At 6 days sampling the chlorophyll a/b ratio in plants treated with 100 and 150 mM NaCl was significantly decreased in comparison with control and 50 mM NaCl (Table 2).

DISCUSSION

Stomata often close in response to drought before any change in leaf water potential and/or leaf water content is detectable (Socías *et al*., 1997). In the present study, with increasing concentration of NaCl in culture medium g_s was decreased. In plants treated with 50 and 100 mM NaCl, g_s was decreased after 6 days of sampling but in 150 mM NaCl after 3 days, g_s decreased (Table 1). It is now well established that there is a drought-induced root- to- leaf signalling, promoted by soil drying and reaching the leaves through the transpiration stream, which induces closure of stomata. This chemical signal has been shown to be abscisic acid (ABA), which is synthesized in the roots in response to soil drying (Davies and Zhang, 1991). In this research RWC in plants treated with 50 and 100 mM NaCl after 12 days of sampling was significantly decreased, however, in plants treated with 150 mM NaCl, after 3 days, RWC was reduced, which resulted in loss of turgor. In fact leaf water status interacts with stomatal conductance and transpiration and under water stress, a good correlation is often observed between leaf water potential and stomata conductance (Medrano *et al*., 2002).

Amongst damages caused by saline stress in plants, the reduction of the photosynthetic processes is one of the most important (Delfine *et al*., 1999). Our results indicated that with decreasing g_s, P_n was also decreased. In 50, 100 and 150 mM NaCl, P_n was significantly decreased after 9, 6 and 3 days sampling respectively (Table 1). It must be emphasized that a high degree of co-regulation of g_s and photosynthesis is usually found (Farquhar *et al*., 2001). In this study, in plants treated with NaCl, chlorophylls contents were decreased with increasing concentrations of NaCl, as compared with control. However, chlorophylls contents in plants grown in 50 and 100 mM NaCl were less impaired (Table 2). The decline in chlorophyll a/b ratio of plants treated with 100 and 150 mM NaCl was significantly higher than that of plants treated in 50 mM NaCl. We therefore confirm that salt- stress conditions led to an oxidative stress in the chloroplasts of pea leaves (Hernández *et al*., 1995, 2001; Gómez *et al*., 2004) and Activated Oxygen Species (AOS) can damage DNA, proteins, chlorophyll and membrane functions (Foyer and Mullineaux, 1994; del- Río *et al*., 2002). The CO_2 compensation concentration in all of NaCl concentrations increased (Table 1) and it agrees with certain findings that Γ increases as RWC falls and the increase is relatively small with initial loss of RWC in some studies, but is greater in others and is very substantial at RWC of 80% and below (Lauer and Boyer, 1992; Tezara *et al*., 1999).

The significant increase of accumulation of free proline in all of plants treated with NaCl was observed (Table 1). As described earlier (Lawlor, 2002) accumulation of amino acids, including proline, is a damage response from the perspective of altered photosynthetic metabolism. The protective function of glutamate and proline at low RWC is then negligible and does not constitute an evolved process with clear benefits. However, a more common rationale for accumulation of amino acids, especially proline, is that it confers advantages, protecting membranes, proteins, etc. when RWC decreases, particularly against increasing ionic concentrations. Our results are in agreement with Fedina and Popova (1996), in which free proline accumulation in leaves of pea increased as the relative water content decreased.

It is concluded that pea plants (*Pisum sativum* cv. Green Arrow) are more resistant to 50 and 100 mM NaCl at the initial stages of imposed salinity, but more sensitive with increasing duration of salinity. This cultivar is strongly sensitive to 150 mM NaCl and damages of salt-stress were significantly observed in initial sampling.

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