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Alleviation of Salt Stress by Phosphorus in Cucumber Microshoots Grown on Rooting Medium

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Abstract: The goal of this work was to test the *in vitro* responses of cucumber microshoots to salt stress as affected by increased phosphorus (P) in the medium. Salt stress was induced by incorporating different levels (0.0, 25, 50, 75 and 100 mM) of NaCl to the medium. Increased P in the medium was efficient to ameliorate the adverse effects of salinity. Shoot and root growth of cucumber microshoots were negatively affected by salt stress, whereas increasing P improved their growth with elevated NaCl levels. NaCl-induced salt stress reduced (more negative) leaf cell sap osmotic potential of cucumber microshoots which was increased (less negative) by P treatments. Total protein content was reduced and proline was accumulated as a result of *in vitro* induced salt stress. However, increased P concentration in the medium enhanced the microshoots content of protein and lowered their content of proline. The microshoots content of K, Ca and P were reduced by salt stress and this reduction was less as P concentration increased in the medium.

Key words: Cucumber · Microshoots · Phosphorus · Proline · Rooting medium · Salt stress

INTRODUCTION

Plants are continuously exposed to numerous biotic and abiotic stresses resulting in altered growth and development and low production [1-3]. Salt stress is among the environmental stress factors that adversely affect crop growth and productivity world-wide [4]. Mitigating the adverse effects of salt stress would have positive impacts on crop growth and yields. Therefore, adopting proper cultural practices during crop cultivation under salt and drought stress conditions is becoming essential [5]. Numerous studies suggested that mineral nutrient-status of plants greatly affect plant adaptation and resistance to salt and osmotic stresses [6]. Phosphorus (P) is one of the essential mineral nutrients and is reported to be involved in ameliorating the negative impact of both salt and drought stresses on plant growth and development [7, 8]. Sawwan *et al*. [9] and Shibli *et al.* [10] demonstrated that P ameliorated the adverse effects of salt stress and water deficit and regulate the osmotic potential in african violet.

The application of plant tissue culture techniques in stress physiological studies, particularly salt and water stress, can be justified by the fact that *in vitro* system has the advantage of relatively little space needed to culture and rigorously control physical environment and nutrient status parameters, which are difficult to regulate with traditional experimental system [8, 11, 12].

Shoot apex culture has been widely used to evaluate plant physiological responses to salinity and osmotic stress in various species, including cherry [13], cucumber [14, 15] and tomato [16]. With regards to the whole plant, a similar response to salt stress could be expected in plantlets grown through *in vitro* shoot apex culture [16], because such explants can be considered mini-replicas of a plant with anatomical organization and ability to root and grow into whole plant.

Although, various *in vitro* studies focused on aspects of plant regeneration in cucumber [17-19], little is known about physiological conditions affecting cucumber *in vitro* cultures. Recently, the responses of cucumber microshoots to salt and osmotic stresses were reported

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[4, 8]. The objective of the present study was to investigate the interactive effects of P with salt stress on growth and physiology of cucumber microshoots.

MATERIALS AND METHODS

Seeds of cucumber (*Cucumis sativus* L. cv Sultan) were used in this study. Modified MS medium [20] was used in all micropropagation steps and in subsequent treatment. All media were supplemented with 1% Bacto agar, 3% sucrose and 0.1 $g.l^{-1}$ myoinositol. The pH of the medium was adjusted to 5.7 prior to autoclaving at 121°C and 15 psi for 20 minutes. Seed germination and shoots were maintained at 16 hr photoperiod using fluorescent light providing a photosynthetic photon flux density of 40 $\mu molm^{-2}s^{-1}$.

Seeds were decoated after soaking in distilled water for 15 minutes. The decoated seeds were soaked in 70% (v/v) ethanol for 1 minute and then surface sterilized by immersing in 1% sodium hypochlorite with 6 drops of Tween-20 per 100 ml for 20 minutes. Finally the seeds were rinsed three times with autoclaved distilled water. The sterilized decoated seeds were germinated on modified MS bioregulators-free medium. Cultures were maintained at 22 °C for 6 days.

The cotyledonary nodes (included both cotyledons, an intact apical bud and 0.5 cm length of intact hypocotyls) from 6 day old seedlings were dissected and inoculated on modified MS medium containing 2 $mg.l^{-1}$ Kn for 3 weeks in order to obtain well-developed plantlets with higher number of nodal explants. Thereafter, single node stem cuttings were taken and inoculated on MS medium containing 1 $mg.l^{-1}$ Kn. Two weeks later, well developed plantlets were obtained. The effectiveness of Kn in promoting shoot development from nodal explants is well documented [4].

Microshoots (3 cm long) were inoculated on rooting medium containing 1.0 $mg.l^{-1}$ IAA to have *in vitro* culture more representative to *in vivo* grown plants [4]. Also rooting medium is more consistent to *in vitro* induced stress than shoot proliferation medium [12]. The medium was supplemented with a combination of NaCl, at 0.0, 25, 50, 75, 100 mM with 0.5, 1.0 or 2.0 mM P using KH_2PO_4 (making 15 combinations). Cultures were grown in 250 ml screw capped bottles containing 45 ml medium.

Data were recorded after one month for shoot length, root number and length and their fresh and dry weights. Dry weights were determined for shoots and roots after drying the samples to a constant weight at 78 °C.

Osmotic potential were measured on leaf samples. Leaf tissues were packed into 1 cm^3 disposable syringes and frozen at -20 °C, allowed to thaw at room temperature for 30 minutes; cell sap was expressed from the culture samples by depressing the syringe plunger. Osmotic potential was measured on 10 µl samples of the extract sap [12] using a Wescor 5500 Vapor Pressure Osmometer. Free proline was extracted and colorimetrically estimated in fresh leaf samples by acid-ninhydrin method of Bates *et al*. [21]. Total protein content in leaves was determined according to Lowry *et al*. [22], using bovine serum albumin as a standard.

For minerals composition, shoots were dried to a constant weight at 78 °C. Two hundred and fifty mg of ground tissue was then digested with 10 ml of concentrated sulfuric acid in presence of selenium reagent mixture and heated on heating digester at 300 °C for 3 hr until clear solution was obtained. The solution was then diluted to 100 ml by addition of distilled water. Potassium was determined by Corning 410C flame photometer and calcium was determined using Varian Spectra AA 200 atomic absorption spectrophotometer. Phosphorus was determined according to Watanabe and Olsen [23] wet ashing procedure and chloride was measured by potentiometric titration [24].

Treatments were arranged in a completely randomized design with 15 replicates. Collected data were subjected to the analysis of variance (ANOVA) and means were separated according to the least significant difference (LSD) at 0.05 level of probability using SAS program.

RESULTS AND DISCUSSION

Generally, growth of cucumber microshoots grown on rooting medium was negatively affected by NaCl–induced salinity; however, supplementary P tended to ameliorate these effects effectively (Table 1).

Although it was reduced with salinity, increasing P, especially to 2 mM concentration, enhanced shoot length at all concentrations of NaCl (Table 1). The longest shoots were obtained at all concentrations of P in absence of NaCl and at 1 and 2 mM P but in presence of 25 mM NaCl. Phosphorus at 1 and 2 mM in absence of NaCl resulted in 1.7 and 5.8% increase in shoot length. At NaCl concentrations greater than or equal to 50 mM, P tended to reduce the severity caused by NaCl (Table 1).

In absence of NaCl, different concentrations of P produced almost similar shoot fresh weights (Table 1); however, the ameliorating effects of P were clear at NaCl concentrations of 25 and 50 mM. Within each of these

Table 1: Interactive effects of P with *in vitro*-induced salinity on vegetative growth of cucumber microshoots grown on rooting medium containing 1 mg.l^{-1} IAA.

	NaCl (mM)				
P (mM)	0	25	50	75	100
	Shoot Length (cm)				
0.5	8.56 a-d[(1)]	8.43 cde	7.50 ef	6.67 g	4.81 i
1.0	8.91 abc	8.56 a-d	7.79 de	7.00 fg	5.13 hi
2.0	9.28 a	9.00 ab	8.43 bcd	7.45 ef	5.62 h
	Shoot Fresh Weight (g)				
0.5	2.60 ab	2.25 c	1.84 ef	1.61 fgh	1.36 h
1.0	2.69 a	2.36 bc	1.94 de	1.69 fgh	1.41 gh
2.0	2.88 a	2.62 ab	2.18 cd	1.89 def	1.49 gh
	Shoot Dry Weight (g)				
0.5	0.185 de	0.146 c	0.117 de	0.095 fg	0.077 h
1.0	0.191 b	0.154 c	0.124 d	0.101 ef	0.083 gh
2.0	0.210 a	0.184 b	0.145 c	0.114 de	0.092 fg

(1) For each parameters, means followed by different letters are significantly different according to LSD test (P<0.05).

Table 2: Interactive effects of P with *in vitro*-induced salinity on adventitious root growth of cucumber microshoots grown on rooting medium containing 1 mg.l^{-1} IAA.

	NaCl (mM)				
P (mM)	0	25	50	75	100
	Root Number				
0.5	8.97 de[(1)]	8.20 efg	7.45 gh	6.85 hi	5.86 j
1.0	9.85 bc	9.28 bcd	8.51 def	7.50 gh	6.15 ij
2.0	10.91 a	9.91 b	9.00 cde	7.78 fg	6.35 ij
	Root Length (cm)				
0.5	9.01 abc	8.42 cd	7.48 ef	6.97 fg	6.40 g
1.0	9.33 ab	8.91 bc	7.88 de	7.25 efg	6.77 fg
2.0	9.79 a	9.32 ab	8.48 bcd	7.50 ef	7.35 ef
	Root Fresh Weight (g)				
0.5	0.258 bc	0.231 cd	0.193 ef	0.155 gh	0.135 h
1.0	0.279 ab	0.258 bc	0.214 de	0.169 fg	0.151 gh
2.0	0.294 a	0.268 ab	0.232 cd	0.173 fg	0.158gh
	Root Dry Weight (g)				
0.5	0.0196 c	0.0163 e	0.0116 h	0.0088 k	0.0078 l
1.0	0.0204 b	0.0181 d	0.0143 g	0.0104 h	0.0090 j
2.0	0.0222 a	0.0195 c	0.0157 f	0.0108 h	0.0096 j

(1) For each parameters, means followed by different letters are significantly different according to LSD test (P<0.05).

concentrations, high shoot fresh weight was obtained by the 2 mM concentration of P compared to 0.5 mM P (Table 1). On the other hand, at 0 mM NaCl, 1 and 2 mM P resulted in 2 and 9% increase in fresh weight, also P was effective in reducing the corresponding percent reduction up to 100 mM NaCl (Table 1). Shoot dry weights were negatively affected by each increase in NaCl concentration. For all concentrations of NaCl, P enhanced shoot dry weight (Table 1) and reduce the severity of the corresponding reduction percentages (Table 1). Heaviest and lightest shoot dry weights were obtained at the 0 mM NaCl X 2 mM P and 100 mM NaCl X 0.5 mM P, respectively (Table 1). Moreover, the former combination resulted in 13% increase in dry weight, while the later resulted in 58% percent reduction (Table 1). At 100 mM NaCl, percent reduction in shoot fresh and dry weights was not significantly different among all concentrations of P.

Generally, adventitious root number was negatively affected by NaCl (Table 2). In absence of NaCl, increasing P from 0.5 to 2 mM, increased the number of adventitious roots from 8.9 to 10.9. Within the same concentration of NaCl less than or equal to 75 mM, P significantly increased adventitious root number (Table 2). Presence of 1 and 2 mM P in absence of NaCl gave about 11 and 23% increase in adventitious root number which was reduced to 5 and 12% at 25 mM NaCl (Table 2). At 100 mM NaCl, reduction percentage in adventitious root number was not affected by P concentration applied.

Although the effect of P on adventitious root length, in absence of NaCl, was not significant, adventitious root length at all concentrations of NaCl less than or equal to 50 mM was increased by P treatment (Table 2). In absence of NaCl percent increase in adventitious root length of about 2.5 and 7.61% was noticed in presence of 1 and 2 mM P, respectively (Table 2). At 75 and 100 mM NaCl, no significant effects of P were noticed on adventitious root length reduction percentages (Table 2).

Adventitious root fresh and dry weights were significantly affected by NaCl X P interactions (Table 2). Though adventitious root weights were generally reduced with salinity, they increased by P treatment (Table 2). Adventitious root fresh weight decreased with NaCl concentration up to 75 mM, At all concentrations of NaCl adventitious root dry weights were significantly reduced (Table 2). At 0 NaCl concentration, 1 and 2 mM P produced the heaviest adventitious root fresh weights, while at 2 mM in absence of NaCl, the highest dry weight was observed, the lightest adventitious root fresh weights were obtained by the 100 mM NaCl at all concentrations of P (Table 2), The least dry weight was noticed at the 75 mM NaCl X 0.5 mM P and 100 mM NaCl X 0.5 mM P (Table 2).

As indicated above, at any imposed NaCl concentration, vegetative and adventitious root growths were enhanced with P (Tables 1 and 2). Similar results were reported earlier on other crops [10, 25]. As P increased, less reduction was observed in shoot length, dry weight, adventitious root number and length of

african violet cultured *in vitro* [10]. Foliar spray of KH_2PO_4 mitigated the detrimental effects of high salinity on spinach [25] According to Mohammad *et al*. [26], P enhanced root growth through increasing both root length and surface area of salt stressed hydroponic grown tomato.

Accumulation of ions for osmotic adjustment and restriction of Na and Cl accumulation in immature tomato leaves appear to be involved in P enhancement of salinity tolerance [27]. Moreover, enhancement of rooting by P might be a key factor for whole improvement of shoot growth and dry matter accumulation [10].

NaCl, P and their interaction exerted significant effects on leaf osmotic potential in cucumber microshoots grown on rooting medium (Figure 1). With increasing NaCl concentration, leaf osmotic potential decreased except for the 0 and 25 mM NaCl, where the presence of higher concentration of P in NaCl-enriched medium, resulted in a significantly higher leaf osmotic potential (Figure 1). Osmotic potential was similar for the 100 mM NaCl X 2 mM P and 75 mM NaCl X 2 mM P. As well, the 50 mM NaCl X 2 mM P and the 25 mM NaCl X 2 mM P gave similar osmolarities (Figure 1).

Leaf osmotic potential of microshoots grown on rooting medium was significantly reduced with increased salinity and P had alleviated this adverse effects. Similarly, Shibli *et al.* [2001] reported P to ameliorate the adverse effects of salinity on leaf osmotic potential of *in vitro* grown african violet. Least leaf osmotic potential of NaCl-stressed tomato was observed at the least P concentration [26]. Furthermore, foliar spraying of spinach stressed plants with KH_2PO_4 increased daily water use to concentrations very close to those in the unstressed plants. These results indicate that P treatment is capable of restoring normal cell water relations, negating the negative effects of salinity [25].

Protein and proline contents of cucumber microshoots grown on rooting medium were significantly affected by NaCl, P and their interaction (Figure: 2 and 3). Generally, protein content was adversely affected by the presence of NaCl in the nutrient medium (Figure 2). At 2 mM P, protein content were not affected in presence of 50 and 75 mM NaCl (Figure 2); however, at 0 and 100 mM NaCl the different combinations with P, resulted in almost similar protein content.

Presence of P at a high concentration, decreased both the reduction of protein content and the increments in proline accumulation associated with NaCl stress; this is likely due to the enhancement of leaf osmotic potential (Figure 1) in presence of P. Salt and osmotic

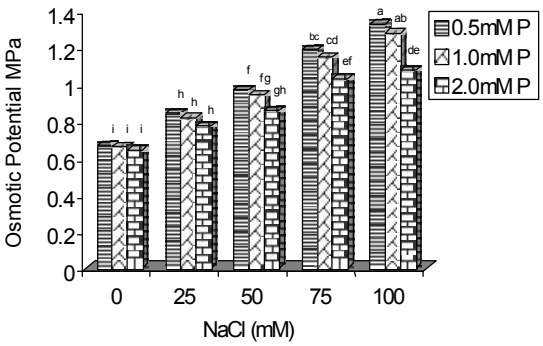

Fig. 1: Interactive effects of P with *in vitro*-induced salinity on leaf osmotic potential of cucumber microshoots grown on rooting medium containing 1mg.l^{-1} IAA. Columns followed by different letters are significantly different according to LSD test, P< 0.05.

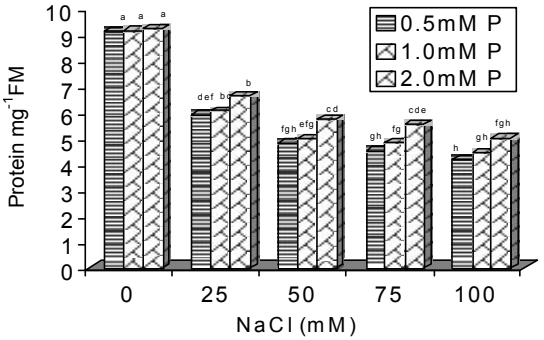

Fig. 2: Interactive effects of P with *in vitro*-induced salinity on protein content of cucumber microshoots grown on rooting medium containing 1mg.l^{-1} IAA. Columns followed by different letters are significantly different according to LSD test, P< 0.05.

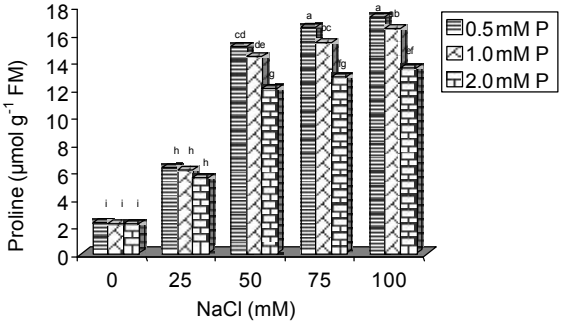

Fig. 3: Interactive effects of P with *in vitro*-induced salinity on proline content of cucumber microshoots grown on rooting medium containing 1mg.l^{-1} IAA. Columns followed by different letters are significantly different according to LSD test, P< 0.05.

stress-induced accumulation of proline has been previously reported in cucumber [14, 15]. Plants exhibit several other mechanisms at the molecular and cellular levels to overcome salt stress conditions and resulting oxidative stresses [8, 28].

Mineral composition of cucumber microshoots grown on rooting medium was significantly affected by NaCl, P and their interaction (Table 3). In general, increasing P within each of 0 and 25 mM NaCl, did not affect Na and Cl contents (Table 3). Otherwise, contents of Na and Cl increased, but contents were significantly higher at the 0.5 mM P than those of the 1 and 2 mM P, at each of this NaCl concentration (Table 3).

Uptake of K was reduced by NaCl-induced salinity. P enhanced K uptake at all concentrations of NaCl and the least K content (21.5 $mg.g^{-1}$ DW) was observed at the 100 mM NaCl in the presence of 0.5 mM P, however at the same concentration of NaCl, 2 mM P resulted in a significantly higher K content compared to 0.5 mM P combination (Table 3). Increasing NaCl concentration caused a marked reduction in Ca content, however, within each concentration of NaCl, increasing P improved Ca uptake, except for the 100 mM NaCl (Table 3). In absence of NaCl, each increase in P concentration resulted in a significant increase in P uptake. Although NaCl-induced salinity decreased P uptake, increasing P concentration within each concentration of NaCl, improved P content up to the 75 mM NaCl (Table 3).

Comparable to responses obtained in cucumber microshoots, Shibli *et al.* [10] using african violet noticed that reduction in P, Ca and K contents with elevated salinity was less as P concentration in the medium increased. Improved Ca and K uptake under salt stress conditions at high P concentrations are possibly due to P enhancement of roots, both number and length, which leads to absorption of more nutrients. Kaya *et al.* [25] also reported that foliar application of KH_2PO_4 on salt-stressed spinach corrected the deficiencies of both P and K and decreased Na content in leaves. They attributed the decrease in Na to the observed increase in biomass production (dilution effects).

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