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Salinity-Induced Changes in Peroxidase Activity and Cell-Wall Polysaccharides in *Vigna*

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We investigated the effect of salinity on the development of seedlings of Vigna unguiculata. At various time intervals, the hypocotyls were measured to estimate the effect of salt concentration on growth parameters. Control plants were tallest and had the greatest fresh weights, whereas these values were lowest in seedlings treated with high levels of salt. Three hydrogen donors -- caffeic acid, ferulic acid, and pyrogalol -- were studied to determine the changes in peroxidase activity for both cytoplasmic and wall-bound fractions. Activity was inversely correlated with hypocotyl elongation. A clear concentration effect was also observed for contents of pectic polysaccharides, low-molecular-weight xyloglucan, and high-molecular-weight xyloglucan, with control seedlings showing lower levels of those wall components than that recorded in the salt-treated seedlings. Here, we also discuss the role of peroxidase and wall components in hypocotyl elongation and growth of *Vigna* when seedlings undergo saline stress.

Keywords: elongation, pectic polysaccharides, peroxidase, salinity, xyloglucan

High salinity is an environmental stress that limits growth and development. Plant responses to excess salt are complex, and involve changes in their morphology, physiology, and metabolism. The majority of plant species are salt-sensitive because they are unable to translocate sufficient Na^+ from the roots to the shoots when the amount of this ion in the roots rises to a level unacceptable to the cytoplasm. This results in overloading of the vacuole and cell damage (Larher et al., 1991). Recent assiduous efforts to increase the salt tolerance of conventional crops, either through selective breeding or genetic manipulation, have not been very successful.

Salt-sensitive plants grow slowly under stress. Their stunted form is associated with water deficits, ion toxicity, nutrient imbalances, and high internal salt concentrations, all leading to altered physiological and biochemical processes (Munns, 1993). However, the underlying mechanism(s) by which salinity inhibits growth is not clearly understood. Crop sensitivity and the degree of injury vary with species, genotype, and growth stage. In general, young seedlings tend to be most salt-sensitive (Gill and Sharma, 1990). Therefore, plants that are better able to grow under saline conditions show certain intrinsic and extrinsic changes during their adaptation.

Peroxidases, a diverse group of heme-containing enzymes, use H_2O_2 as an electron acceptor to oxidize a wide variety of molecules. Widely distributed in plants, these enzymes have been implicated in many physiological processes. This complexity requires the participation of many biochemical circuits. Cell walls play an important role in mediating physiological events. For example, during elongation or expansion, the existing cell-wall architecture must be modified to permit incorporation of new material, thus increasing the surface area of the walls and inducing water uptake by protoplasts. Numerous growth-regulating agents, including hormones and environmental factors, apparently affect water uptake by modifying the ability of the primary cell wall to extend irreversibly.

The survival of plants in adverse environments depends on their ability to withstand the extreme stresses that affect developmental, physiological, and biochemical processes. We must strive to better understand the mechanisms involved in the inhibition of plant growth under saline conditions as well as the means by which some species tolerate salt stress. Such information may help accelerate the introduction of environmental and genetic manipulations aimed at increasing crop salinity resistance. Therefore, the objective of our study was to evaluate the effects of NaCl concentration on two aspects of elongation and growth in *Vigna unguiculata* hypocotyls: 1) peroxidase activity with various hydrogen donors and 2) levels of cell-wall polysaccharides (i.e., pectic polysaccharides, and low- and high-molecular-weight xyloglucans).

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MATERIAL AND METHODS

Seeds of *V. unguiculata* were soaked in tap water for 3 h. They were then thoroughly washed with tap water, followed by three or four rinses with distilled water. Afterward, they were transferred to moistened filter paper and held in the dark for 24 h. Uniformly germinated seeds were then transferred to sieve culture dishes containing nutrient media (Doddema and Telkamp, 1979) and distilled water. Some dishes were kept as the control while others were exposed to two different concentrations of NaCl -- 0.10 M and 0.25 M. (Previous studies had shown that seedlings do not survive at higher concentrations.) The dishes were placed in a light room (ca. 200 mM $m^{-2}s^{-1}$ from six fluorescent tubes at 0.5 m overhead). The time at which these dishes were transferred to the light room was considered the 'zero hour'.

Growth Analysis

At 8-h intervals initially((24 h later on), 20 seedlings from each treatment were selected for growth analysis over the 96-h test period. The seedlings were rinsed with distilled water, then excised into root, hypocotyl, leaf, and cotyledon portions. Root and hypocotyl lengths were measured and the fresh weights (fw) of the roots, hypocotyls, and cotyledons were recorded.

Biochemical Analysis

To estimate the amount of cell-wall polysaccharides, the hypocotyls first were killed by boiling them in methanol for 5 min, then stored in methanol. Chilled hypocotyls were used to determine peroxidase activity.

Extraction of Soluble and Bound Peroxidases

The hypocotyls were homogenized with a pinch of sand in a pre-chilled mortar and pestle, using 0.02 M K-phosphate buffer (pH 6.4). This mixture was centrifuged at 10,000*g* for 10 min. The supernatant was used for estimating soluble peroxidase activity. After the cytoplasmic enzymes were extracted, the residual wall material was thoroughly washed with distilled water and centrifuged until the washings were free of the peroxidase reaction with guaiacol. The wall fraction was then shaken regularly with 10 mL of 1 M NaCl for 1 h at room temperature (RT) to release the ionically bound enzymes. After centrifugation at 10,000*g* for 10 min, the supernatant served as the source of bound enzyme.

Peroxidase Assay

Peroxidase activity was measured by recording changes in absorbance at 400 nm (ΔA_{400}). Various hydrogen donors included ferulic acid, caffeic acid, and pyrogalol. The brown color that developed when these donors were oxidized in the presence of H_2O_2 was spectrophotometrically measured. Our assay mixture consisted of 12 mM K-phosphate buffer (pH 6.4), 4 mM hydrogen donor, enzyme, and 1 mM H_2O_2. Activity was expressed as ΔA_{400} g fw^{-1} min^{-1}.

Extraction of Pectic Polysaccharides

Approximately 500 mg of the methanol-boiled hypocotyls were crushed with a pinch of sterilized sand in ice-cold water, and centrifuged at 10,000*g* for 5 min. Supernatant that contained the cytoplasmic enzymes was then discarded. The pellet was washed 12 to 15 times with distilled water until it was free of cytoplasmic enzymes. Afterward, 10 mL of 1 M NaCl was twice added, for 1 h each time, to remove the wall-bound enzymes. Following centrifugation, the supernatant was discarded and the pellet was washed successively with ice-cold water, acetone, and a 1:1 (v/v) chloroform-methanol mixture. After this was done twice, the pellet was air-dried at RT. The dried pellet was treated with 15 mL of dimethyl sulfoxide for 12 h to dissolve the starch and facilitate the extraction of pectic substances (Wada and Ray, 1978). After the DMSO was removed via centrifugation, the pellet was extracted three times with 20 mM ammonium oxalate - oxalic acid buffer solution (pH 4.0) at 70°C for 1 h to remove the pectic polysaccharides. The supernatants of all three washes were combined and the volume was made up to 20 mL. This served as the source of the pectic polysaccharides (Nishitani and Masuda, 1981).

Extraction of Xyloglucans

To fraction the hemicellulosic xyloglucans, the pectin-free wall pellet was extracted twice (2 h each time) with 4% KOH solution to obtain the low-molecular-weight (LMW) xyloglucans. The residue was then extracted over 24 h with 24% KOH solution to obtain high-molecular-weight (HMW) xyloglucans. Although these alkali extracts were acidified (pH 5.0) with 5% and 33% acetic acid, respectively, this acidification caused no precipitation of the hemicellulosic polysaccharides.

Determination of Total Polysaccharides and Xyloglucan Content

The total polysaccharide content in the pectic fraction was determined according to the phenol sulfuric acid method, and was expressed as ΔA_{490} g fw^{-1}. Afterward, 0.5 mL of the extract was mixed with 0.5 mL of 5% phenol and 2.5 mL of 98% sulfuric acid with constant stirring. After 10 min, the tubes were placed on a water bath at 30 C for 20 min. The yellow-orange color that developed was read at 490 nm. Xyloglucan contents were determined by iodine staining, via the modified method of Kooiman (1960). Briefly, 1.0 mL of the acidified extract was mixed with 0.25 mL of I_2KI solution (0.5% I_2 and 1% KI); the reaction mixture was kept in the dark for 1 h at 4 C. The resulting bluish-green color was read at 640 nm.

RESULTS AND DISCUSSION

Growth

When saline stress was imposed through the nutrient medium, *Vigna* growth was inhibited (Fig. 1). A cubic polynomial was the best fit (r = 0.99) to apply to all three concentration treatments (control, 0.1 M, and 0.25 M). Hypocotyl lengths for the controls steadily increased before stabilizing at 72 h. A similar effect was observed with the 0.1-M concentration, whereas little elongation was seen after 24 h when plants were grown with 0.25 M NaCl. At all measured times, seedlings exposed to the highest salt concentration were shortest; maximum inhibition was seen at 96 h. Lengths of the control seedlings were significantly different from those measured with the salt treatments ($p < 0.001$). This adverse effect produced by high salinity may have reduced growth because of the extra energy expended for osmotic adjustment or the plant's repair system. Salt stress may also have negatively affected metabolic processes, such as the translocation and partitioning of assimilates toward a sink. Reddy et al. (1997) have reported that high NaCl concentrations can increase respiration, thereby reducing the net photosynthetic fixation, a potential causative factor in decreased overall growth for some species. Likewise, inhibited physiological processes may cause lower accumulations of dry biomass. Although ions such as Na^+ are helpful in osmotic adjustment, their excess may lead to ionic toxicity, nutritional and cationic imbalances, and reduced plant growth.

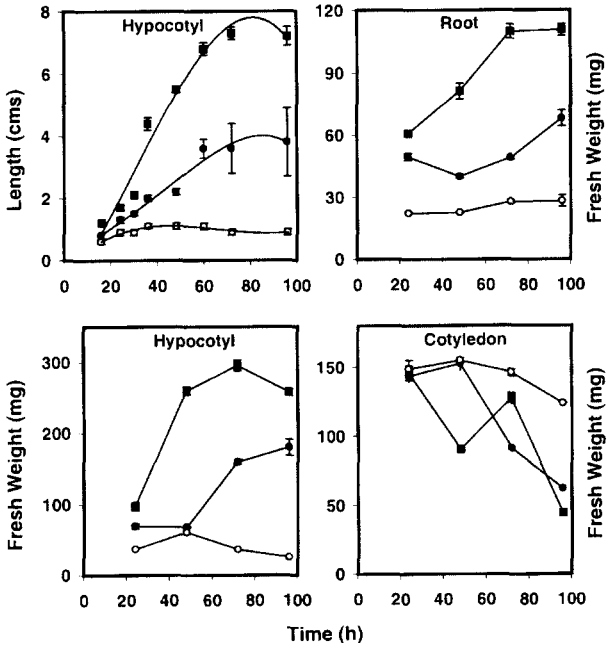

Figure 1. Effect of different concentrations of NaCl on lengths and fresh weights in different organs of *Vigna* seedlings. Distilled water control (■), 0.1-M NaCl (●), and 0.25- M NaCl (○). Vertical bars represent ± standard deviation.

Variations in fresh weight were seen in the roots, hypocotyls, and cotyledons (Fig. 1) - a clear indication of the effect of salt stress in plant organs. The fresh weights of the control roots and hypocotyls steadily increased for 72 h, then stabilized in the former and slightly decreased in the latter. Those seedlings treated with 0.1- M NaCl showed a nearly similar response, whereas fresh weights hardly changed at all from 24 to 96 h with the higher salt concentration. At any given hour, the maximum weights were recorded for the distilled-water controls. The lowest weights were found with the 0.25-M salt-treated seedlings, while those of the 0.1-M treatment being found in between. In contrast, cotyledon fresh weights were highest with the 0.25-M concentration and lowest with the controls.

These results support the contention that, in germinating seeds, major food reserves must be hydrolyzed and transformed in order to be transported and utilized for seedling growth. In fact, Davies and Slack (1981) have shown that the embryonic axis has a direct influence on the breakdown of seed reserves. Therefore, we conclude that high salinity affected metabolic activities and adversely influenced plant growth, with the maximum inhibition being found in our 0.25-M treatment. Under stress conditions, the mobilization of reserves from the endosperm to the developing organs, e.g., shoots and roots, was affected because the plants were utilizing those reserves to

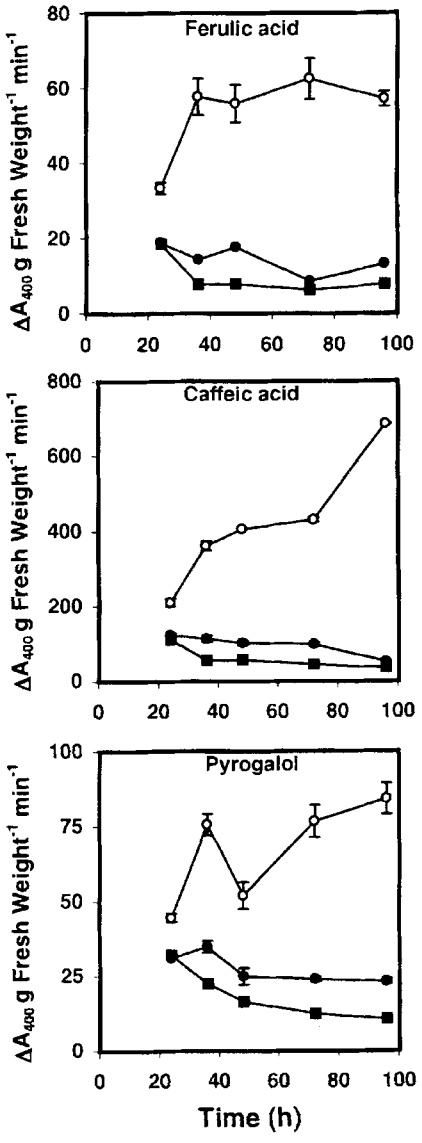

Figure 2. Effect of different concentrations of NaCl on peroxidase activity in hypocotyl cytoplasm of *Vigna*, based on different hydrogen donors. Other details as per Figure 1.

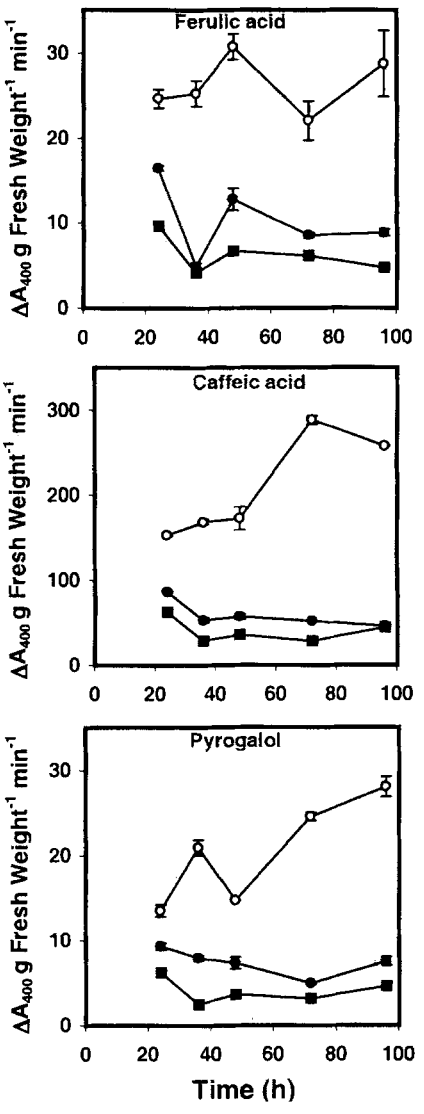

Figure 3. Effect of different concentrations of NaCl on wall-bound peroxidase activity with different hydrogen donors in hypocotyls of *Vigna* seedlings. Other details as per Figure 1.

maintain osmotic balance. Therefore, the poor growth of our *Vigna* plants exposed to saline stress may have been caused by a variety of factors, including low water uptake, high internal salt concentrations, or toxicity.

Peroxidases play a large role in plant responses to stress conditions (Sherf and Kolattuudy, 1993). Because peroxidase isozymes are involved in cell-wall structure and expansion (Brett and Waldron, 1990), we measured the changes in cytoplasmic peroxidase activity using three hydrogen donors (Fig. 2). Maximum activity was found for caffeic acid; the minimum were detected with ferulic acid and pyrogalol. Activities of all three donors were highest in the presence of 25-M NaCl, and lowest for the distilled-water control. Moreover, hypocotyl length was inversely correlated with peroxidase activity. Venketesan and Chellapan (1999) have also reported that peroxidase activity is stimulated in plants under extreme saline conditions, with levels being two-fold higher than that measured in control plants.

Ionically wall-bound peroxidase activity also changed with the three hydrogen donors (Fig. 3), with the trend being similar to that observed for the cytoplasmic enzyme. Maximum activity was found with caffeic acid, and was 10 times greater than that calculated for ferulic acid and pyrogalol. Again, a salt concentra-

tion of 0.25-M prompted the maximum activity at all growth stages, the greatest difference among treatments being detected at 96 h. Peroxidase activity may be related to the maintenance of cell-membrane integrity when H_2O_2 is detoxified to water, thereby modifying the effect of free radicals under stress (Levitt, 1980). The rise in activity in our present study, which may have been the result of either increased enzyme synthesis or decreased degradation, might prove useful when plants are adapting to conditions under which peroxidation of the membrane lipids must be prevented (Kalier et al., 1984). The induction of peroxidase under saline conditions has also been reported in *Distichlis spicata* (Danies and Gould, 1995) and *Suaeda nudiflora* MoQ (Cherian and Reddy, 2000).

Cell walls are an important component in plants, not only because they comprise a large proportion of the cell biomass, but also because their metabolism aids in determining wall extensibility for cell enlargement (Zhong and Lauchi, 1998). In our study, pectic polysaccharides and LWM and HMW xyloglucans were quantitatively determined in the control and stressed plants (Fig. 4). Maximum pectic-polysaccharide contents were detected in the seedlings treated with 0.25-M NaCl, followed by those grown with the 0.1-M concentration. Hypocotyl elongation was inversely correlated with these measured contents. The analysis of variance showed significant differences between the pectic - polysaccharide contents of the control and the treated seedlings ($p < 0.001$).

Plants that are able to survive in stressed environments do so because of their ability to express preexisting defense mechanisms or adjustments. Under stress conditions, their adaptive response is elicited mainly through changes in the endogenous levels and balance of phytohormones. Re-establishing hormonal equilibrium in this new environment probably plays a central role in their survival (Amzallag and Lerner, 1995). However, under severe stress, plants occasionally fail to adapt, probably because of high catabolism. We also observed that the pectic-polysaccharide content was inversely correlated with hypocotyl length; growth was arrested in the presence of NaCl. This relationship has also been reported in *Phaseolus* (Bagatharia and Chanda, 1998), further supporting the theory that pectic polysaccharides are involved in cell-wall loosening.

The amount of LWM xyloglucan was almost three times higher than that of the HMW xyloglucan (Fig. 4). Content of the former was at its maximum after 16 h in all treatments, then steadily and sharply decreased to a low level that was maintained between 72 h and 96 h. At all stages of growth, the 0.25-M treated seedlings had the highest level of LWM xyloglucan, followed by those grown with 0.1-M NaCl. An inverse correlation was found with hypocotyl elongation, and measured levels of HMW xyloglucan followed a parallel trend. Fry (1989) and Gasper et al. (1991) have demonstrated that auxin-induced cell enlargement accompanies xyloglucan degradation and solubilization, and that hypocotyl elongation is inversely correlated with xyloglucan content. In our study, growth was inhibited in the presence of NaCl, and xyloglucan content was greatest in the seedlings treated with the highest salt concentration.

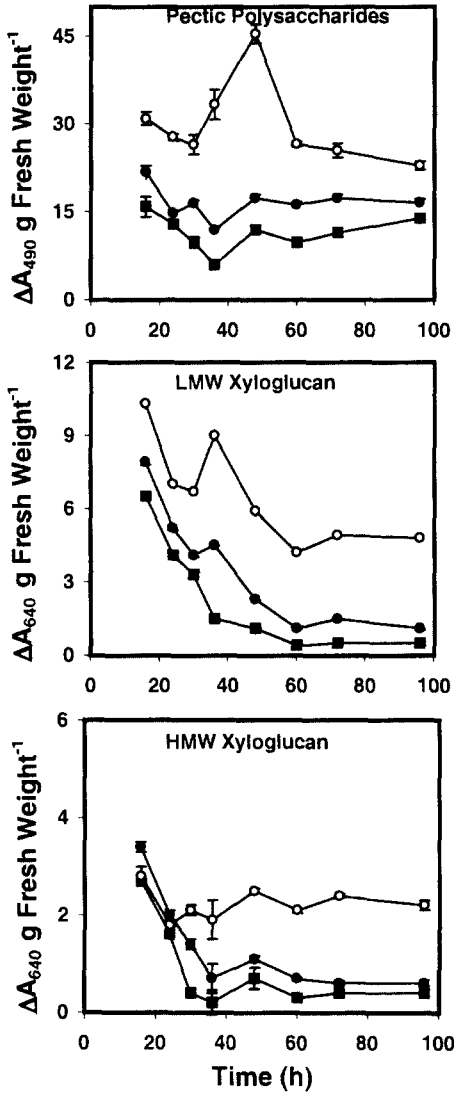

Figure 4. Effect of different concentrations of NaCl on contents of pectic polysaccharides, and low-molecular-weight (LMW) and high-molecular-weight (HMW) xyloglucans in *Vigna* seedlings. Other details as per Figure 1.

The ability of plants to grow under high salinity is a

complex phenomenon requiring mechanisms that operate at the gene, membrane, cell, tissue, and whole-plant level. Based on the results reported here, we conclude that peroxidases play several important roles in response to salt stress: 1) providing resistance to the formation of H_2O_2; 2) protecting cells from harmful concentrations of hydro peroxides; and 3) protecting cell membranes against active oxidants. Saline stress affected hypocotyl elongation in our *Vigna* seedlings, with higher NaCl concentrations causing maximum inhibition and promoting greater accumulations of pectic polysaccharides and xyloglucan. We also believe that the degradation of cell-wall components is involved in cell-wall loosening and, in turn, reduced plant development.

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