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CHANGES IN ARGININE, PAL ACTIVITY, AND NEMATODE BEHAVIOR IN SALINITY-STRESSED CITRUS

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Key Word Index—*Citrus limon*; Rutaceae; PAL; arginine; *Tylenchulus semipenetrans*; phenolics; defense.

Abstract—*De novo* arginine biosynthesis has been described as a response of citrus to a range of stresses. It is often noted that stress in plants enhances susceptibility to herbivory and pathogenic attack. Using a citrus and nematode (*Tylenchulus semipenetrans*) system, the effects of salinity stress on nematode behavior, amino acids (particularly arginine), and phenylalanine ammonia lyase (PAL) activity was investigated. The hypothesis was tested that under salinity stress, citrus grows more slowly and produces arginine in response to high levels of *in vivo* ammonia, resulting in lower PAL activity and increased susceptibility to nematode attack. After 30 days of high salinity (0.1 M NaCl), plants exhibited a 38% reduction in growth, 35% reduction in PAL activity, and had 54% higher infection rates. PAL activity was inversely correlated ($P \leqslant 0.05$) with salinity level and with increase in arginine concentration.

INTRODUCTION

Phenolic compounds in plants arise from cinnamate produced by the deaminase action of the enzyme phenylalanine ammonia lyase (PAL, EC 4.3.1.5). Regulation of PAL channels phenylalanine either into proteins (low activity) or phenolics (high activity). Activity is controlled in some cases by substrate availability [1–3]. The glycolytic intermediate, phosphoenol-pyruvate, is the precursor for both phenolics and amino acids [3, 4]. Decline in the organic acids of carbohydrate catabolism (an indication of demand) has been demonstrated in stressed plants with high ammonia nitrogen that are synthesizing amino acids [4]. Rabe and Lovatt [5, 6] have shown *de novo* arginine biosynthesis as a response to stress-induced ammonia intoxication in plant tissue. The metabolic branch-point between arginine and phenolic biosynthesis suggests that high demand for arginine in stress may negatively affect availability of metabolic precursors for phenolic defense.

Three frequently reported responses of plants to stress are slowed growth, increased tissue levels of free amino acids, and enhanced susceptibility to herbivory and pathogenic attack [7–9]. Stress conditions causing slowed growth may result in ammonia accumulation in plant tissue, especially in "nitrogen-amended" situations that occur in agriculture [6]. Phenypropanoid metabolism also generates ammonia that is recycled in plant tissue [10]. Citrus responds with an arginine increase in numerous stress situations [11,12]. Mashela *et al* [13] have demonstrated increased susceptibility of salinity-stressed citrus to attack by the citrus nematode (*Tylenchulus semipenetrans*).

We undertook this study to determine the effect of salinity-stress on plant defense and growth of citrus. Using PAL activity as a putative measure of commitment to chemical defense, we tested the hypothesis that as salinity stress induced slowed growth and increased arginine levels, PAL activity would be diminished, thus creating a more favorable rhizosphere environment for attraction and infection of the nematode to the citrus root. Other amino acids were quantitated to note any shifts in their distribution.

RESULTS AND DISCUSSION

Effects of salinity on citrus growth and nematode behavior.

After 30 days of stress, plant heights under salinity treatments (0.10 M and 0.025 M Na Cl) were sig-

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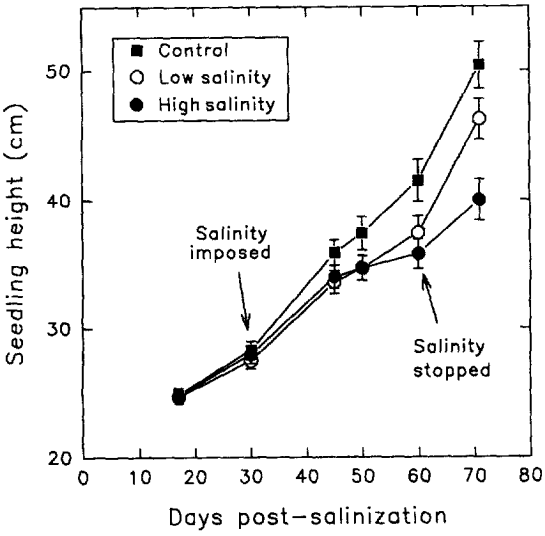

Fig. 1. Growth responses of citrus (Volkamer lemon) to 2 rates of salinity over time; low salinity, 0.025 M NaCl; high salinity, 0.1 M NaCl.

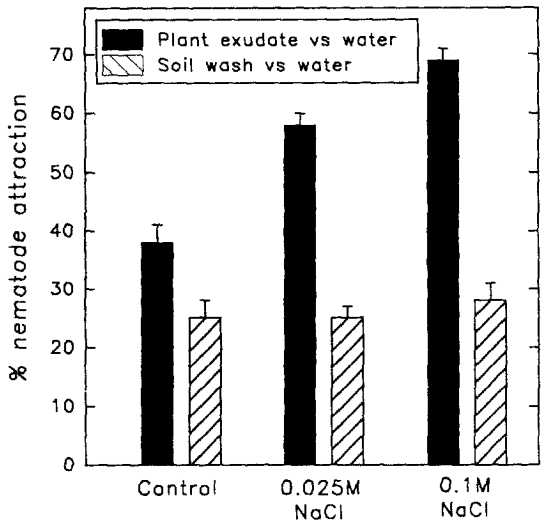

Fig. 2. Nematode bioassay: 50% denotes no preference; <50% repellency; >50% attraction; Assay was in sand using composites of 5 each treatment rhizosphere soil exudates or soil-only washes.

nificantly lower than those of controls. There was a steady decrease in growth throughout the time course of the experiment (Fig. 1). Accompanying an overall 38% reduction in plant height, root and shoot wet weights were reduced 14% and 27%, respectively.

The burden of salinity for plants is osmotic retention of water by the soil and, thus, decreased accessibility to plants. Ion-induced changes in membrane permeability and nutrient uptake also occur [14]. Growth processes are especially sensitive to the effects of salt, providing reliable criteria for assessing degree of stress [15].

At 30 days after salinity imposition, preference for root exudates by the citrus nematode in a sand assay, correlated with the salinity (Fig. 2). Factors affecting plant growth can change exudate composition. For example, increased "leakiness" of roots in stressed plants is frequently reported [16]. Although calcium, an important factor in the maintenance of membrane integrity and ion transport regulation [17], was included in the salinity treatments to mitigate possible ionic effects on cell leakage, high salinity treated root exudates, nonetheless, showed increased nitrate, potassium, and chloride (186, 133, and 209%, respectively) possibly due to decreased uptake or exclusion by the roots. Low salinity treatment produced no significant nutrient leakage.

Nematode inoculations were added to the salinity-stressed/leached seedlings at the 30 day salinity point in order to take advantage of slowed growth and exudate attractiveness (indicators of optimum stress). Final nematode infections at 6 months post-inoculation showed a dose response with 54% higher females per gram of root tissue in salinity-treated plants (data not shown), but no differences in counts of nematodes in soil, indicating successful colonization of the roots. Infection data were not significant; however, this effect has been demonstrated previously [13]. Although constant salinity in soil reduces nematode populations in most cases, salt stress followed by leaching mimics the intermittent drying wetting cycles of field conditions, and has been found to predispose citrus to higher infections by the citrus nematode [13]. Nematode dose responses, in both attraction to the root exudates and successful colonization, suggest a break down in chemical defense.

Effect of salinity on arginine concentration and PAL activity

Arginine as a percent of total amino acids in citrus feeder root tissue increased significantly (30 days salinity) only at the higher 0.1 M Na Cl (Table 1, Fig. 3). Since there was a strong dose response of nematode attraction to exudates (Fig. 2), arginine is not likely to be the sole factor responsible for the changes in nematode responses to the roots. The arginine responses here are consistent with previous work [5] where stress-induced arginine in citrus peaks at 4–6 weeks, more slowly than in herbaceous annuals which can respond in as little as 7–10 days. Rabe and Lovatt [18] reported arginine accumulation in phosphorus deficient citrus due to increased *de novo* synthesis rather than to reduced catabolism or increased protein degradation, and correlated this with ammonia accumulation. This is consistent with the hypothesis that stress-induced *de novo* arginine biosynthesis provides a mechanism for detoxification of excess tissue ammonia [19].

PAL activity of the salinity-stressed feeder roots of

Table 1. Effect of salinity on arginine concentration and PAL activity in Volkamer lemon seedling feeder roots after 30 days 0.1 M Na Cl

Salinity dose	Feeder root responses	
	Arginine concentration (% total amino acid)	PAL activity* (nKat/100 mg protein)
Control	3.6 (±0.65)[a]	115 (±8.7)
0.025 M Na Cl	3.6 (±0.84)[a]	101 (±9.2)
0.10 M Na Cl	7.5 (±1.0)[b]	75 (±15.9)

Data are means of 5 replications, means in a column with the same letters are not different ($P \leq 0.05$).

*Correlation between salinity dose and PAL = 0.52 ($P \leq 0.05$).

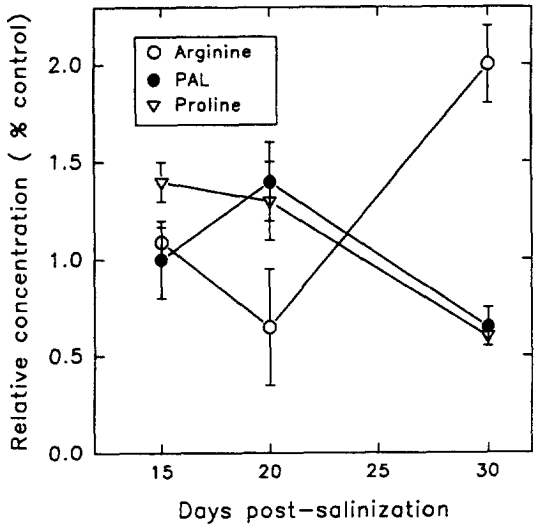

Fig. 3. Relative changes in PAL activity, arginine and proline over time at high salinity (0.1 M Na Cl). (Tissue extraction for PAL was acetone powder from days 15 and 20, fresh tissue for day 30).

citrus (at 30 days salinity) decreased relative to controls in a significant correlation with salt concentration (activity = 69.2–9.4 concentration; $r^2 = 0.267$; $P < 0.05$) (Table 1). The inverse relationship between PAL activity and attraction of nematodes to root exudates suggests a PAL-related decrease in resistance.

An inverse correlation ($P \leq 0.01$) between PAL activity and arginine concentration was observed when relative changes in PAL and arginine over time (15, 20, and 30 days salinity) were assessed (Fig. 3). Values as percent of controls were used to compensate for growth changes and differences in extraction method. Since PAL is sensitive to the physiological state of the plant [20], the metabolic position of this enzyme, relative to amino acid synthesis, makes it a reasonable hypothesis that *de novo* arginine biosynthesis could affect PAL activity. At 30 days salinity, however, total phenolics (as gallic acid equivalents) for root tissue showed no differences (7.3, 7.4, and 7.9% (w/w) for controls, 0.025 M Na Cl, and 0.1 M Na Cl treatments, respectively). Total phenolics in exudates likewise were not significantly changed for the same 3 treatments. Measurable decreases in PAL are not always reflected in a decrease in total phenolics [21, 22], although inhibition of PAL concomitant with decreased phenolic defense and increased susceptibility to disease is well-documented [23, 24]. The broad range of potential end products of the PAL pathway as well as the possible presence of substances which interfere with the Folin's test (sugars, nucleic acid fragments and the readily oxidized substances, ascorbic acid, sulfur dioxide, or ferrous ions) may have prevented detection of changes in phenolics [25]. The differential accumulation of the various aromatic pathway end products at different subcellular sites (lignins in cell wall; flavonoids in vacuoles; coumarin derivatives on epidermal cell surfaces) and thus potential differences in extraction efficiency and reactivity could explain this as well [26, 27]. Citrus roots produce a range of bioactive phenolic compounds, including coumarins, flavonoids, alkaloids, flavones, and sesquiterpenes [28–30]. Future work should investigate changes in specific families of compounds and tissue specific compartmentation.

Proline, often reported as a stress-metabolite, also increased with salt stress (14–50% of total amino acids, data not shown), supporting its well-documented role in stressed plants where it acts as an osmoticum and increases protein solvation [31, 32]. A maximum increase occurred 15 days after stress and then decreased as PAL activity decreased and arginine concentration increased (Fig. 3). Arginine is converted to proline [33]. Our data suggest that arginine may have some metabolic priority in this scenario.

A slight increase in ammonia (0.1 M salinity) (30 days) was noted, however, due to insufficient plant material, roots stabilized for ammonia analysis were not available. There were no other significant differences in either proteins, total amino acids or individual amino acids.

In summary, the data presented here show that arginine peaks in response to salinity in citrus. This arginine increase corresponds to a decrease in PAL activity indicating possible competition between the two metabolic pathways. These metabolic changes are accompanied by changes in the root rhizosphere that produce exudates highly attractive to the citrus nematode. This may be due to a qualitative change in phenolic defense compounds, nutrient leakage of roots due to salinity, some other unknown change in the rhizosphere, or a combination of factors. If *de novo* arginine biosynthesis is a means of detoxifying nitrogen excesses during stress and is an "a priori" path-

way, chemical defenses could be compromised due to affects on PAL activity.

EXPERIMENTAL

Salinity time course

Three month-old Volkamer lemon citrus seedlings were potted into 75% Candler sand and 25% Canadian sphagnum in 10 cm clay pots, watered daily, and fertilized weekly using Peters Professional 20-10-20 (N-P-K), 1.5 g-L, 100 ml/plant. After 4 weeks, the irrigation water was salinized to 0.025 M or 0.10 M NaCl, (plus 3 mM $CaCl_2$ added to lessen ionic effects). Each plant received 100 ml every 1–3 days as needed [13]. Plant heights were measured 15, 20, and 30 days after salinity treatment began and 10 days after salinity cessation and leaching of salts. Five replicate plants were randomly selected from each treatment for chemical analyses and exudate bioassays on these same days. Pots were leached of salts by adding 1 L water in four 250 ml increments, repeated 3 times at 12 hr intervals. Electrical conductivity after leaching and before exuding was near that of tap water. Exudates were collected 24 hr after pots were leached.

Exudates and soil washes

Plants were gently removed from pots and all soil except that within 0.5–1.0 cm of root surfaces worked away. Remaining rhizosphere soil was shaken free from the roots and 50 cc was equilibrated to 30% w/w moisture and stored at 5–10°C overnight. Exudates were removed from soils by centrifugation (2700 RPM, 10 min), filtered through a 0.45 μm nylon filter, and frozen. Washes from soil-only control pots were similarly extracted. Treatments, controls, and soil washes were run against water to prevent an inflation of effect due to the repellent nature of soil washes and non-stressed root exudates. Nutrient analysis of exudates (for Cl^-, NO_3^+, PO_4^{-3}, SO_4^{-2}, NH_3 and K^+) was done with an automated Dionex-300 series chromatography system, using an AS-4A column for anions and a CS-12 column for cations [34].

Nematode bioassay

Procedures of Duncan and Abou-Setta were followed [35]. Acid-washed Candler sand (6% moisture) was placed into standard (100 × 15 mm) petri dishes. 300 μl aliquots of each of the test solutions (e.g., exudates vs. water) were placed alternately around the dish at 3 equidistant points. Ca. 3000 juveniles and males of *Tylenchulus semipenetrans* in 300 μl H_2O were pipetted onto the sand at the dish center. Composites of exudates from 5 plants or 5 soil washes vs. water were compared in 8 replicate petri plate units. Units were kept in the dark for 48 hr at 25°C after which time 1.5 cm diameter cones of treatment sites were removed with a cork borer, combined with 15 ml water, and the nematodes counted.

Amino acids and phenolics

Feeder roots from 5 replicate plants were ground in liquid nitrogen. Root were extracted 3X in 2 ml methanol:chloroform:water 12:5:1 in a rotating mixer (with sand as an abrasive), and phase separated 1 hr using 1 ml chloroform and 1.5 ml water [36]. After centrifugation for 5 min at 3000 RPM and separation, the aqueous phase was dried, and samples were resuspended in 1 ml water and analyzed. Amino acids were analyzed with a Beckman system 6300 high performance analyzer (ion exchange separation with post-column derivatization with ninhydrin). Column used was a Beckman high performance column # 338073 [37].

Total phenolics were quantified using 200 μl of the amino acid extract, 1 ml 1:10 diluted Folin's Ciocalteau Reagent, and 800 μl sodium carbonate (75 g/L). After heating 1 hr at 50°C, absorbance was measured at 765 μl on an LKB Biochrom Ultraspec II spectrophotometer [25]. Gallic acid was used as a standard. 200 μl of exudates were also analyzed for phenolics.

PAL extraction and assay

Assays were performed with either acetone powder [38] or fresh frozen tissue [39] (stored at −80°C) extracted with 0.1 M sodium borate buffer pH 8.8 (SBB). Acetone powders were made with 4 g fresh tissue frozen at −80°C, ground in liquid nitrogen in a mortar and pestle, then homogenized 2X in 15 ml ice cold acetone for 30 sec. This was vacuum-filtered, air dried, then sifted through a 100 mesh sieve and stored at −15°C. Fresh tissue was frozen at −80°C, ground in liquid nitrogen in a mortar and pestle, then stored at −80°C till used. For the assay, a 1.5 g fresh tissue equivalent of acetone powder or frozen tissue was homogenized 30 sec in 15 ml SBB (0.1 M pH = 8.8) with 10 mM β-mercapto-ethanol and kept on ice for 30–60 min. The tissue suspension was centrifuged (12,000 RPMs) at 0°C, and the supernatant was used in the assay. Assay conditions were: 3 ml sample extract, 5 ml SBB, and 2 ml of SBB for blanks or 2 ml SBB plus 0.05 M L-phenylalanine for the reaction. Cold reaction tubes were placed in a 40° water bath and absorbance at 290 μm was read at 15, 30, 45, 60, and 90 min. Activity values were based on 90 min minus 15 min absorbance readings. Commercially purchased PAL (from *Rodotorula glutinis*) was used to control for activity under these assay conditions. Proteins were quantified with 50 μl of the PAL extract using a Coomassie R. Protein assay kit with bovine serum albumin as standard. Absorbance was read at 495 μm [40].

Nematode inoculations

After 30 days of salinity, plants were leached as for exudate recovery and inoculated with *Tylenchulus semipenetrans*. Inoculum was collected from infested trees in Bartow, FL. Nematodes were scrubbed from roots, treated with $CuSO_4$ (1000 PPM, 30 min) to sanitize them, and added to the pots. Non-nematode filtrate was added to the controls. Inoculum totaled 500,000 eggs and 235,000 juveniles and males per pot. Infections were quantified at 6 months post-inoculation by blender extraction of females from roots [41]. Juvenile nematodes were recovered from soil using Baerman funnels in which nematodes migrate through a filter from soil into water [42].

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