SEPARATION OF THE EFFECTS OF DROUGHT AND INFECTION BY *PHYTOPHTHORA CINNAMOMI* ON 'HASS' AVOCADO

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Abstract

Water-deficit stress resulted in a statistically significant increase in the NH_3-NH_4^+ content of leaves of 'Hass' avocado scions on Mexican seedling or clonal 'Duke 7' rootstocks, 6 months from budding, when xylem pressure potentials were less than -1.8 MPa. Three months after inoculation, recovery of *Phytophthora* from well-watered Mexican seedling and clonal 'Duke 7' rootstocks was 60 and 40%, respectively. Xylem pressure potentials and leaf NH_3-NH_4^+ concentrations of these trees were the same as those of the well-watered, uninoculated (control) trees. Water-deficit stress reduced the recovery of *P. cinnamomi* 20 and 50% for Mexican seedling and clonal 'Duke 7' rootstocks, respectively. For these trees, root infection by *P. cinnamomi* did not increase the severity of water-deficit stress nor the accumulation of NH_3-NH_4^+ in the scions. The results provide evidence that root infection by *P. cinnamomi* does not cause increased NH_3-NH_4^+ production by a direct action of the pathogen on the metabolism of the roots prior to pathogen-induced water-deficit stress. In detached leaves of the 'Hass' avocado, NH_3-NH_4^+ accumulation stimulated ethylene biosynthesis. Evidence is presented suggesting that leaf damage, in part, results from an interaction of ethylene with NH_3-NH_4^+.

1. Introduction

In the field, 'Hass' avocado trees (*Persea americana* Mill.) in the early stages of infection with *Phytophthora cinnamomi* (Rands) exhibit symptoms similar to water-deficit stress. Consequently, growers often increase irrigation resulting in over-watering and increased *P. cinnamomi* infection. Previous research (Nevin and Lovatt, 1987) demonstrated that water-deficit stress caused the accumulation of toxic levels of NH_3-NH_4^+ in the leaves of 'Hass' avocado, resulting in dieback of young shoot tips, browning of the leaf tip and margin, and leaf abscission. When these symptoms are the result of root infection by *P. cinnamomi*, it is not known: (i) if they are also due to the accumulation of toxic levels of NH_3-NH_4^+ and (ii) whether they are caused by *P. cinnamomi*-induced water-deficit stress, or a direct action of the pathogen on nitrogen metabolism in the roots. The accumulation of free ammonia (NH_3) in leaves of plants infected by fungi or bacteria is well-known (Farkas and Kiraly, 1961; Lamar *et al.*, 1969; Durbin, 1971; Goodman, 1972; Sadler and Scott, 1974; Bashan *et al.*, 1980; Turner, 1981; Novacky and Ullrich-Eberius, 1982).

In the present study, *P. cinnamomi* infected and uninfected (control) trees of 'Hass' avocado on *P. cinnamomi*-sensitive Mexican seedling rootstocks and *P. cinnamomi*-tolerant clonal 'Duke 7' rootstocks were maintained under well-watered (control) conditions or subjected to water-deficit stress. Early

differences in the metabolic response of the trees to root infection by *P. cinnamomi* and water-deficit stress were discerned, and the biochemical basis for the symptoms was identified in each case.

2. Materials and Methods

'Hass' avocado scions of uniform height and leaf number on clonal 'Duke 7' and Mexican seedling rootstocks *Persea americana* Mill. (80 trees each), 6 months from budding, maintained in a glasshouse at 30 ± 1°C were subjected to four treatments (20 trees per treatment): (1) well-watered (100% daily water use) and uninoculated with *P. cinnamomi*; (2) well-watered and inoculated with *P. cinnamomi*; (3) water-deficit stress (water withheld) and uninoculated with *P. cinnamomi*; and (4) water-deficit stress and inoculated with *P. cinnamomi*. Half the trees were harvested at the end of 4 weeks and the remainder at the end of 8 weeks from the initiation of water-deficit stress to identify the earliest difference in metabolic response between treatments. Inoculation preceded the initiation of water-deficit stress by 2 months. For each harvest, the following parameters were determined: (1) root infection by *P. cinnamomi*; (2) tree water status; (3) tree growth; (4) leaf NH_3-NH_4^+ content; (5) leaf proline content; (6) rate of arginine biosynthesis *de novo* by leaves; and (7) rate of ethylene biosynthesis by leaves.

2.1. *P. cinnamomi*

White millet seed was boiled 30 to 40 minutes to remove the seed coat. 750 cc of boiled millet seed was mixed with 150 ml of Campbell's V-8 juice and 100 cc of University of California Soil Mix (UC Mix) and autoclaved two times 24 hours apart. The cooled medium was maintained under asceptic conditions, inoculated with an avocado isolate of *P. cinnamomi*, incubated 10 days at room temperature, and then used as inoculum. Fifty ml of inoculum was mixed with 40 liters of UC Mix to give 10 to 20 propagules per liter of soil. Each tree was potted in 2 liters of soil. Six weeks after inoculation, the trees were immersed in water to the top of the soil for 24 hours.

Infection was determined in sections of roots surface sterilized for 1 minute in 10% bleach solution containing one drop detergent per 100 ml. Twenty root sections per tree were asceptically transferred to two plates of PARH medium (Tsao and Ocana, 1969; Masago *et al.*, 1977), incubated in the dark at room temperature for 4 days, and checked for *P. cinnamomi* daily for a total of 8 days. Plates still negative at the end of 8 days, were assumed to be negative.

2.2. Tree physiology

Xylem pressure potential was measured using a Scholander pressure chamber.

Tree height from the bud union, number of branches, and number nodes per shoot were recorded.

Leaf NH_3-NH_4^+ content was determined in an aliquot (1 g fr wt.) of young, fully expanded leaves homogenized in 5 ml 10% TCA using a Polytron tissue homogenizer (PCU-2, Brinkman Instruments) at speed 6. The probe was rinsed with 5 ml 10% TCA, which was added to the homogenate. The homogenate was centrifuged at 10 000*g* at 2°C for 10 minutes. The NH_4^+ content of the acid-soluble supernatant fraction, containing the combined pool of NH_3-NH_4^+ as NH_4^+, was determined using a Wescan Ammonia Analyzer (Carlson, 1978). The assay was linear for NH_4^+ concentrations from 0 to 100 µg per ml. Samples were diluted to give values in this range.

Leaf proline content was determined in an aliquot (500 mg fresh wt.) of young, fully expanded leaves frozen in 5 ml 3% aqueous 5-sulfosalicylic acid for at least 24 hours. Free proline was extracted by shaking for 2 hours at 30°C. One ml of extract was transferred to 1 ml glacial acetic acid and 1 ml acid ninhydrin (2.5 g ninhydrin, 60 ml glacial acetic acid, 40 ml 6 M H_3PO_4) and incubated for 1 hour at 100°C. The reaction was terminated by transferring the sample to an ice bath. The chromophore was extracted with 4 ml toluene and absorbance was read at 520 nm after 20 minutes (Levy, 1980). Proline content was linear from 2.5 to 8.0 µg proline per ml. Samples were diluted before reaction with acid ninhydrin to give values within this range.

Activity of the *de novo* arginine biosynthetic pathway was assessed in the intact cells of young, fully expanded leaves (500 mg fresh wt.) pooled for each treatment by measuring the incorporation of radiolabeled carbon supplied as $NaH^{14}CO_3$ (5 mM, 37.5 µCi) into arginine and urea during a 3-hour incubation period in Shive's nutrient solution. The amounts of [guanido-^{14}C]arginine and [^{14}C]urea synthesized from $NaH^{14}CO_3$ by the leaves were determined using commercial arginase and urease as described previously by Lovatt and Cheng (1984).

Ethylene production was determined hourly on 1-ml samples taken from 930-ml jars containing five leaves using a Varian Aerograph model 1440 flame ionization gas chromatograph equipped with a 2 m x 3.2 mm column packed with 60 to 80 mesh activated alumina. The carrier was N_2 at a flow rate of 50 ml per minute. Temperature of the injector, column, and detector were 80, 90, and 180°C, respectively (Eaks, 1980). At each sampling, the column was calibrated with 1 ml samples of a standard ethylene-nitrogen mixture.

3. Results

Well-watered 'Hass' avocado trees inoculated with *P. cinnamomi* had a higher recovery rate of the fungus than trees subjected to water-deficit stress (Table 1). Mexican seedling rootstocks exhibited a greater incidence of recovery than the clonal 'Duke 7' rootstocks. Roots of uninoculated trees were infection-free at both harvests. Uninoculated 'Hass' avocado trees on Mexican seedling rootstocks subjected to water-deficit stress exhibited significantly lower xylem pressure potentials than trees with roots that were infected with *P. cinnamomi*. For trees on clonal 'Duke 7' rootstocks, the presence or absence of root infection by *P. cinnamomi* did not influence the severity of the water-deficit stress treatment. Plant water status of the well-watered 'Hass' avocado trees on either Mexican seedling or clonal 'Duke 7' rootstocks was not affected by root infection by *P. cinnamomi* at harvest 1 (Table 2) or at harvest 2. Thus, only the results for harvest 1 are reported in this communication.

Table 1 - Recovery rate of *Phytophthora cinnamomi* (%).[z]

	Inoculated with *P. cinnamomi*			
	Yes		No	
Rootstock	$+H_2O$	$-H_2O$	$+H_2O$	$-H_2O$
Mexican	60	50	0	0
Duke 7	40	20	0	0

[z] Harvest 1 - One month after the initiation of the water-deficit stress treatment. Trees were inoculated 2 months before the initiation of the water-deficit stress treatment.

Table 2 - Tree water status (MPa).[z]

	Infected with *P. cinnamomi*			
	Yes		No	
Rootstock	$+H_2O$	$-H_2O$	$+H_2O$	$-H_2O$
Mexican	-0.6 a	-1.6 b	-0.6 a	-2.6 c
Duke 7	-0.7 a	-1.8 b	-0.4 a	-2.0 b

[z] Harvest 1 - One month after the initiation of the water-deficit stress treatment. Trees were inoculated 2 months before the initiation of the water-deficit stress treatment. Values followed by different letters are significantly different at $p < 0.05$.

Neither root infection by *P. cinnamomi* or water-deficit stress had a statistically significant effect on growth of 'Hass' avocado scions on Mexican seedling or clonal 'Duke 7' rootstocks (Table 3). Despite the fact that statistically significant differences in tree height, number of branches, and number of nodes per branch were not obtained, the effects of the treatments were easily discerned visually even at the first harvest. Uninoculated 'Hass' avocado trees on both Mexican seedling or clonal 'Duke 7' rootstocks subjected to water-deficit stress exhibited the most damage, followed by water-deficit stressed trees with roots infected by *P. cinnamomi*. Only the trees subjected to water-deficit stress exhibited wilting, epinasty, leaf tip burn and leaf abscission. In each of these cases, 'Hass' on Mexican seedling rootstocks exhibited more intense symptoms than 'Hass' on clonal 'Duke 7'. Well-watered, uninoculated (control) trees were healthy.

Table 3 - Effect of root infection by *Phytophthora cinnamomi* and water-deficit stress on tree height (cm).[z]

	Infected with *P. cinnamomi*			
	Yes		No	
Rootstock	$+H_2O$	$-H_2O$	$+H_2O$	$-H_2O$
Mexican	34	30	35	25
Duke 7	30	29	27	25

[z] Harvest 1 - One month after the initiation of the water-deficit stress treatment. Trees were inoculated 2 months before the initiation of the water-deficit stress treatment. Data are not significantly different at $p < 0.05$.

An increase in NH_3-NH_4^+ in leaves of 'Hass' avocado scions on either rootstock was an early and statistically significant symptom of water-deficit stress, but not of root infection by *P. cinnamomi* (Table 4). Since root infection by *P. cinnamomi* did not significantly reduce xylem pressure potential at the first harvest, the results clearly support that NH_3-NH_4^+ accumulation is a response to water-deficit stress and that NH_3-NH_4^+ does not accumulate as a result of direct action of *P. cinnamomi* on root metabolism prior to pathogen-induced water-deficit stress.

Water-deficit stress caused a reduction in the activity of the pathway for *de novo* biosynthesis of arginine in intact leaf tissue coincident to the accumulation of NH_3-NH_4^+ (Table 5). The *de novo* arginine biosynthetic pathway is involved in the removal of ammonia to prevent ammonia from accumulating to toxic levels that result in leaf damage, i.e., leaf tip burn and necrosis of the leaf

margin. Inhibition of this pathway during water-deficit stress would result in increased levels of free NH_3-NH_4^+.

Table 4 - Leaf NH_3-NH_4^+ content (μg/g fresh wt.).[z]

	Inoculated with *P. cinnamomi*			
	Yes		No	
Rootstock	$+H_2O$	$-H_2O$	$+H_2O$	$-H_2O$
Mexican	14 a	17 a	14 a	23 b
Duke 7	16 a	27 b	20 a	28 b

[z] Harvest 1 - One month after the initiation of the water-deficit stress treatment. Trees were inoculated 2 months before the initiation of the water-deficit stress treatment. Values followed by different letters are significantly different at $p < 0.05$.

Table 5 - *De novo* arginine biosynthesis (nmoles $NaH^{14}CO_3$ incorporated into arginine plus urea/g fresh wt. · 3 hours).[z]

	Inoculated with *P. cinnamomi*			
	Yes		No	
Rootstock	$+H_2O$	$-H_2O$	$+H_2O$	$-H_2O$
Mexican	2.9 ± 0.1	2.2 ± 0.1	3.4 ± 0.4	1.8 ± 0.1
Duke 7	1.5 ± 0.3	0.2 ± 0.2	1.8 ± 0.2	0.8 ± 0.6

[z] Harvest 1 - One month after the initiation of the water-deficit stress treatment. Trees were inoculated 2 months before the initiation of the water-deficit stress treatment.

Increased ethylene biosynthesis by leaves of a number of plant species is a common response to water-deficit stress. Ethylene causes leaf epinasty and leaf abscission (Reid, 1985) which is an observed characteristic of the 'Hass' avocado during water-deficit stress. However, not all treatments resulting in water-deficit stress (Table 2) caused increased rates of ethylene biosynthesis. Differences in the rates of ethylene biosynthesis between treatments were not statistically significant ($p < 0.05$) (Table 6).

Recently, Corey and Barker (1989) provided evidence that the symptoms of NH_3-NH_4^+ toxicity in tomato were related to increased ethylene biosynthesis. The results of our research are equivocal. Not all treatments resulting in increased leaf concentrations of NH_3-NH_4^+ caused increased of ethylene biosynthesis (Tables 4 and 6).

Table 6 - Ethylene biosynthesis (nl/g fresh wt. · 2 hours). [z]

	Inoculated with *P. cinnamomi*			
	Yes		No	
Rootstock	$+H_2O$	$-H_2O$	$+H_2O$	$-H_2O$
Mexican	3.0	0.7	1.9	3.4
Duke 7	2.0	2.2	3.3	5.1

[z] Harvest 1 - One month after the initiation of the water-deficit stress treatment. Trees were inoculated 2 months before the initiation of the water-deficit stress treatment. Data are not significantly different at $p < 0.05$.

In an attempt to clarify the possible relationship between NH_3-NH_4^+ accumulation, ethylene biosynthesis, and leaf damage, 'Hass' avocado leaves (with the lower 1.25 cm of the leaf cut off to provide a 2.5 cm-wide surface for solution uptake by transpiration) were inserted to a depth of 2 cm into aerated solutions containing NH_4Cl at concentrations of 0, 12.5, 25, 50, 100, and 200 mM with and without 1 mM aminoethoxyvinyl-glycine (AVG), an inhibitor of ethylene biosynthesis, or 1, 5, and 25 mM 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene. The leaves were incubated for 24 hours under continuous light (500 $\mu\Sigma/m^2 \cdot$ sec) at 30°C.

Leaf damage increased in a manner paralleling the NH_4^+ concentrations of the treatment solutions. Ethylene biosynthesis increased with increasing leaf NH_3-NH_4^+ concentrations up to 25 mM NH_4^+ (Table 7). Ethylene biosynthesis in leaves incubated with 25, 50, or 100 mM NH_4^+ was the same, while leaf concentrations of NH_3-NH_4^+ increased linearly (data not shown). Leaves incubated with 200 mM NH_4^+ had low rates of ethylene biosynthesis not significantly different from the control. This was perhaps due to excessive tissue damage and cell death at this concentration of NH_4^+. The addition of AVG, a potent inhibitor of ethylene biosynthesis, to solutions containing 25 and 50 mM NH_4^+, reduced ethylene biosynthesis more than 80% and the symptoms of leaf damage by approximately 50%, but had no effect on leaf NH_3-NH_4^+ content.

Table 7 - Ethylene biosynthesis (nl/g fresh wt. · 4 hours).

	-1 mM AVG	+1 mM AVG
NH_4^+, none-control	3.2 ± 2.7	
12.5 mM	8.1 ± 3.2	
25 mM	17.6 ± 9.6	1.7
50 mM	15.1 ± 5.0	3.2
100 mM	12.6 ± 4.7	
200 mM	4.3 ± 2.1	
ACC, 1 mM	262 ± 52	
5 mM	376 ± 151	
25 mM	236 ± 15.5	

These results suggest that the accumulating NH_3-NH_4^+ stimulates ethylene biosynthesis in 'Hass' avocado leaves up to a maximum rate and that ethylene plays a role in the browning of the leaf tissue. However, ACC, the immediate precursor of ethylene, dramatically increased the rate of ethylene biosynthesis (Table 7), but did not cause any tissue damage, suggesting that ethylene must interact with NH_3-NH_4^+ to cause damage.

Proline accumulation is a common symptom of water-deficit stress in a number of plant species. For citrus, a linear relationship between the free proline content of the leaves and xylem pressure potential has been reported (Levy, 1980). Proline did not accumulate in the leaves of the 'Hass' avocado scions budded on either the Mexican seedling or clonal 'Duke 7' rootstocks in response to water-deficit stress at harvests 1 (Table 8) or 2. Whether the statistically significant reduction in leaf proline content of 'Hass' avocado scions on *P. cinnamomi* infected Mexican seedling rootstocks subjected to water-deficit stress and *P. cinnamomi* infected clonal 'Duke 7' rootstocks under well-watered conditions is physiologically significant is not known at this time.

Table 8 - Leaf proline content μg/g fresh wt.).[z]

	Inoculated with *P. cinnamomi*			
	Yes		No	
Rootstock	$+H_2O$	$-H_2O$	$+H_2O$	$-H_2O$
Mexican	100 a	53 b	92 a	102 a
Duke 7	49 b	79 a	112 a	101 a

[z] Harvest 1 - One month after the initiation of the water-deficit stress treatment. Trees were inoculated 2 months before the initiation of the water-deficit stress treatment. Values followed by different letters are significantly different at $p < 0.05$.

4. Discussion

Water-deficit stress resulted in a statistically significant increase in the NH_3-NH_4^+ content of leaves of 'Hass' avocado scions on Mexican seedling or clonal 'Duke 7' rootstocks, 6 months from budding, when xylem pressure potentials were less than -1.8 MPa. Xylem pressure potentials from -1.8 to -2.6 MPa are indicative of moderate stress, and the levels of NH_3-NH_4^+ that accumulated, although statistically significant, were low relative to concentrations reported previously to accumulate during water-deficit stress or fungal infection. This may reflect the fact that the trees were young and received no nitrogen fertilizer during the experiment.

The activity of the pathway for the *de novo* biosynthesis of arginine was significantly reduced (>50%) in trees with xylem pressure potentials less than -1.8 MPa. This is the fourth system in which a stress (salinity or water-deficit stress of intact plants or osmotic stress of cells in suspension culture) resulted in inhibition of the activity of pathway for the *de novo* biosynthesis of arginine (Lovatt, unpublished).

Root infection by *P. cinnamomi* did not influence plant water status or leaf NH_3-NH_4^+ content. Although all inoculated trees appeared infected three months after inoculation, recovery of *Phytophthora* from well-watered Mexican seedling and clonal 'Duke 7' rootstocks was 60 and 40% , respectively. Xylem pressure potentials and leaf NH_3-NH_4^+ concentrations of the 'Hass' avocado scions were the same as those of the well-watered, uninoculated (control) trees. Water-deficit stress reduced the recovery of *P. cinnamomi* 20 and 50% for Mexican seedling and clonal 'Duke 7' rootstocks, respectively, but root infection by *P. cinnamomi* did not increase the severity of water-deficit stress nor the accumulation of NH_3-NH_4^+ in the scions. The results provide evidence that root infection by *P. cinnamomi* does not cause increased NH_3-NH_4^+ production by a direct action of the pathogen on the metabolism of the roots prior to the pathogen-induced water-deficit stress.

Increased ethylene production and proline accumulation are common responses of leaves of water-stressed plants. These responses were not observed in the present study with 'Hass' avocado scions on Mexican seedling or clonal 'Duke 7' rootstocks, infected or uninfected, during water-deficit stress. In detached leaves of the 'Hass' avocado, NH_3-NH_4^+ accumulation stimulated ethylene biosynthesis. Thus, the preliminary results of this study provide evidence that leaf damage, in part, results from an interaction of ethylene with NH_3-NH_4^+. The physiological significance of this interaction in the field remains to be determined.

The results of this research provide evidence that NH_3-NH_4^+ accumulation in leaves is not an early symptom of root infection by *P. cinnamomi*. The research will be continued to determine whether pathogen-induced water-deficit stress will result in increased leaf concentrations of NH_3-NH_4^+ and concomitant leaf damage characteristic of uninfected, water-deficit stressed trees.

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