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Demonstration of ammonia accumulation and toxicity in avocado leaves during water-deficit stress

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SYNOPSIS

The authors have demonstrated that ammonia (measured, as the combined pool of NH_3-NH_4+) increases in leaves of woody perennials and herbaceous annuals in response to stress. In addition, the results provide evidence that ammonia accumulates to toxic levels resulting in leaf necrosis of stress-sensitive plants, while stress-tolerant plants detoxify their cells of ammonia through the de novo synthesis of arginine. The use of water-deficit or low temperature stress to induce flowering in citrus varieties is well known. The authors have demonstrated that the degree of flowering is dependent on the severity of stress and on the accumulation of ammonia. Maximum flower number can be achieved under conditions of minimal stress by increasing the ammonia content of the tree through foliar application of low biuret urea. Preliminary experiments with Hass avocado on clonal Duke 7 rootstock provide evidence that NH_3-NH_4+ accumulates in avocado leaves in response to water-deficit and low temperature stress. However, the results are too preliminary to predict the success of inducing flowering in avocado by water-deficit or low temperature stress. The results suggest that the photorespiratory nitrogen cycle is the major source of ammonia that is produced during stress, suggesting that carbohydrate depletion is not a pre-requisite for, but a consequence of, ammonia accumulation.

INTRODUCTION

The authors have been investigating accumulation of ammonia (measured the hypothesis that any stress that as the combined pool of NH_3-NH_4+) in stunts the growth of a plant or causes carbohydrate depletion will result in the young and old leaves as an early response of the plant to stress. It was demonstrated that NH_3-NH_4+ accumulates in young and mature leaves of woody perennials or herbaceous annuals in response to water-deficit stress, low temperature, salinity, and phosphorus deficiency. It was also demonstrated (1) that stress-sensitive plants are unable to detoxify their cells of NH_3-NH_4+, which soon reaches toxic levels and results in leaf symptoms, eg tip burn and margin necrosis; and (2) that stress-tolerant plants remove NH_3-NH_4+ through *de novo* synthesis of arginine, thus preventing NH_3-NH_4+ to build up to a toxic level (Rabe& Lovatt, 1986; Lovatt, 1986). Currently the specific case of avocado scion varieties is being tested to determine if ammonia accumulation in leaves is an early response of avocado to stress, including water-deficit, low temperature, and salinity. The purpose of this research is three-fold.

Water costs are now as high as 50 per cent of the gross revenue in some avocado-growing areas of California. To cut costs, growers have reduced the number of irrigations applied later in the season. Results of preliminary studies suggest that this leads to the accumulation of levels of ammonia toxic to the leaves, resulting in leaf damage, reduced photosynthesis, and leaf abscission. The concentration of ammonia in the youngest, fully expanded leaves of the fall flush of 16-year-old avocado trees *(Persea americana* Mill cv Hass) under commercial production increased during water-deficit stress: compare 163 µg NH_3-NH_4+ per g dry wt leaf tissue from well-watered control trees to 268 µg per g dry wt leaves from water-deficit stressed trees. Secondly, the availability of irrigation water of good quality is becoming increasingly limited. High boron and saline soils are becoming impediments to avocado production in California.

The authors have demonstrated that salinity alters nitrogen metabolism and causes NH_3-NH_4+ to accumulate to toxic levels in leaves of herbaceous annuals (Lovatt, 1986). For example, transferring eight-day-old squash plants *(Cucurbita pepo* L cv Early Prolific Straightneck) from aerated hydroponic culture in Shive's nutrient solution to aerated Shive's nurtient solution plus 30 mM or 60 mM NaCl-aCl_2 (2:1 molar ratio; the salt is added at the rate of 1/3 the total amount every other day) resulted in a marked reduction in the growth of the plants. Despite the fact that the nitrate content of the young leaves (five-days-old) declined 50 per cent ($P < 0,05$), there was a dramatic increase in the concentration of ammonia in the leaves of the stressed plants at the end of only 10 days of treatment ($P<0,05$). In addition, the amount of ammonia that accumulated increased in parallel with the increased amount of salt added. Mature leaves, which were exposed to the stress five days longer than young leaves, accumulated a greater net amount of ammonia. When compared to leaves from the healthy control plants, there was a net accumulation of 200 and 250 µg NH_3-NH_4+ per g dry wt youngest, fully expanded leaves from the 30 and 60 mM treatments, respectively. A net increase of 250 and 350 µg NH_3-NH_4+ occurred per g dry wt mature leaves for the two salt treatments, respectively. It was observed for squash that symptoms of ammonia toxicity appear when the concentration of NH_3-NH_4+ in the leaf exceeds the normal level for the tissue by more than 150 pg NH_3-NH_4+ per g dry wt. Removal of ammonia through the synthesis of arginine decreased as the severity of the salt treatment increased. This work needs to be extended to woody perennials, especially to avocado, which is very sensitive to salinity.

Supplying nitrogen for crop production by application of commercial nitrogenous fertilisers represents a significant expense to the grower. The accumulation of NH_3-NH_4+ in leaves of avocado trees grown under reduced irrigation or under saline conditions suggests that nitrogen fertilisation probably needs to be managed differently if growers are going to reduce irrigation or if salinity problems exist in a grove.

Thirdly, the authors want to determine if ammonia accumulation is an essential component of flower induction in avocado varieties. They have recently established that this is the case for citrus. For citrus scion varieties, water-deficit or low temperature stress increases the NH_3-NH_4+ content of the leaves and induces flowering. Zheng & Lovatt (1987, Plant Physiol, Abst, in press) and Hake & Lovatt (1987, Plant Physiol,

Abstr, in press) have demonstrated that the intensity of flowering can be regulated by the duration of severity of the stress or by increasing the NH_3-NH_4+ content of leaves of minimally stressed trees by foliar application of urea at the rate of 1,5 g or 0,1 kg low biuret urea, per tree for five-year-old rooted cuttings of the 'Washington' navel orange or 16-year-old trees of Frost Lisbon Lemon on Troyer citrange rootstock under commercial production, respectively. If this proves to be the case in avocado, we will be able to take the first steps in learning to manipulate the time of avocado bloom and the resulting harvest to bring varieties to market when the price is the highest, as is currently done for lemons and lime.

The research, when completed, will demonstrate whether ammonia accumulates to toxic levels in avocado leaves during water-deficit, low temperature, or salinity stress of both field-grown and controlled-environment-grown avocado scion varieties of Topa Topa and on clonal Duke 7 rootstock; determine the level of ammonia that is toxic to avocado leaf tissue, *ie* causes leaf symptoms; and identify the source of accumulating ammonia. It is essential to determine if the ammonia that accumulates during stress comes from the reduction of nitrate fertiliser, from the photorespiratory nitrogen cycle, or from protein degradation in order to develop the best cultural practice to prevent the accumulation of ammonia to a toxic level and to use stress to induce flowering.

MATERIALS AND METHODS

Water-deficit stress - Hass avocado on clonal Duke 7 rootstock one and two years from budding, grown under controlled environmental conditions in a growth chamber or a glasshouse and 16-year-old trees under commercial production are treated as follows: (1) well-watered control; (2) water is withheld, trees are stressed to less than -30 bars over 20 days and maintained at less than -20 bars for 40 days, and then rewatered quickly; (3) water is withheld, trees are stressed to less than -20 bars in 10 days and then irrigated at 25 per cent normal irrigation and rewatered quickly after 50 days; and (4) same as treatment (3) with a foliar application of low biuret urea at the rate of 1,5g per budding and 0,1 kg per commercial tree at the end of the 10 days without water.

For field-grown trees under commercial production, treatments will be initiated in mid-June. Water-deficit stress is monitored as pre-dawn water potential by pressure bomb.

Low temperature stress - Under controlled environmental conditions using growth chambers, Hass avocado trees on clonal Duke 7 rootstock one or two years from budding are subjected to low temperature treatment consisting of 8-h days (500 $\mu E/m^2$ scc) at 15 to 18°C (59-64°F) and 16-h nights at 10 to 13°C (50-55°F) for eight weeks and then transferred to 12-h days (500 $\mu E/m^2$.sec) at 24°C (75°F) and 12-h nights at 19°C (66°F). Control trees are maintained under the warmer conditions for the total length of the experiment. Trees are watered once a week with half-strength Hoagland's nutrient solution and as needed with H_2O.

Salinity stress - Hass avocado trees on clonal Duke 7 rootstock one and two years from budding, grown under glasshouse conditions, are irrigated with half-strength Hoagland's nutrient solution with and without 30 mM NaCI:$CaCl_2$ (2:1 molar ratio).

In all experiments, leaf NO_3^-, NH_3-NH_4+, amino acid profile, protein content, starch content, and activity of the *de novo* arginine bio-synthetic pathway are quantified according to the methods of Rabe & Lovatt (1984, 1986), polyamine titers are determined after benzoylation (Friedman, Levin & Altman, 1986) by HPLC (Flores & Galston, 1982), and photosynthesis and transpiration are monitored by dual isotope porometer (Johnson, Rowlands & Ting, 1979). Leaf nutrient status is determined by the University of California Cooperative Extension Diagnostic Laboratory.

Photorespiration - Activity of the photorespiratory nitrogen cycle of avocado scion varieties is assessed in detached leaves by immersing their petioles for 36 hours in aerated solutions of (1) H_2O; (2) 10 mM methionine sulfoximine (MSO); or (3) 10 mM MSO and 10 mM isonicotinic acid hydrazide. Each set of treatments is incubated at 30°C at 500 µE/m^2 sec continuous light with and without 60 mM NaCI:$CaCl_2$ (2:1 molar ratio), at 15 to 18°C (59-64°F) at 500 µE/m^2 sec continuous light, or at 30°C in the dark. Leaves were rated for NH_3-NH_4+ toxicity symptoms, and NH_3-NH_4+ content was determined as described above.

Glutamine synthetase was assayed in cell-free extracts prepared from control leaves treated with H_2O and from leaves treated with MSO to determine the effectiveness of MSO in inhibiting glutamine synthetase in each variety (Lovatt, 1983).

RESULTS

Water-deficit stress preliminary results - All data are the average ± standard deviation where n is six well-watered control trees or 12 water-deficit stressed trees, unless otherwise stated.

Hass avocado trees on clonal Duke 7, one year from budding, were grown under 12-h days (310 µE/m^2.sec) at 24°C (75°F) and 12-h nights at 19°C (66°F) with and without irrigation. Preliminary results demonstrated that well-watered control trees maintained a water potential of -3,0 ± 1,2 bars over the 30-day experiment. The water potential of droughted trees decreased gradually to a minimum of -29 ± 11 bars at the end of 30 days. Pre-dawn water potentials were not significantly different from those obtained at other times during the day.

For the well-watered control trees, stomatal conductance was 0,025± 0,015 cm/sec, photosynthesis was 0,55±0,3mg CO_2 fixed/dm^2.h (this value is low due to the low light intensity of the chamber), and transpiration was 0,09±0,06 g H_2O/dm^2.h. Maximum photosynthesis and transpiration occurred two hours after the chamber lights came on and remained high for approximately two hours.

Three weeks after water was withheld, water potential, stomatal conductance, photosynthesis, and transpiration were significantly reduced three-fold, 72, 88 and 69 per cent, respectively, in leaves of water-stressed avocado plants compared to the well-watered controls (Table 1).

TABLE 1 Effect of water-deficit stress on young Hass avocado trees on clonal Duke 7 rootstock[a].

	Control (well-watered)	Water-deficit stress (water withheld 21 days)
Water potential (bars)	-0,6±1,0	-19,3±9,7
Stomatal conductance (cm/sec)	0,025±0,015	0,007±0,004
Photosynthesis (mg CO_2 fixed/dm2 h)	0,55±0,3	0,07±0,05
Transpiration (g H_2O/dm^2 h)	0,09±0,06	0,028±0,014
NH_3-NH_4+ (µg dry wt leaf tissue)	1 508±102	2 061±248
NO_3^- (µg dry wt leaf tissue)	<100	<100
Starch (mg glucose equivalents/ g dry wt leaf tissue)	10,46	10,62±1,3

[a] Average values±standard deviation (n=6 control trees, 12 water-deficit stressed trees),

Water-deficit stressed trees exhibited browning of young shoot tips and necrosis of the leaf tip and margin. With time, this browning progressed across the blade of the leaf to the petiole. There was considerable leaf abscission for the water-deficit stressed trees. At the end of the 30-day experiment, four trees had no viable leaves.

Water-deficit stressed trees were rewatered after 30 days of stress. Browning of the leaves and shoot tips continued for up to 10 days after rewatering.

Low temperature stress preliminary results - Hass avocado trees on clonal Duke 7 rootstock one year from budding were subjected to average temperatures of 20,7 ± 3,6°C (69,2 ± 12°F) day (approximately eight hours) and 6,9 ± 0,6°C (44,5 ± 4°F) night in a lathhouse at the Citrus Research Center and Agricultural Experiment Station of the University of California, Riverside, from November 21 until December 21, and then transferred to 12-h days (310 µE/m^2.sec) at 24°C (75°F) and 12-h nights at 19°C (66°F). The NH_3-NH_4+ content of the trees remained high [1 692 ± 630 µg per g dry wt leaf tissue (0 ± standard deviation, n = five weeks)], until the trees began to flower, five weeks after transfer to the warmer temperature. At this time, the NH_3-NH_4+ content of the leaves averaged 247±17 (0 ± standard deviation, n = two weeks). Leaf $N0_3^-$ and starch content did not change in response to temperature. Leaf $N0_3^-$ content was less than 100 µg per g dry wt leaf tissue, and starch content averaged 15,9 ± 8 mg glucose equivalents per g dry wt leaf tissue during the experiments.

Photo respiration preliminary results - The addition of methionine sulfoximine (MSO), a known inhibitor of glutamine synthetase, caused browning on the leaves of all scion varieties. Browning begin at the leaf tip and progressed along the leaf margin and down the blade toward the petiole. Supplementing the MSO-containing medium with isonicotinic acid hydrazide, an inhibitor of glycine synthase, the enzyme that catalyses the NH_3-generating reaction in photorespiration, reduced the amount of leaf damage observed with MSO alone. No browning occurred when leaves were maintained in the dark. Salt caused browning to occur in the presence of isonicotinic acid hydrazide.

For all treatments, the sensitivity of the varieties tested was Gwen> Pinkerton > Hass > Bacon, The NH_3-NH_4+ content of the leaves closely paralleled the degree of leaf damage (Table 2).

TABLE 2 Accumulation of ammonia from the photorespiratory nitrogen cycle at the end of 36 hours[a].

Variety	H_2O (control)	MSO (10 mM)	MSO (10 mm) INA (10 mM)
	30°C, continuous light (500 µE/m^{2-} sec)		
Hass	93	926	646
Gwen	103	1 596	1 063
Pinkerton	49	679	660
Bacon	58	1 013	508
	30°C, dark		
Hass	28	431	467
Gwen	57	226	387
Pinkerton	61	392	288
Bacon	44	388	345
	15-18°C, continuous light (500 µE/m2·sec)		
Hass	45	812	673
Gwen	71	1 291	1 051
Pinkerton	61	1 065	778
Bacon	53	1 011	677
	30°C, continuous light (500 µE/m^2-sec) 60 mM NaCl:$CaCl_2$ (2:1 molar ratio)		
Hass	71	1 019	1 491
Gwen	94	1 881	1 701
Pinkerton	67	1 469	1 726
Bacon	84	944	1 592

a Ammonia as lag NH_3-NH_4+ per g fr wt leaf tissue. The leaf content of NH_3-NH_4+ (µg per g fr wt) at the initiation of the experiment (T_o) was for Hass, Gwen, Pinkerton and Bacon, respectively: 24±3, 25±2, 30±5 and 29±2 (0±standard deviation, n=5). The effectiveness of 10 mM MSO to inhibit glutamine synthetase for 36 hours was 10, 70, 100 and 0 per cent for Hass, Gwen, Pinkerton and Bacon avocados, respectively.

DISCUSSION

While only preliminary, results suggest that avocado varieties may respond to stress in a manner similar to that of citrus varieties, ie ammonia accumulation in leaves in response to water-deficit and low temperature stress. The results are far too preliminary to speculate as to whether water-deficit or low temperature stress can be used to successfully induce flowering in avocado varieties.

The NO_3^- and starch content of leaves did not change during water-deficit or low temperature stress of Hass avocado. This is also the case for citrus. These two observations, taken together with the results of the preliminary photorespiration

experiment, suggest that the ammonia accumulating during stress is not from the reduction of nitrate supplied by fertilisation, but from increased activity of the photorespiratory nitrogen cycle and/or failure to refix NH_3 generated by glycine synthase. Inhibition of protein synthesis with concomitant degradation of amino acids may also contribute to the pool of NH_3-NH_4+ accumulating during stress. In addition, the results indicate, contrary to previous belief, that reduced carbohydrate availability is not an essential pre-requisite for NH_3-NH_4 + to accumulate.

The authors are currently investigating the hypothesis that increased activity of the photorespiratory nitrogen cycle is an early response of plants to stress, excluding mineral nutrient deficiencies, and the key factor initiating altered carbon metabolism leading to carbohydrate depletion.

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