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Leaf Net CO_2 Assimilation and Electrolyte Leakage and Alcohol Dehydrogenase Activity in Roots of Mamey Sapote (*Pouteria sapota*) Trees as Affected by Root Zone Oxygen Content

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Net CO_2 assimilation (A) of leaves and root electrolyte leakage (EL) and alcohol dehydrogenase enzyme (activity) (ADH) in roots of mamey sapote (*Pouteria sapota*) trees were assessed in response to different oxygen concentrations in the root zone. In separate experiments, 'Pantin' and 'Magaña' scions on mamey sapote seedling rootstocks were grown hydroponically with an oxygen concentration of 7–8 mg $O_2 \cdot L^{-1}$ H_2O in the root zone maintained by bubbling air into the hydroponic medium (aerated treatment) or with an oxygen concentration of 0–1 mg $O_2 \cdot L^{-1}$ H_2O maintained by purging O_2 from a hydroponic medium with N_2 gas (O_2-purged treatment). Net CO_2 assimilation of 'Magaña' leaves from the O_2-purged treatment declined over time and was at or near zero 8 days after treatment (DAT). Net CO_2 assimilation of 'Pantin' leaves in the aerated treatment was higher than that of leaves in the O_2-purged treatment at 2–6 DAT only. Electrolyte leakage from roots was significantly greater in the O_2-purged treatment than in the aerated treatment. Two days after treatments, root ADH activity in both cultivars tended to be consistently higher in the O_2-purged than aerated treatment. The ADH activity of mamey sapote roots appears to be up-regulated as a result of root-zone hypoxia. However, increased ADH activity alone is apparently not sufficient to limit low soil oxygen stress of mamey sapote trees as evidenced by decreased A of leaves and increased EL from roots of trees exposed to low oxygen content in the root zone.

Mamey sapote [*Pouteria sapota* (Jacq.) H.E. Moore and Stearn] is a tropical tree native to the humid lowlands of southern Mexico, and south through parts of Central America to northern Nicaragua (Balerdi and Shaw, 1998; Verheij and Coronel, 1992). In southern Florida, soils are subjected to periodic flooding during high water table conditions that coincide with periods of heavy rainfall and/or tropical storms. Flooding of mamey sapote orchards in this area often results in tree decline and death (Crane et al., 1997).

Oxygen concentration in flooded or poorly drained soil may become deficient (hypoxic) enough to inhibit normal aerobic root respiration. Hypoxia generally occurs at soil concentrations less than 2 mg $O_2 \cdot L^{-1}$ H_2O, although the O_2 content at which plants become hypoxic may differ among plant species (Gibbs and Greenway, 2003). Curtis (1949) found that roots of avocado trees (*Persea americana*) in a hydroponic medium were able to withstand oxygen levels of 1 mg $O_2 \cdot L^{-1}$ H_2O for 10 d without root damage, whereas concentrations below 1 mg $O_2 \cdot L^{-1}$ H_2O, or complete lack of O_2 in the medium (anoxia) caused root damage. Most plant species are able to survive only brief periods of anoxia (Drew, 1997). Plant roots rarely are exposed to sudden anoxia under natural conditions. Rather, there is a gradual transition from normoxia (an adequate supply of oxygen in the root zone) to hypoxia and anoxia, which allows plants to acclimate to low soil oxygen levels before conditions become lethal (Drew, 1997).

There has been a considerable amount of research on physiological responses of plants to hypoxia or anoxia caused by flooding or poor soil drainage (Bailey-Serres and Voesenek, 2008; Drew, 1997; Gibbs and Greenway, 2003; Givan, 1999; Kozlowski, 1997; Schaffer et al., 1992, 2006). For woody, perennial species including fruit crops, many studies have focused on assessing leaf gas exchange (Kozlowski, 1997; Schaffer et al., 1992). One of the earliest responses of woody perennial species to low soil oxygen content is a reduction in net CO_2 assimilation of leaves (Kozlowski, 1997; Schaffer et al., 1992). Significant reductions in net CO_2 assimilation occur before visible stress symptoms. Thus, leaf gas exchange measurements have been useful for quantifying stress in tree species in response to soil hypoxia or anoxia.

The immediate biochemical precursor to ethanol, acetaldehyde, is considerably more toxic to plant cells than ethanol and may be a factor in plant cell death during anaerobic metabolism (Drew, 1997; Vartapetian and Jackson, 1997). The conversion of acetaldehyde to ethanol is catalyzed by the enzyme alcohol dehydrogenase (ADH). Research with herbaceous plant species

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has shown that increased ADH activity can improve a plant's tolerance to anoxia (Chung and Ferl, 1999; Gibbs et al., 2000; Kato-Noguchi, 2000; Morimoto and Yamasue, 2007; Preiszner et al., 2001). Root ADH levels have been reported for only a few mesic forest trees such as swamp tupelo (*Nyssa sylvatica* var. *biflora*) (Angelov et al., 1996) and melaleuca (*Melaleuca cajuputi*) (Yamanoshita et al., 2005). However, to the authors' knowledge ADH activity in response to root anoxia or hypoxia has not been reported for any commercial fruit tree species.

The objective of this study was to determine the effects of root hypoxia and anoxia on net CO_2 assimilation of leaves and electrolyte leakage ADH activity in roots of the two major mamey sapote cultivars grown in flood-prone areas of southern Florida.

Materials and Methods

Two experiments were conducted in a greenhouse with 2-year-old mamey sapote (*Pouteria sapota*) scions grafted onto *Pouteria sapota* seedling rootstocks. 'Pantin' was used as the scion in the first experiment and 'Magaña' was used as the scion in the second experiment. Trees were grown in 19-L polyethylene containers filled with a mix of peat moss, coarse sand, pine bark, and perlite.

Each experiment consisted of two treatments, each with roots placed in a hydroponic medium to allow sampling of root tips without disturbing the rest of the root system. In one treatment, an oxygen concentration of 7–8 mg O_2 L^{-1} H_2O was maintained in the root zone by bubbling air into the hydroponic medium (aerated treatment). In the other treatment, the oxygen concentration in the root zone was maintained at 0–1 mg O_2 L^{-1} H_2O by purging O_2 from the medium with N_2 gas (O_2-purged treatment). Dissolved oxygen (DO) content in the hydroponic medium was monitored using an Oakton DO 100 handheld dissolved oxygen meter (Oakton Instruments, Vernon Hills, IL). The O_2-purged treatments were initiated by purging tap water with N_2 gas until a DO concentration of 0 mg O_2 L^{-1} water was achieved prior to submerging roots into the water. The DO concentration was maintained at 0–1 mg O_2 L^{-1} H_2O. The medium was aerated with ambient air for the aerated hydroponic treatment at 0.16 L air·min^{-1}·L^{-1} water with an air pump (Whisper® Model 100, Tetra®, Blacksburg, VA). Preliminary experiments (M.T. Nickum, unpublished data) showed that mamey sapote seedlings, of similar age to those in this study, can be removed from soil and grown hydroponically in tap water for up to 40 d with no observable nutrient stress. Therefore, during the short duration of this study (10 d), no nutrients were added to the hydroponic medium. The experiments were terminated after 10 d because by that time leaves of plants in the O_2 purged medium ceased photosynthesizing and their leaves began to abscise. Temperatures in the hydroponic root medium were monitored with a HOBO® Water Temp Pro temperature logger (Onset Computer Co., Bourne, MA). Temperatures in the hydroponic medium ranged from 19 to 31 °C.

Air temperature in the greenhouse during the experiments was monitored with a StowAway® TidBit® temperature logger (Onset Computer Co.) placed in a plant canopy at a height of 1.5 m. Air temperature in the canopy ranged from 19 to 43 °C during the experiments.

Net CO_2 assimilation in each experiment was measured with a CIRAS-2 portable leaf gas exchange system (PP Systems, Amesbury, MA). Leaf gas exchange measurements were made on one recently matured leaf (3rd to 5th leaf distally from the stem apex) per plant at a photosynthetic photon flux of 1000 µmol·m^{-2}·s^{-1} (light saturation), a reference CO_2 of 375 µmol·mol^{-1} and a flow rate of 200 mL·min^{-1} into the leaf cuvette. Measurements were made between 1000 and 1400 HR, 0, 3, 6 and 0, 2, 4, and 8 DAT began for 'Pantin' and 'Magaña' trees, respectively.

Electrolyte leakage from 'Pantin' roots was determined 0, 4, and 9 DAT by harvesting 2 g fresh weight of terminal sections of the healthiest roots. Root sections were rinsed with deionized (DI) water, blotted dry, and placed into a 60-mL tube with 30 mL of DI water. The tubes were capped and placed on a shaker containing 0.9 g of root tissue for 24 h. The electrical conductivity was determined with a Fisher Scientific Accumet® AR50 pH/ION/conductivity meter (Fisher Scientific, Waltham, MA) for each solution after the roots were removed. Roots were then frozen for 24 h at –80 °C to lyse the cells, removed from the freezer and the corresponding solution returned to each tube, and the samples were shaken for 24 h. The roots were removed from the solution and electrical conductivity of the solution was determined. Root electrolyte leakage was calculated as a percentage by dividing the electrical conductivity prior to freezing by the electrical conductivity after freezing (Crane and Davies, 1987; Stergios and Howell, 1973).

The total amount of electrolytes present in the root cells per unit dry weight was also determined. While electrolyte leakage assesses permeability of the root cell membrane, the total electrolyte level assesses the amount of electrolyte maintained in the root cell. Roots were frozen at –80 °C and then shaken for 24 h in 30 mL of DI water as previously described. The resulting electrical conductivity was measured and levels standardized for 1 mL DI water. Roots were then oven dried and weighed.

Root ADH activity was determined in each experiment 0, 2, 4, 6, 8, 10 DAT. Root tips were harvested from each plant, rinsed with DI water, placed into a 1.5-mL microcentrifuge tube and immediately placed on ice (Bailey-Serres and Voesenek, 2008; Gibbs and Greenway, 2003). Samples were then stored at –80 °C for 1 to 2 d until enzyme extraction. Root samples were processed and ADH activity measured as described by Russell et al. (1990) and Chung and Ferl (1999). Roots were ground on ice with sea sand in 1.5 to 2 mL of an extraction solution containing 50 mM Tris-HCl and 15 mM DTT at pH 8.8. Samples were centrifuged at 12,000 g for 12 min at 4 °C. The enzyme reaction was initiated by placing 500 µL of reaction solution (50 mM Tris-HCl, 1.7 mg/mL NAD^+, pH 8.5), 100 µL 50% ethanol, and 100 µL extract in disposable cuvettes that were inverted seven times. The cuvettes were allowed to sit for 1 to 2 min and then placed in a Beckman DU-640 Spectrophotometer (Beckman Instruments, Fullerton, CA). Absorbance was zeroed and recorded at A340 every 15 s for 2 min. Protein content of the extract was determined with a protein assay kit (Bio-Rad Laboratories, Hercules, CA) and ADH enzyme activity was calculated as nmol NADH·min^{-1}·mg $protein^{-1}$.

Plants in each experiment were arranged in a completely randomized design with seven single-plant replicates per treatment. Net CO_2 assimilation data were analyzed using repeated measures analysis. For all other variables difference in treatment means on each measurement date were compared using a non-paired *t*-test. All data were analyzed with SAS Version 9.1 Statistical Software (SAS, Inc., Cary, NC).

Results

Net CO_2 assimilation in the O_2-purged treatment declined steadily for 'Pantin' and 'Magaña' trees, respectively (Fig. 1). Net CO_2 assimilation in the O_2-purged treatment was at or near 0 µmol CO_2·m^{-2}·s^{-1} by 6–8 DAT (Fig. 1). Net CO_2 assimilation of

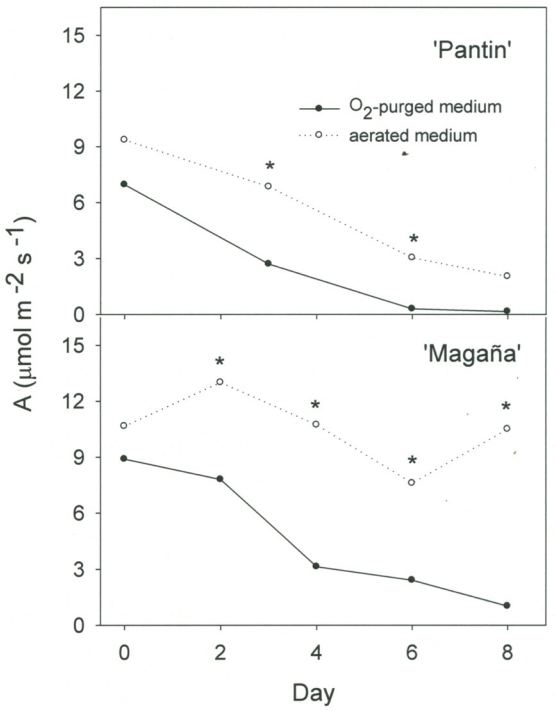

Fig. 1. Net CO_2 assimilation (A) of 'Pantin' and 'Magaña' mamey sapote scions on mamey sapote seedling rootsotcks grown in O_2-purged or aerated hydroponic media. Asterisks represent significant difference ($P < 0.05$) between treatments determined by a non-paired *t*-test, n = 7.

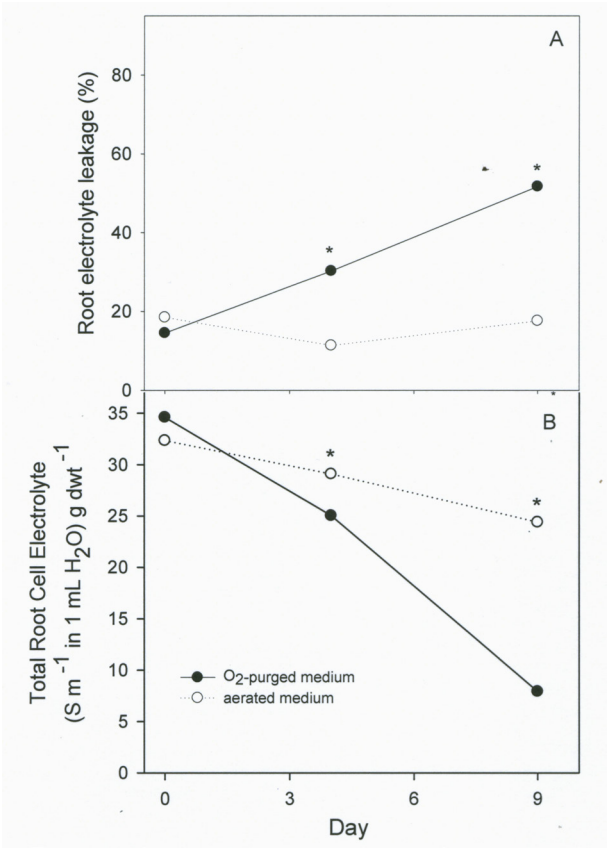

Fig. 2. Percent root electrolyte leakage media (**A**) and (**B**) total root electrolyte content from 'Pantin' mamey sapote scions on seedling mamey sapote rootstock grown in O_2-purged or aerated hydroponic media. Units measured as amount of electrical conductivity (S·m^{-1}) concentrated into 1 mL of DI water per gram root dry weight. Asterisks indicate significant difference ($P < 0.05$) between treatments determined by a non-paired *t*-test, n=7.

'Magaña' trees in the aerated treatment was consistently higher than that of trees in the O_2-purged treatment beginning 2 DAT. Although net CO_2 assimilation of 'Pantin' trees in the aerated treatment were higher than that of trees in the O_2-treatment 2–6 d after treatments, by day 8 there was no significant difference between treatments. Net CO_2 assimilation of 'Pantin' trees in the aerated treatment declined at about the same rate as that of trees in the O_2-purged treatment.

The percentage of electrolyte leakage was greater and the total amount of electrolytes was less in the O_2-purged than the aerated treatment 4 and 9 DAT (Fig. 2). The percentage of electrolyte leakage declined for the trees in the O_2-purged treatment and the total amount of electrolytes increased linearly over time.

Alcohol dehydrogenase enzyme activity was detected in root tips of trees in all treatments (Fig. 3). Mean root ADH activity in both the O_2-purged and aerated treatments ranged from 5 to 125 nmol NADH·min^{-1}·mg protein^{-1}. The ADH activity was considerably higher for trees in the O_2-purged treatment than those in the aerated treatment beginning 2 d after treatments were begun. These differences were not statistically different ($P > 0.05$), presumably due to the between-tree or between-root variability.

Discussion

Leaf gas exchange for 'Pantin' and 'Magaña' trees in the O_2-purged hydroponic treatment declined similarly as observed in a previous study of the same cultivars subjected to soil flooding (Nickum et al., 2010). In that study, trees in a gravelly loam soil, typical of the soils found the mamey sapote production areas of southern Florida, were subjected to several days of continuous soil flooding and net CO_2 assimilation of trees significantly declined after 3 d of flooding and continued to decline to their lowest points after 7 to 10 d (Nickum et al., 2010). In this study, the decline in net CO_2 assimilation for trees in the O_2-purged treatment indicated that plant metabolism was negatively affected as a result of reduced oxygen availability in the root zone 2–4 d after flooding. However, in the present study, net CO_2 assimilation of 'Pantin' trees in the aerated treatment also declined over time, but at a slower rate than trees in O_2-purged treatment. Thus, for 'Pantin', the amount of O_2 bubbled into the hydroponic medium may not have been sufficient to sustain net CO_2 assimilation at their maximum values. It was observed for both cultivars by 10 DAT that leaves of several trees in the O_2-purged treatment began to abscise, whereas leaves of trees in the aerated treatment remained intact. Thus, although net CO_2 assimilation of 'Pantin' in the aerated treatment declined somewhat, those trees were experiencing less hypoxia than trees in the O_2-purged treatment.

Trees in the O_2-purged treatment exhibited a rapid increase in the percentage of electrolytes lost over time, whereas there was no increase of electrolyte leakage for trees in the aerated treatment. Similarly, Crane and Davies (1987) found an increase in root electrolyte leakage from flooded rabbiteye blueberry (*Vaccinium ashei*) plants after 6 d of flooding. In addition, Ojeda et al. (2004) found that root electrolyte leakage was greater for flooded than non-flooded seedling trees of *Annona glabra* L. (pond apple) and *Annona muricata* L. (soursop).

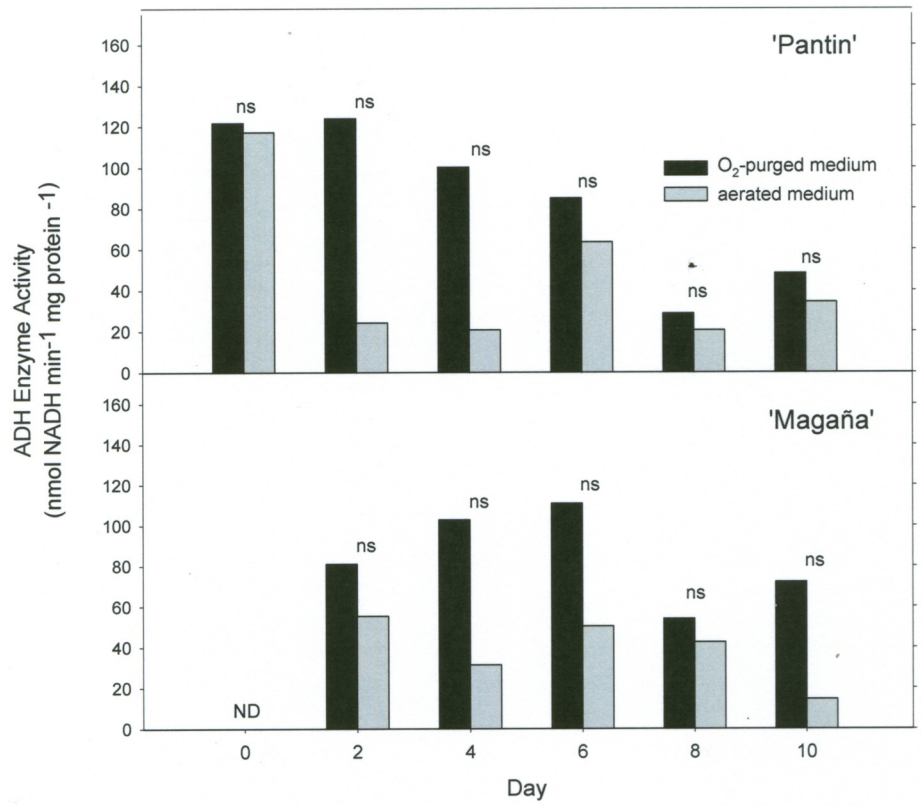

Fig. 3. Alcohol dehydrogenase enzyme activity in roots of 'Pantin' and 'Magaña' mamey sapote scions on seedling mamey sapote rootstock grown in O_2-purged or aerated hydroponic media during 0 to 10 days. ND indicates that enzyme activity was not detected; NS indicates no significant difference ($P > 0.05$) between treatments determined by a non-paired *t*-test, n=7.

Significant levels of ADH activity in the root were detected on all sampling dates, except on day 0 for 'Magaña' trees, where ADH activity, if present, was below our detection limits. Levels of root ADH activity in *Pouteria sapota* were similar to those reported for maize (*Zea mays*), 60 to 360 nmol NADH·min^{-1}·mg protein^{-1} (Kato-Noguchi, 2000), *Lepidium latifolium*, 150 to 500 nmol NADH·min^{-1}·mg protein^{-1} (Chen and Qualls, 2003), and *Arabidopsis thaliana*, 50 to 480 nmol NADH·min^{-1}·mg protein^{-1} (Chung and Ferl, 1999). Root ADH activity in non-flooded swamp tupelo [*Nyssa sylvatica* var. *biflora* (Walt.) Sarg.] seedlings ranged from 100 to 125 nmol NADH·min^{-1}·mg protein^{-1}, which increased to 200 to 300 nmol NADH·min^{-1}·mg protein^{-1} after 30 d of flooding (Angelov et al., 1996). Flood-tolerant *Melaleuca cajuputi* seedlings exhibited non-flooded ADH levels of 500 to 900 nmol NADH·min^{-1}·mg protein^{-1}, and flooded levels up to 1700 nmol NADH·min^{-1}·mg protein^{-1} (Yamanoshita et al., 2005). The ADH enzyme activity observed in roots of *Pouteria sapota* in this study lies well within the ranges of these species.

Root ADH activity appeared to be up-regulated as a result of oxygen depletion in the root zone, beginning 2 DAT. The lack of statistical significance for either scion was presumably due to within-tree or within-root variability. This variability may have been reduced if we used a larger sample size but due to the amount of time required to sample tips and extract and analyze ADH, a larger sample size may have introduced temporal variability into the data set. Nevertheless, for both cultivars the trend was consistent for each sampling period, beginning 2 DAT. Thus, root ADH activity appears to be up-regulated in mamey sapote trees grafted onto seedling rootstocks The capacity for increased ADH activity in mamey sapote is similar to observations with other plant species, including mesic adapted forest species (Angelov et al., 1996; Yamanoshita et al., 2005). However, increased ADH activity alone is apparently not sufficient to limit stress of mamey sapote trees under very low soil oxygen conditions as evidenced by decreased net CO_2 assimilation, leaf abscission and increased root electrolyte leakage of trees exposed to low oxygen content in the root zone.

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