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Low Temperature Induced Azide-insensitive Oxygen Uptake in Grapefruit Flavedo Tissue

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Abstract. **O_2 uptake by tissues of grapefruit (*Citrus paradisi* Macf.) was measured using manometric Warburg techniques. The highest rate of uptake was by flavedo tissue and the lowest rate was by intact juice vesicles. O_2 uptake by flavedo tissue increased exponentially when temperature increased, and the proportion of O_2 uptake insensitive to azide (N_3), an inhibitor of the cytochrome path, was greater at low temperatures than at high temperatures. Salicylhydroxamic acid (SHAM), an inhibitor of the alternative path, was only marginally inhibitory at all temperatures. Arrhenius-type plots of O_2 uptake showed discontinuities at 12°C in the presence and absence of respiratory inhibitors. O_2 uptake increased in flavedo tissue of grapefruit during the low temperature hardening of young trees. The increase was primarily in the N_3-insensitive component. Low temperatures also induced N_3-insensitivity in detached stored fruit, and induction was greater in chilling-resistant interior canopy fruit than in chilling-susceptible exterior fruit. Thus, N_3-insensitive O_2 uptake may be related to chilling resistance of sensitive fruit.**

Grapefruit harvested in late February or early March after the trees have been exposed to low night temperatures generally are more resistant than early or late harvested fruit to chilling injury (22, 23, 24). Grapefruit harvested from the interior canopy of the tree also are more resistant than exterior canopy fruit to chilling injury (20, 21), The nature of the resistance mechanism is not known, although chilling-sensitive and chilling-resistant plant species differ in their respiratory responses to low temperature. Arrhenius-type plots of respiratory rates of chilling-sensitive species show breaks or discontinuities at temperatures where chilling injury occurs (13, 14). Similar plots of respiratory activity for chilling-resistant species show no breaks or discontinuities. Chilling-sensitive plant species also exhibit accelerated respiratory rates immediately after transfer from chilling to warmer temperatures, whereas chilling-resistant species show little or no increase in respiratory activity (3, 4, 5).

The peculiar respiratory behavior of chilling-sensitive species is believed to result from low temperature decreasing the flexibility of mitochondrial membranes (13). However, differences in the respiratory pathways of chilling-resistant and chilling-sensitive species also may exist. Plant mitochondria can possess 2 paths of electron transport: the conventional cyanide-sensitive cytochrome path and a cyanide- and N_3-insensitive alternative path (26). Cultivars of wheat with high alternative path activity are more frost hardy than those with low alternative path activity (1, 15). The respiration of buds of early blooming pear species at low temperatures also is less sensitive to cyanide than the respiration of buds of late blooming species (2). Flavedo tissue (colored portion of the rind) of chilling-resistant orange is less sensitive than similar tissue of chilling-sensitive grapefruit to cyanide (19). However, it is not known whether the difference in response to respiratory inhibitors contributes to the resistance of plant tissues to chilling injury.

The purpose of this study was to investigate the effects of temperature on the respiratory activity and respiratory pathways of various grapefruit tissues that differ in their resistance to chilling injury.

Materials and Methods

Plant material. 'Marsh' grapefruit harvested from research plots at the Univ. of Florida Citrus Research and Education Center, Lake Alfred, Fla., were utilized for all experiments except the controlled low temperature hardening experiment. For this latter experiment, 4-year-old containerized 'Marsh' grapefruit trees were hardened in controlled environmental chambers as previously described (24). The hardening regime consisted of consecutive 7-day exposures to 20°, 15°, 10°, and 5°C. Temperatures during hardening were maintained within ± 0.5°, and the photosynthetic photon flux during the 12-hr photoperiod was 400 μmol $s^{-1}m^{-2}$ at the top of the 3-feet-tall trees.

Respiratory measurements were begun on tissues of fruit within an hour after harvest or removal from storage. Storage was in fiberboard cartons in rooms maintained at 5° ± 0.5°C.

Respiratory measurements. O_2 uptake was determined for tissues of individual fruits, each fruit considered a sampling unit. Flavedo tissue was removed in 5-mm wide strips, 1–2 mm thick, and cut into 10 mm lengths. The strips were rinsed in deionized water, blotted with paper towelling, and kept on ice until weighings were made and O_2 uptake determinations were begun using manometric Warburg techniques. Ten pieces of tissue, weighing about 1 g, were used for each determination. Strips of the spongy albedo tissue were removed and prepared in a manner similar to that used for flavedo tissue. However, the albedo tissue was not rinsed prior to use, since it readily absorbs water. Juice vesicles were removed from the segment membranes and walls without rupture. Intact vesicles were unrinsed.

The tissue was weighed to the nearest mg and placed in 2 ml of 50 mM potassium phosphate buffer, pH 6.5, with or without the respiratory inhibitors, N_3 and/or SHAM, each at a concentration of 2 mM. N_3 was used as an inhibitor of the cytochrome path, since it is less volatile than cyanide and does not require special manometric techniques (12). Preliminary results with N_3 were similar to those obtained with cyanide (19). SHAM was used as an inhibitor of the alternative path (26). The center wells of the Warburg vessels contained fluted filter paper wicks with

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Table 1. O_2 uptake by flavedo and albedo tissue and intact juice vesicles of grapefruit.

Tissue	μl O_2 g dry wt^{-1} hr^{-1}	
	O_2 uptake at 5°C	O_2 uptake at 25°C
Flavedo	123 ± 9[z]	383 ± 25
Albedo	110 ± 9	352 ± 29
Juice Vesicles	15 ± 6	115 ± 53

[z]Mean ± SD of 8 samples.

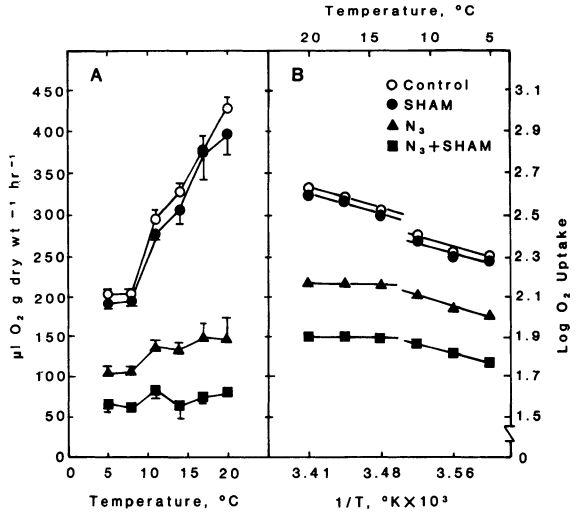

Fig. 1. **A.** Temperature effect on O_2 uptake by grapefruit flavedo tissue in the presence of inhibitors. ○——○, no inhibitors; ●——●, 2 mM SHAM; ▲——▲, 2 mM N_3; ■——■, 2 mM SHAM + 2 mM N_3. **B.** Arrhenius-type plots of O_2 uptake rates in A. Each point represents the mean ± SD of 6 samples from 2 separate experiments.

0.5 ml of 4 N KOH to absorb CO_2 evolved by the tissue. O_2 uptake was determined at temperatures from 5° to 25°C. A thermobarometer was used to correct for variation due to minor pressure changes. The Warburg vessels and contents were temperature-equilibrated for 15 min with the stopcocks open and another 15 min with the stopcocks closed prior to measurements of O_2 uptake. Manometers were read at 5 and 20 min intervals for high and low temperature experiments, respectively. Rates were calculated from linear uptake rates during the first hr at high temperatures and the first 2 hr at low temperatures.

Following measurements of O_2 uptake, the tissue was removed from the vessels, dried for 24 hr at 70°C, and weighed. Results are expressed on a dry weight basis. All treatments were replicated in 3 vessels with tissue from 3 different fruits, and each experiment was repeated at least twice except when otherwise noted.

Results

At both 5° and 25°C, flavedo tissue had the highest rate of O_2 uptake, and intact juice vesicles had the lowest rate of uptake (Table 1). Wound respiration was considered to be related to the normal respiratory rates of the intact tissues. Since chilling injury symptoms appear initially and primarily in flavedo tissue, only O_2 uptake by flavedo tissue was examined in subsequent experiments.

O_2 uptake increased as the temperature increased from 5° to 20°C (Fig. 1A). SHAM was marginally inhibitory at all temperatures. In contrast, the relative inhibition by N_3 either alone or in combination with SHAM, was greater at nonchilling than at chilling temperatures. The proportion of O_2 uptake sensitive to N_3 increased from 50% to 70% as temperature increased from 5° to 20°. Arrhenius-type plots of the O_2 uptake rates showed discontinuities at about 12° in the presence and absence of inhibitors (Fig. 1B).

Rates of O_2 uptake tended to increase during low temperature hardening of young trees (Fig. 2A). The percentage of O_2 uptake insensitive to N_3 also increased (from 30% to 65%) as hardening temperatures were reduced from 20° to 5°C (Fig. 2B). At the same time, the percentage of O_2 uptake insensitive to both N_3 and SHAM increased from 10% to 30%.

During the first week of storage at 5°C, respiratory rates in the absence of inhibitors did not change (Fig. 3A, B). However, O_2 uptake became insensitive to N_3 during storage of the fruit at 5°, and the interior fruit was less sensitive than the exterior

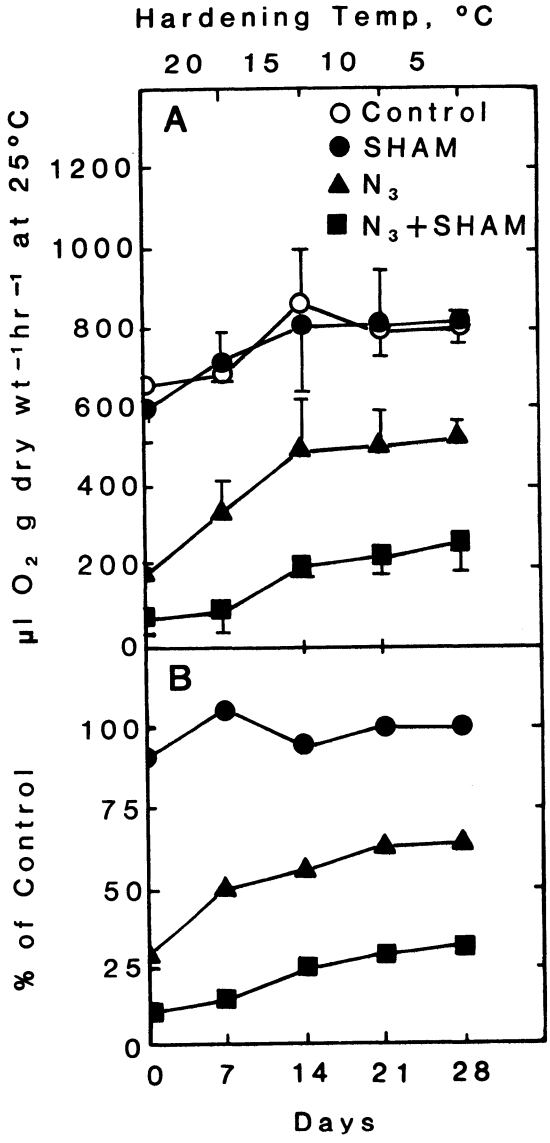

Fig. 2. **A.** Induction of N_3-insensitive O_2 uptake in grapefruit flavedo tissue during low temperature hardening of young trees. **B.** Percentage of uninhibited O_2 uptake. Inhibitor concentrations same as in Fig. 1. Each point represents the mean ± SE in 3 samples from one experiment.

fruit to N_3. After 3 weeks at 5°, 70% to 80% of the O_2 uptake at 5° by interior fruit was insensitive to N_3, whereas only 35% to 40% of the O_2 uptake by exterior fruit was insensitive to N_3 (Fig. 3C, D).

At 25°C the N_3-insensitive component of O_2 uptake was apparent only in tissue of interior fruit stored at 5° for 2 weeks or longer (Fig. 4A, B). At 25°, the N_3-insensitive component represented only 40% of the total O_2 uptake by interior fruit and about 25% of the O_2 uptake by exterior fruit (Fig. 4C, D).

Discussion

Results of this study tend to support the idea that chilling sensitivity may be related to the respiratory activity of the tissue. The flavedo tissue is the first and frequently the only tissue of grapefruit to manifest symptoms of chilling injury (7, 18, 20). It also has a higher rate of respiration than other tissues of the grapefruit. Apparently, the outermost tissues of fruits characteristically have higher rates of metabolism than other tissues of the fruit (6, 8, 17).

By contrast, the respiratory rate of whole grapefruit increases during the winter months (25), and, in this study, respiration of fruit tissues increased during the low temperature hardening of young trees. Yet, the seasonal trend of grapefruit to resist chilling injury occurs during the time when respiratory rates appear to be highest (22, 23). However, the relative proportion of O_2 uptake insensitive to N_3 increased in the tissue of fruit exposed to low temperatures, either on the tree or in storage, and the increase in interior fruit was greater than in exterior fruit. The relative proportion of O_2 uptake insensitive to cyanide (the alternate path) also increased in tissues of other plant species exposed to low temperatures (1, 2, 9, 10). Thus, a greater proportion of O_2 uptake is insensitive to N_3 and/or cyanide at chilling than at nonchilling temperatures (2, 15, 27, 29).

It is not clear from this study whether the N_3-insensitive alternative path was active in the absence of N_3. SHAM was only marginally inhibitory to O_2 uptake at all temperatures except when N_3 was present; and in other studies (26, 28), this observation has been interpreted to mean that the alternative path, although present, is not functioning. However, temperatures below 15°C diverted the bulk of respiratory electron flow to the cyanide-insensitive alternative path in chilling-sensitive *Cornus stolonifera* callus (29). Similarly, when roots of *Plantago lanceolata* were transferred from 21° to 13°, cytochrome path activity decreased to about 60% of the initial activity, and alternative path activity increased by a similar amount (27).

In general, the role of the N_3- and cyanide-insensitive alternative path in most plant tissues is not clear. Although the temperature of tissues of the aroids increases several degrees when the alternative path is operating (16), it is unlikely that in most other species, the alternative path, even when it is maximally operating, generates enough heat to protect them against frost damage. Lambers (11) has postulated that the alternative path serves as an energy overflow and is active when there is an

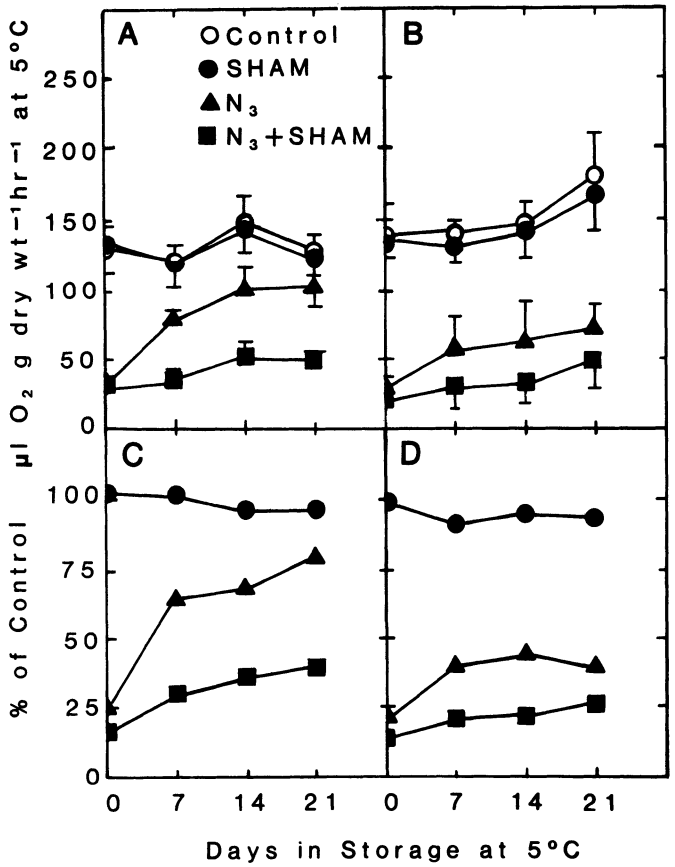

Fig. 3. Respiration at 5°C of grapefruit flavedo tissue during storage of detached fruit at 5°. **A.** Interior canopy fruit. **B.** Exterior canopy fruit. **C.** Percentage of uninhibited O_2 uptake by interior canopy fruit. **D.** Percentage of uninhibited O_2 uptake by exterior canopy fruit. Inhibitor concentrations same as in Fig. 1. Each point represents the mean and SD of 6 samples from 2 separate experiments.

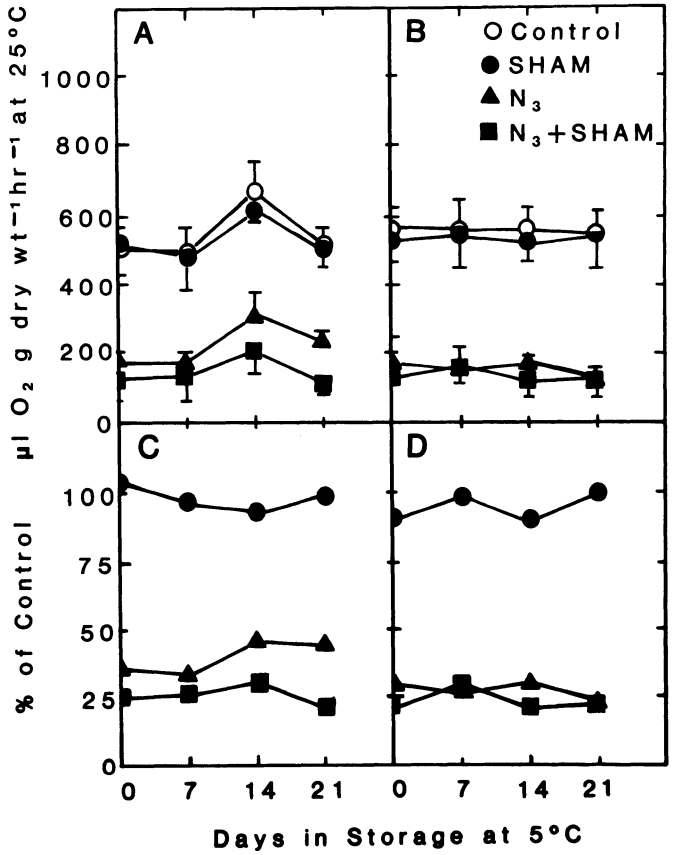

Fig. 4. Respiration at 25°C of grapefruit flavedo tissue during storage of detached fruit at 5°. **A.** Interior canopy fruit. **B.** Exterior canopy fruit. **C.** Percentage of uninhibited O_2 uptake by interior canopy fruit. **D.** Percentage of uninhibited O_2 uptake by exterior canopy fruit. Inhibitor concentrations same as in Fig. 1. Each point represents the mean and SD of 6 samples from 2 separate experiments.

imbalance between the supply of carbohydrates and the requirement of carbohydrates for structural growth, energy production, storage and osmoregulation. It is interesting to note that the level of reducing sugars increases in the flavedo tissue of grapefruit exposed to chilling temperatures (22, 23, 24, 25).

Whether N_3-insensitive respiration contributes to the resistance of interior canopy fruit to chilling injury is not clear. Nevertheless, differences in the effects of chilling temperature on paths of respiratory electron transfer exist between chilling-resistant interior and chilling-susceptible exterior grapefruit. However, further studies are necessary to determine the role of N_3-insensitive respiration in grapefruit tissues and how it is regulated.

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