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Physiological Factors Affecting Response of Mature 'Valencia' Orange Fruit to CMN–Pyrazole. II. Endogenous Concentrations of Indole-3-Acetic Acid, Abscisic Acid, and Ethylene

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Abstract. **Endogenous concentrations of IAA and ABA in the peel, pulp, seed, and abscission zone of mature 'Valencia' oranges [*Citrus sinesis* (L.) Osbeck] were determined by high-performance liquid chromatography and enzyme-linked immunosorbent assay from early November 1998 to mid-June 1999. Ethylene production of mature 'Valencia' oranges during the same period was determined by gas chromatography. IAA concentrations in the pulp and seed were three to five times lower than those in the peel over the 7-month observation period. IAA concentration in the abscission zone and peel was high from late April to mid-May, the period of less responsiveness to abscission chemicals. ABA concentration in the pulp was low over the entire observation period. ABA concentration in the abscission zone and peel was low during the less responsive period. Ethylene production was always low except for a slight increase during late December and early February. The IAA to ABA ratio was high in the fruit abscission zone during the less responsive period. Fruit detachment force of CMN–pyrazole-treated fruit was positively correlated with the ratio of endogenous IAA to ABA or endogenous IAA, but negatively to endogenous ABA in the fruit abscission zone. These data suggest the balance between IAA and ABA in the fruit abscission zone may be an important factor in determining sensitivity and thereby the response of mature 'Valencia' orange fruit to abscission chemicals. Chemical names used: abscisic acid (ABA); indole-3-acetic acid (IAA); 5-chloro-3-methyl-4-nitro-1*H*-pyrazole (CMN–pyrazole).**

Most of the 'Valencia' oranges (*Citrus sinesis*) grown in Florida are used for processing and are harvested from early April through June (Wilson et al., 1981). Abscission chemicals can effectively reduce detachment force of mature 'Valencia' orange fruit and promote fruit abscission from mid-February through April. Then there is a period of 2 to 4 weeks during which the response of mature fruit to abscission chemicals is markedly reduced. Abscission chemicals become effective again after this period (Hartmond et al., 2000a; Holm and Wilson, 1976, 1977; Wheaton et al., 1977). However, little is known about the factors responsible for the less responsive period.

Oranges are nonclimacteric fruit and mature fruit abscise only at the abscission zone in the calyx (Brown, 1997; Goren, 1993). Endogenous plant hormones are involved in fruit abscission. The concentration of endogenous auxin in the abscission zone must decrease below a certain threshold to promote abscission (Osborne, 1989). In oranges, endogenous auxin and ethylene are two major hormones controlling the fruit or leaf abscission process (Goren, 1993). Application of 2,4 dichlorophenoxyacetic acid (2,4-D), a synthetic auxin, effectively reduced preharvest drop of orange fruit (Gardner et al., 1950; Zur and Goren, 1977). Sprays of abscisic acid (ABA) at 200 $mg \cdot L^{-1}$ enhanced both ethylene production and fruit loosening of mature 'Valencia' oranges induced by the abscission chemical cycloheximide (CHI) (Cooper and Henry, 1972). Using explants of 'Pineapple' orange fruit and 'Valencia' orange fruit, Rasmussen (1974) found that ABA introduced through the stem was more effective than spray application in promoting cellulase activity and reducing fruit detachment force. During the harvest season, new flushes, young fruit for the following year's crop, and roots grow rapidly. These young tissues are rich sources of endogenous plant hormones (Goldschmidt, 1976; Hofman, 1990; Plummer et al., 1991). Therefore, it has been speculated that endogenous plant hormones from these young tissues account for the reduced response of mature fruit to abscission chemicals in 'Valencia' oranges (Holm and Wilson, 1976; Rasmussen, 1973; Wheaton et al., 1977).

In a companion paper, we investigated seasonal variation in the response of mature 'Valencia' orange fruit to the abscission chemical CMN–pyrazole particularly with respect to the influence of young fruit, shoot, and root growth on this response (Yuan et al., 2001). The objective of the present study was to 1) determine the levels of endogenous plant hormones, especially indole-3-acetic acid (IAA), ABA, and ethylene from early November to mid June, and 2) evaluate the relationships between these endogenous hormones and the responsiveness of mature 'Valencia' orange fruit to the abscission chemical CMN–pyrazole.

Materials and Methods

Plant materials. Nine uniform 10-year-old 'Valencia' orange trees grafted on rough lemon (*Citrus jambhiri* Lush) rootstock, were selected from a previously described grove located at the Citrus Research and Education Center, Lake Alfred, Fla. (Yuan et al., 2001), and separated into three replicate groups of three trees each. Fifteen fruit having pedicels 3 to 4 cm in length were collected from each tree at 1- or 2-week intervals beginning 12 Nov. 1998 and ending 15 June 1999. Forty-five fruit from each group at each sampling time were pooled and immediately

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separated into peel, pulp, seed, and abscission zone. The fruit abscission zones were removed according to the method of Kazokas and Burns (1998). Promptly after the separation of fruit, samples were frozen in liquid nitrogen, and lyophilized. All plant materials except the abscission zone were ground in a Wiley mill to pass a 40-mesh (0.635-mm) screen, and used for measurement of free IAA and ABA. An additional 15 fruit were collected on each sampling date and used for determination of fruit ethylene production. Ten fruit from each replicate were also collected, washed, and used for measurement of fruit color.

Extraction, purification, and measurement of free IAA and ABA. The method of Bertling and Lovatt (1996) was modified to extract and partially purify plant materials for free IAA and ABA. Briefly, 3 g of ground peel, pulp, or seed, were placed in an Erlenmeyer flask. To each Erlenmeyer flask, 80 mL of 80% methanol containing butylated hydroxytoluene (BHT) at 40 $mg \cdot L^{-1}$ as an antioxidant was added, and the tissue was extracted overnight at 4 °C. The sample of abscission zone tissue was placed in a plastic centrifugation tube with 30 mL of 80% methanol containing BHT at 40 $mg \cdot L^{-1}$, pulverized using a Brinkmann rotary homogenizer (Brinkmann Instruments Co., Westbury, N.Y.), transferred to an Erlenmeyer flask holding another 50 mL of 80% methanol containing BHT at 40 $mg \cdot L^{-1}$, and then extracted overnight at 4 °C. One hundred microliters of ^{3}H-IAA (≈67 Bq) and ^{14}C-ABA (≈83 Bq) were added as internal standards to the plant samples to determine the recovery rate. The crude extract was filtered through Whatman No. 2 filter paper and the residue was reextracted twice overnight at 4 °C in 80 mL of 80% methanol containing BHT at 40 $mg \cdot L^{-1}$. The filtrates were combined and evaporated to the aqueous phase in vacuo at 35 °C. The aqueous phase was stored at –20 °C overnight, thawed, and centrifuged at 15,000 g_n for 20 min. The supernatant was adjusted to pH 8.0 with 5% NH_4OH and loaded onto a column system that was first preconditioned and washed with 15 mL of 1.0 M ammonium acetate (pH 8.0) and subsequently with 15 mL of 0.01 M ammonium acetate (pH 8.0). The column system consisted of an insoluble polyvinyl pyrrolidone (PVP) (10 × 1.5 cm) and a diethylaminoethyl (DEAE) Sephadex anion exchange column (10 × 1.5 cm). The column system was eluted with 0.01 M ammonium acetate (pH 8.0). A Sep-Pak C_{18} cartridge (Waters, Milford, Mass.) that was first preconditioned with 100% methanol and subsequently with 0.1 M acetic acid was attached to the DEAE column of the column system. IAA and ABA were eluted with 1.5 M acetic acid, and collected at the Sep-Pak C_{18} cartridge, which was rinsed with 10 mL of distilled water before IAA and ABA were eluted with 5 mL of 50% methanol. The eluates were dried in vacuo and dissolved in 2 mL of 100% high performance liquid chromatography (HPLC) grade methanol.

The samples were further purified by HPLC according to the method of Miller et al. (1987) with some modification. In brief, a 500 µL-sample containing IAA and ABA was applied to a 250 × 4.6 mm Whatman Partisil ODS-3 C_{18} reverse phase column (5-µm particle size) at a flow rate of 1.0 $mL \cdot min^{-1}$ with a 15% to 80% (v/v) gradient over a period of 50 min in a solution of 1% acetic acid, followed by an increase to 100% methanol over 5 min. Fractions corresponding to IAA and ABA were collected and dried in vacuo. ABA fractions were diluted with 25 mM Tris buffer (pH 7.5) before quantification by an enzyme-linked immunosorbent assay (ELISA). Tris buffer was prepared by diluting 3.03 g Trizma base, 5.84 g NaCl, 0.2 g $MgCl_2.6H_2O$, and 0.2 g NaN_3 in 1 L of distilled water and adjusted to pH 7.5 with HCl. IAA fractions were resuspended in 250 µL of methanol, methylated with diazomethane, dried in vacuo, and redissolved in Tris buffer (pH 7.5) for assaying by ELISA. IAA and ABA were quantified by ELISA using monoclonal antibodies against IAA and ABA (Agdia Inc., Elkhart, Ind.) and the ELISA procedures were conducted as recommended by the manufacturer. The recovery rate was ≈70.2% for IAA, and 52.4% for ABA.

To determine the less-responsive period, groups of 20 uniform mature fruit were selected , marked, and sprayed with CMN–Pyrazole at 150 $mg \cdot L^{-1}$ on each of four replicate trees on each date

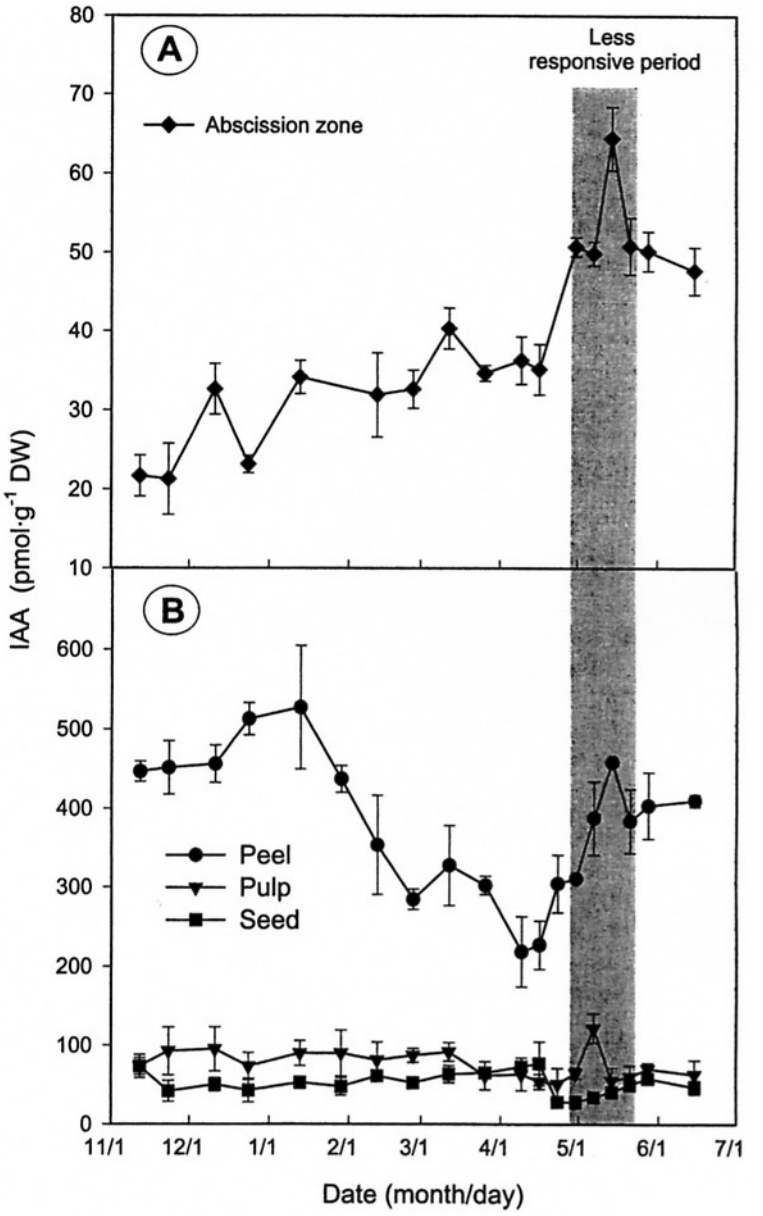

Fig. 1. Endogenous IAA concentrations in the (**A**) abscission zone and (**B**) peel, pulp, and seed of 'Valencia' orange fruit in the 1998–99 season. Data are means ± SE (n = 3).