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The Influence of Ethylene Treatment of Immature Fruit of Prune (*Prunus domestica* L.) on the Enzyme-mediated Isolation of Mesocarp Cells and Protoplasts[1]

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Abstract. **Immature fruit of 'French' prune were treated in air or ethylene and mesocarp tissues incubated with crude cell wall degrading enzymes to release cells and protoplasts. Ethylene treatment substantially reduced the release of cells and protoplasts and increased the proportion of pectic polymers in the cell walls.**

It is well established that cell enlargement is associated with increases in turgor pressure (9). Cell enlargement, however, is also dependent on the extensibility of the cell wall (3, 16), and changes in cell enlargement rate have been correlated with alterations in cell wall composition (12, 13). Changes in wall composition are strongly correlated with the decrease in flesh firmness of ripening fruit (10, 11).

The ethylene generating compound ethephon causes rapid cessation of peach and prune fruit enlargement (18, 22). Martin et al. (14) found that ethephon applied a month following anthesis resulted in an increase in the firmness of prune flesh at harvest. This may have resulted from ethylene-induced modification of cell size [as ethephon was applied after cessation of cell division, (5)] or cell wall composition.

The objective of this work was to determine whether ethylene affects walls of mesocarp cells of immature prune fruit. Our assessment was based on (a) altered lability of tissue slices to the action of crude cell wall degrading enzyme mixtures commonly used in the making of protoplasts and (b) compositional changes in mesocarp cell wall preparations.

Materials and Methods

Ethylene treatment of plant material. Fruited branches of 'French' prune were removed from trees between 25 and 60 days after anthesis. The detached branches were exposed to air or to 15 µl/liter ethylene for 60 hr as previously described (18, 21).

Cell and protoplast isolation. Immediately following treatment, mesocarp of air- and ethylene-treated fruit was sliced 1-2 mm thick. One g samples of the freshly-sliced tissue were incubated at 30°C in a mixed enzyme solution containing sorbitol-d-hydrate (0.7 M), Mg $Cl_2 \cdot 6H_2O$ (1 mM), KH_2PO_4 (2 mM), cysteine (5 mM), 0.5% Macerase (Calbiochem) and 1% Cellulysin (Calbiochem). The medium was adjusted to pH 5.8 with KOH. The tissue was vacuum-infiltrated with the enzyme solution for three 2-min periods and agitated in 15 ml of the same solution on a rotary shaker (100 cycles/min) at 30°C. Thirty minutes later the solution was poured through 2 layers of cheesecloth and the filtrate, containing damaged cells and cell debris, was discarded. Freshly mixed enzyme solution was added and, after 3 hr, cells and protoplasts were collected by centrifugation (8 min at 100 x g), washed, and transferred to White's medium. Cell and protoplast viability were assessed on the basis of (a) general appearance under the phase contrast microscope, (b) observation of cyclosis, (c) fluorescein diacetate hydrolysis (17, 23) and (d) exclusion of methylene blue dye by intact membranes (8, 23).

The isolated protoplasts and cells were counted in a haemocytometer at 250 x magnification.

Cell wall isolation and polysaccharide analysis. Fruited branches were treated with air or ETH as previously described. Mesocarp tissue was frozen in liquid N_2, lyophilized, and ground in a Wiley mill. The ground mesocarp was washed with water and centrifuged 3 x at 1200 x g. The pellet was then washed in a high salt buffer (0.5 M phosphate buffer, pH 7) to remove adsorbed electrolytes. Following centrifugation the pellet was incubated for 30 min at 30°C in 1 CH_3Cl:1MeOH to extract lipids. Finally, the dried CH_3Cl:MeOH-washed pellet was incubated in 2 changes of alpha-amylase (from *B. subtilis*; ICN Pharmaceuticals, Inc.) (in 50 mM phosphate buffer, pH 7; 1 mg enzyme/ml) over 24 hr. The residue constituted the "cell wall" preparation.

The non-cellulosic neutral sugar composition of the cell walls was measured, following hydrolysis in 2N trifluroacetic acid (TFA), by gas-liquid chromatography (2). Gas chromatographic peaks were analyzed on the Autolab 6300 digital integrator interfaced to the chromatograph (Varian Model 1520B) flame ionization detector. The column used was a 120 x 0.3 cm (outside diameter) copper column containing a liquid phase mixture of 0.2% ethylene glycol adipate, 0.2% ethylene glycol succinate and 0.4% silicone XF-1150 on a solid support of Gas Chrom P (100-120 mesh) (20). Temperature programming for alditol acetates was from 125° to 170°C at a rate of 1°/min with a nitrogen flow rate of 20 ml/min.

Uronic acid composition was determined colorimetrically using the technique of Ahmed and Labavitch (1).

Results and Discussion

Cell and protoplast isolation. A pronounced turbidity appeared in the mixed enzyme solution containing tissue slices of air- but not ethylene-treated fruit within the first hr of incubation. Altered lability of tissue slices to enzymatic digestion was verified by counting the isolated cells and protoplasts. Ethylene treatment reduced the combined protoplast plus cell yield between 90% and 30% in 3 experiments conducted 39 to 51 days after full floom (AFB), (Table 1). Thirty-nine days AFB the standard isolation medium (see Methods) liberated primarily protoplasts, whereas cell walls 47 and 51 days after full bloom (AFB) were apparently incompletely hydrolyzed by the same medium. As a result, the cell:protoplast ratio was increased in the latter 2 isolations. No differential effects of treatment on the qulity of isolated cells and protoplasts were apparent within 24 hr after isolation. Raising the concentration of Cellulysin to 2.0% increased protoplast yield 60 days AFB. Since protoplast release from plant tissues depends on the dissolution of cell walls, the effect of ethylene was presumed to be on some component(s) of the wall complex. Cell walls were prepared from air- and ethylene-pretreated fruit tissues, and samples of these walls were incubated with the enzyme mixture used to prepare protoplasts. No differences in either the amounts

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Table 1. Effect of ethylene treatment on enzymic release of cells from mesocarp tissue.

Days AFB	Treatment	No. of cells and protoplasts released/gram fresh wt[z] ($\times 10^{-5}$)[y]
39	Ethylene	1.75 ± 0.25
	Air	3.25 ± 0.10
47	Ethylene	2.80 ± 0.10
	Air	4.00 ± 0.26
51	Ethylene	1.17 ± 0.12
	Air	1.85 ± 0.14

[z]Each value is the mean of 2 duplicates ± 1 SE.
[y]Determinations made after 3 hr in mixed enzyme solutions; however, visual differences (turbidity) between treatments were most dramatic within 1 hr.

of carbohydrate solubilized (determined by anthrone, 6) or the amounts of reducing groups generated (determined according to the technique of Nelson (15) as modified by Somogyi, 19) were observed.

Ethylene treatment resulted in cell walls that contained 50% more uronosyl residues than in walls prepared from air-treated (control) tissues (Table 2). In addition, mesocarp cell walls from ethylene-treated tissues contain more arabinose. This indicates a higher level of pectic polysaccharides in treated fruit since arabinans have been shown to be covalently bound to uronic acid-rich polysaccharides in walls of vegetative cells (20) and from peaches and pears (Labavitch, unpublished results). Whether these data indicate that there is increased synthesis of pectic polymers in response to ethylene or that the hormone reduces the loss of pectins during fruit growth or cell wall preparation is not clear.

The relation between increased amounts of cell wall pectin and decreased production of protoplasts is open to speculation. Both Cellulysin and Macerase contain large amounts of polygalacturonase so it is unlikely that pectolytic activity is limiting. Cassells and Barlass (4) correlated the difficulty in preparing protoplasts from tomato leaf with the cell wall content of calcium pectate. Presumably the cross-linking of pectin carboxyl groups by Ca^{++} makes pectin degradation difficult. However, no differences in calcium content of walls from ethylene- or air-treated tissues were observed using flame photometry (data not presented). *In vivo* differences in Ca^{++} content could have been obscured by the extraction technique.

It is not clear whether the relative reduction of xylose in walls from ethylene-treated fruit (Table 2) is biologically significant or due to the increase in arabinose and uronic acids.

Table 2. Carbohydrate composition of cell walls prepared from ethylene- and air-treated mesocarp tissues.

Carbohydrate composition	Treatment	
	Air	Ethylene
Non-cellulosic neutral sugars		
Rhamnose[z]	4.7 ± 0.1	6.0 ± 0.1
Fucose[z]	1.1 ± 0.0	1.6 ± 0.1
Arabinose[z]	29.8 ± 0.3	37.4 ± 0.4
Xylose[z]	33.5 ± 1.0	21.1 ± 0.7
Mannose[z]	1.8 ± 0.1	2.1 ± 0.2
Galactose[z]	26.3 ± 0.4	28.5 ± 1.2
Glucose[z]	2.8 ± 0.0	3.3 ± 0.1
Total[y]	35.9	39.6
Uronic acids[y]	22.2 ± 2.4	32.8 ± 0.8

[z]Data for individual sugars are expressed as a percentage of the total 2N TFA hydrolyzable sugars measured by GLC. Values are the average of 2 determinations ± SE.
[y]Data are expressed as a percent of initial cell wall dry weight.

Other wall sugars do not show decreases, however.

Ethylene suppression of auxin stimulated pea stem elongation and induction of cell swelling has been attributed to disorientation of cellulose microfibrils (7). Our cell wall analysis did not include measurement of wall cellulose content.

Enlargement of immature prune fruit is curtailed by ethylene (18). This effect is correlated with a) decreased yields of isolated cells and protoplasts following incubation of tissue slices with crude cell wall degrading enzymes and b) compositional changes in the cell wall. The functional relation, however, between ethephon-induced fruit thinning and cell wall metabolism has not been defined. Similarly, the relation between the level and nature of pectic polymers in the wall and the lability of the cell wall to enzymatic digestion and protoplast isolation awaits further definition.

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Relationship of Rootstock to Leaf and Juice Lipids in Citrus[1]

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Abstract. **Major leaf alkanes (C29-C33) of 2 scions on 10 rootstocks of citrus were examined by gas chromatography. A small but definite effect of the rootstock on the alkane profiles of the scions was observed. The effect of rootstock on alkane patterns in juice sacs was very small. Rootstock affected the fatty acid patterns of total and neutral lipids as well as of triglycerides and sterol esters.**

Information on the chemical composition of the epicuticular wax layer of citrus leaves is important if efficient use of sprays of growth regulators, leaf nutrients, pesticides, and antitranspirants is to be attained. Long-chain hydrocarbons (alkanes), the major component of this wax layer, vary among the citrus species both quantitatively (1) and qualitatively (1, 11). Of the many classes of compounds present in waxes, the alkanes are the least wettable. Since water repellency of plant surfaces affects chemical deposition, leaf waxes with high contents of alkanes and/or different hydrocarbon compositions would be expected to affect the deposition of chemicals and their absorption by the leaf. Another epicuticular wax surrounds the juice vesicles, and holds them together. The quality of citrus sections is related to the composition of this wax layer of which alkanes are a major component (12). Fatty acids are major lipid constituents found within the juice vesicle.

The majority of citrus trees grown today are budded on rootstocks, and there are numerous examples of scion-rootstock interactions (2, 3, 4, 5, 6, 13). Our previous studies (9, 10, 11) were conducted on seedling trees to avoid any rootstock effects on the hydrocarbon, fatty acid, and sterol profiles of the scions. The present experiment was conducted to study the effects of rootstock on leaf and juice lipids.

Materials and Methods

The leaves and fruit used in these studies were from Whitmore Foundation Farm (U. S. Horticultural Research Laboratory, Orlando, Florida). Leaves were from trees consisting of 2 scions, 'Orlando' tangelo (*Citrus paradisi* Macf. x *C. reticulata* Blanco) and 'Owari' satsuma (*C. reticulata*), on 10 rootstocks; Rusk (RSK) citrange [*C. sinensis* (L.) Osbeck] x [*Poncirus trifoliata* (L.) Raf.], Troyer (TROY) and Carrizo (CAR) citranges (*P. trifoliata* x *C. sinensis*), Large Flower trifoliate orange (LFTO) (*P. trifoliata*), Seville sour orange (SO) (*C. aurantium* L.), Cleopatra mandarin (CLEO) (*C. reticulata*), Sanquine Grosse Ronde sweet orange (SANG) (*C. sinensis*), Orlando tangelo (ORL) (*C. paradisi* x *C. reticulata*), Estes rough lemon (RL) [*C. limon* (L.) Burm. f.], and Milam (MIL) (rough lemon hybrid?).

The trees, planted on Astatula fine sand in 1965, were spaced 4.5 x 5.4 m in a randomized block with 3-tree plots. The trees had regular and uniform horticultural attention and were vigorous and fruitful. Six replicates of each scion-rootstock combination were taken from these 3-tree plots. A sample consisted of 20 to 30 mature leaves picked from each of the 3 trees on both September 8 and October 13, 1976.

The epicuticular wax was removed from the leaves by dipping them for 3 min in 200 ml chloroform. The alkanes were isolated from this extract by thin-layer chromatography (11). Fruit for studies on the alkanes and fatty acids of juice were obtained on December 14, 1976, from 18 of the same trees ('Orlando' on 6 rootstocks) used in the leaf alkane study. A sample consisted of 12 fruit picked from the south side of the tree. The fruit were cut in half, and the intact juice sacs were carefully separated from core, peel, seeds, and carpellary membrane with the aid of a citrus spoon. The lipids were extracted with Folch's reagent from a Celite pad (8) and the major portion of the lipid extracts fractionated into neutral lipids (NL), glycolipids (GL), and polar lipids (PL) (9). Triglycerides (TG) and sterol esters (SE) were isolated from a portion of the NL fractions by thin-layer chromatography (9). Methyl esters of the fatty acids (FAMEs) from total lipids (TL), NL, GL, and PL lipids, as well as from TG and SE, were prepared and purified along with the alkanes by thin-layer chromatography (9) and stored in hexane for gas-liquid chromatography (9). All alkanes and FAMEs were analyzed on a 182 cm x 0.6 mm glass 3% SP1000 on 100/120 Gas Chrom Q gas-liquid chromatographic column that was run isothermally at 160°C for FAMEs (9), and temperature programmed for juice sac (10) and leaf (11) alkanes. Duplicate gas-liquid chromatographic analyses were run on each leaf alkane sample. A single gas-liquid chromatographic analysis was run on each of the six juice-sac alkane samples obtained (3 from TL, 3 from NL) from each scion-rootstock combination. Duplicate gas-liquid chromatographic analyses were run on the TL, NL, TG, and SE FAME samples, whereas a single analysis was run on each of the GL and PL FAME samples.

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