

## Mechanical wounding and abscission in citrus

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Fruit detachment force (FDF), ethylene evolution, fruit and leaf drop were determined in *Citrus sinensis* for periods up to 96 h after mechanical wounding. Injury by removing a thin section of mature fruit flavedo reduced FDF, increased ethylene evolution and promoted abscission. Injuring flavedo 1 cm below the calyx was more effective at reducing FDF than injuring flavedo at the equator or the blossom-end of mature fruit. Injuring the calyx or peduncle of mature fruit, or injuring three leaves closest to the mature fruit did not reduce FDF. Immature fruitlets either did not abscise or underwent low rates of abscission in response to mechanical wounding, depending on age. Inhibiting ethylene binding in wounded mature fruit with 1-methylcyclopropene (1-MCP) increased ethylene evolution compared with wounded fruit alone, but the reduction in FDF was similar. When an ethylene biosynthesis inhibitor (aminoethoxyvinylglycine, AVG) was used, reduction in FDF of wounded mature fruit exposed to AVG was

similar to that of wounded fruit alone but ethylene production was markedly reduced. Wounding mature leaf blades in the presence or absence of 1-MCP resulted in elevated but equal ethylene evolution up to 48 h after wounding, however, no leaf drop occurred. Thereafter, ethylene evolution was higher in 1-MCP-treated wounded leaves. Removing up to 77% of the total mature leaf area did not cause leaf drop, nor did wounding tissue across the laminar or petiolar abscission zones. Leaflets of 5 mm length reached nearly 100% abscission after mechanical wounding, whereas wounding leaves 20 mm length resulted in 15% abscission. The data suggest that mechanical wounding of flavedo results in mature fruit abscission, and ethylene binding may not be mandatory to initiate abscission in citrus fruit. The differential response of fruit and leaves at different ages to wounding may be related to potential contribution to carbohydrate accumulation, and production and sensitivity of tissues to an abscission signal(s).

### Introduction

Abscission is a naturally occurring process that enables plants to shed their organs. A number of molecular and physiological events occur during abscission that begin with perception and propagation of an appropriate signal and culminate in organ separation (Roberts et al. 2002). Abscission can be triggered by a range of conditions, including excessive water loss and various forms of wounding (Cheong et al. 2002). Mechanical injury can result in abscission of a damaged organ (Blundell and Peart 2000, Klingeman et al. 2001). The probability of abscission occurring as a result of a mechanical wound may depend on plant species and organ being wounded, the developmental age of the affected organ, as well as its potential contribution to carbohydrate accumulation and seed dispersal.

Most research exploring the role of wounding on fruit abscission has focused on the effect of insect damage on immature fruit drop. Increased insect damage is

associated with increased immature fruit loss in some cases (Childers and Bullock 1999) but not in others (Mascarenhas et al. 1999, Follett and Gabbard 2000). Damage inflicted by insects is often a combination of mechanical injury and subsequent injury promoted by injection of chemical elicitors into afflicted tissue. Thus, damage induced by herbivore feeding is not necessarily similar to damage induced by mechanical injury (Walling 2000). Mechanically damaged immature leaves can be more prone to abscise than mature leaves, although this is not always the case (Blundell and Peart 2000). Immature leaves may be more susceptible to water loss when injured and consequently shed to prevent excessive dehydration. In addition, immature leaves have limited lignin and other secondary metabolites, and therefore may have fewer defences against pathogens. Shedding of damaged, immature leaves would quickly minimize the opportunity for

pathogen colonization. Little work has been done to examine the effect of mechanical wounding on mature fruit abscission. A better understanding of mechanism(s) by which mechanical wounding affects fruit and leaf abscission could provide a strong basis for developing a system that can better control economic loss in agricultural crops.

Plant tissue wounding induces ethylene biosynthesis, and ethylene is considered to be a key component in propagating wound-related signals leading to a biological response (Wang et al. 2002). Ethylene has been associated with the induction and acceleration of abscission (Goren 1993, Brown 1997). Ethephon, an agrochemical that releases ethylene after contact with alkaline cellular environments, has been successfully used to promote fruit loosening in cherry and defoliation in cotton as an aid to harvest (Abeles et al. 1992, Warner and Leopold 1969). Auxin and abscisic acid are also involved with abscission, and cross talk between multiple hormonal signals can determine the location and timing of cell separation (Goren 1993, Ortolá et al. 1997, Taylor and Whitelaw 2001, Roberts et al. 2002). Although ethylene can promote abscission, work with *Arabidopsis* ethylene perception and delayed floral abscission mutants has demonstrated that ethylene is not needed to induce abscission (Bleecker and Patterson 1997, Patterson and Bleecker 2004).

In this study, we used the response of various aged citrus leaves and fruit to mechanical injury at several locations on organs to explore the relationship between wounding and abscission in 'Valencia' and 'Hamlin' orange. The results demonstrate that mechanical injury of the pigmented portion of the peel (flavedo) initiated mature fruit abscission, even in the presence of the ethylene binding inhibitor 1-methylcyclopropene (1-MCP). Mature leaves did not abscise in response to either mechanical injury or partial blade removal. Mechanical injury of immature leaves and fruitlet peel induced abscission of the wounded organ, but only within the first 2 weeks following shoot emergence and anthesis, respectively.

## Materials and methods

### Plant material

Field-grown, 13-year-old 'Valencia' orange trees (*Citrus sinensis* Osbeck) on rough lemon rootstock and 4- to 6-year-old potted 'Hamlin' or 'Valencia' trees were used in this study. All treatments were applied directly to tissues on the tree. Treatments were randomly applied on four to eight uniform field-grown or potted trees in each experiment. Organs selected for wounding had no visible sign of decay or injury, and each was washed with distilled water and blotted dry before experiments were begun. No visible sign of decay was observed on organs throughout the course of the trials.

### Mechanical wounding of fruit and associated tissues

The peel of citrus fruit is composed of the coloured outer flavedo and the inner white spongy albedo. Flavedo of

immature (5 mm, 20 mm and 45 mm in diameter average) and mature (74 mm in diameter average) fruit were mechanically injured using a scalpel affixed with a no. 10 surgical blade (Feather Safety Razor Co, Ltd, Japan). Three replicates of 30 mature or immature fruit were used in field studies, and at least three separate experiments were conducted. For potted tree studies, three replicates of 10 mature fruit in two separate experiments were conducted. For most experiments involving the effect of wounding, the treatment consisted of removing a 4-mm wide and 2-mm deep section of flavedo encircling the fruit approximately 1 cm below the calyx (Fig. 1, position 1). To determine the effect of flavedo wounding position, treatments included the removal of a 4-mm wide and 2-mm deep section of flavedo along the fruit equator (Fig. 1, position 2), or 1 cm above the blossom scar on the blossom end (Fig. 1, position 3). Calyxes of mature fruit were mechanically injured (Fig. 1, position 4) using a no. 10 blade affixed to a scalpel. Three replicates of 10 calyxes were used in three separate experiments. In this case, seven longitudinal cuts were made, beginning closest to the peduncle and extending downward towards the flavedo. Each cut was approximately 4 mm in length and about 2 mm deep. The peduncle of the mature fruit was mechanically injured (Fig. 1, position 5) using a no. 10 blade affixed to a scalpel. Seven longitudinal cuts approximately 5- to 7-mm in length were made immediately proximal to calyx. Three replicates of 10 peduncles were used in at least two separate experiments. Fruit detachment force was measured as indicated below.

### Mechanical wounding of leaf tissues

Citrus leaves contain a main blade separated from the petiole by a laminar abscission zone. A second abscission zone, the petiolar abscission zone, is located between the

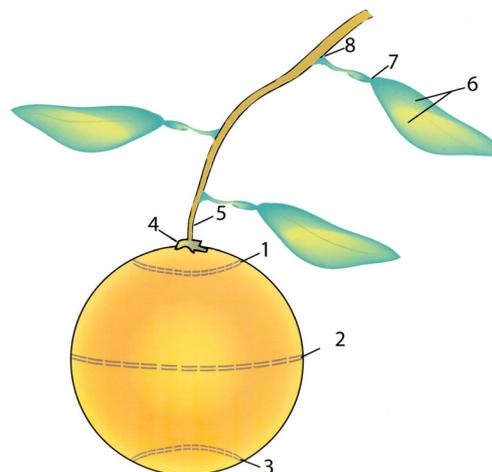


Fig. 1. Schematic diagram of relative positions of wounding in citrus fruit and leaves. 1, stem-end peel; 2, equator peel; 3, blossom-end peel; 4, calyx; 5, peduncle; 6, leaf blade; 7, laminar abscission zone; 8, petiolar abscission zone.

petiole and the branch. Using a no. 10 blade affixed to a scalpel, mature and immature leaf blades were mechanically injured by making a series of three longitudinal cuts through the leaf parallel to the midrib on each side (Fig. 1, position 6). In mature and immature leaf blades, approximately 90% of the total blade length was injured. For mature leaves in the field, three replicates of 15 leaves were used in three separate experiments, whereas two replicates of 20 leaves were used in two separate experiments with potted trees. Two replicates of 20 leaflets in two separate experiments were used in studies involving immature leaves. Alternatively, approximately 25, 50, or 75% of the mature leaf blade area was removed using scissors. In separate treatments, the laminar or petiolar abscission zone was mechanically injured (Fig. 1, positions 7 and 8, respectively) using a no. 10 blade affixed to a scalpel. Five equidistant 3-mm cuts were made perpendicular to the abscission zone fracture plane. To determine the effect of leaf wounding on mature fruit loosening, three leaves closest to the mature fruit were injured on the blade as described above. Fruit detachment force was measured as indicated below.

### 1-MCP and AVG treatments

Potted 'Hamlin' or 'Valencia' trees were placed in 18.4 m<sup>3</sup> insulated walk-in chambers with temperature and humidity control (26 ± 1°C; 85 ± 5% RH). A solution of SmartFresh (3.3% 1-methylcyclopropene, 1-MCP, Rohm & Haas, Philadelphia, PA) was prepared according to the manufacturer's instructions. Briefly, SmartFresh was dissolved in distilled water and placed directly into the chamber with experimental plants immediately prior to wounding fruit and leaves. The final concentration of 1-MCP was estimated to be 1000 nL<sup>-1</sup>. An aqueous solution of aminovinylglycine (AVG) at 0.5 mM was prepared and applied directly to the cut surface of the mature fruit flavedo immediately after wounding using an absorbent cotton wrap. Every 24 h, solution on the absorbent wrap was refreshed with 1 ml 0.5 mM AVG to insure constant supply throughout the duration of the experiment. For both 1-MCP and AVG studies, four to six potted trees per treatment were used in two separate experiments.

### Ethylene determination

For determination of ethylene evolution in fruit, 6–15 fruit from each tree were selected at each sampling time and enclosed in 0.87-l Rubbermaid plastic containers while on the tree (Yuan et al. 2001). Briefly, at various times up to 96 h after wounding, the open containers with fruit inside were closed with the plastic lid and sealed for 2 h. One ml of gas sample was withdrawn from the sealed container through the rubber septum affixed to the lid and ethylene concentration measured with a gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with an activated alumina column and flame ionization detector. For measurement of leaf ethy-

lene evolution, at least eight single leaf replicates were selected from each tree. The petiole of each leaf was carefully slipped through a slit into a 5 mm in diameter hole in a rubber stopper. The open spaces between the petiole and the rubber stopper were sealed with non-phytotoxic 3145 Mil-A-46146 RTV adhesive/sealant (Dow Corning Co., Midland, MI). Each leaf was placed into a 60-ml syringe by inserting the rubber stopper to the end of syringe and affixing a rubber septum to the syringe top. Each tube was sealed for 2 h at various times up to 96 h after wounding. A one ml gas sample was withdrawn from the sealed syringe through the rubber septum 2 h after container closure, and ethylene concentrations were measured as described above.

### Determination of removed leaf area

Approximately 25, 50, or 75% of leaf blade area was removed using sharp scissors from fully expanded mature leaves approximately 6 months of age. Twenty leaves in each of three replicates were used for each treatment in two separate experiments. The removed area was determined by measuring the removed leaf pieces with a portable area meter Model LI-3000 (Lambda Instruments Co. Lincoln, NE). At the end of the experiment, the remaining attached blade was harvested and its area determined. The percentage leaf area removed was calculated by dividing the leaf area initially removed at the beginning of the experiment by the sum of the initial area plus the harvested leaf area at the end of the experiment.

### Determination of fruit detachment force and percent leaf drop

Mature and immature fruit (45 mm in diameter) were harvested from the trees with 15–20 cm attached stems at various times up to 6 days after wounding. Detached fruit were transported to the laboratory and fruit detachment force (FDF) was determined by using a digital force gauge ('Force Five', Wagner Instruments, Greenwich, CT). Fruit stems were clipped to 4.0 cm in length, inserted into the gauge and the stem pulled parallel to the fruit axis until it separated from the fruit. FDF was expressed either in kilograms or percent of control. Leaf drop was counted at various times after wounding and expressed as a percentage of the total number of leaves at the beginning of each test.

### Results

Wounding mature fruit peel and mature leaf blades resulted in increased ethylene evolution (Fig. 2). An initial peak of ethylene evolution occurred 12 h and 24 h after wounding in mature fruit and leaves, respectively. Exposure of wounded mature fruit flavedo to the ethylene perception inhibitor 1-MCP markedly increased fruit ethylene evolution when compared with wounded flavedo alone. In contrast, treatment with 1-MCP did

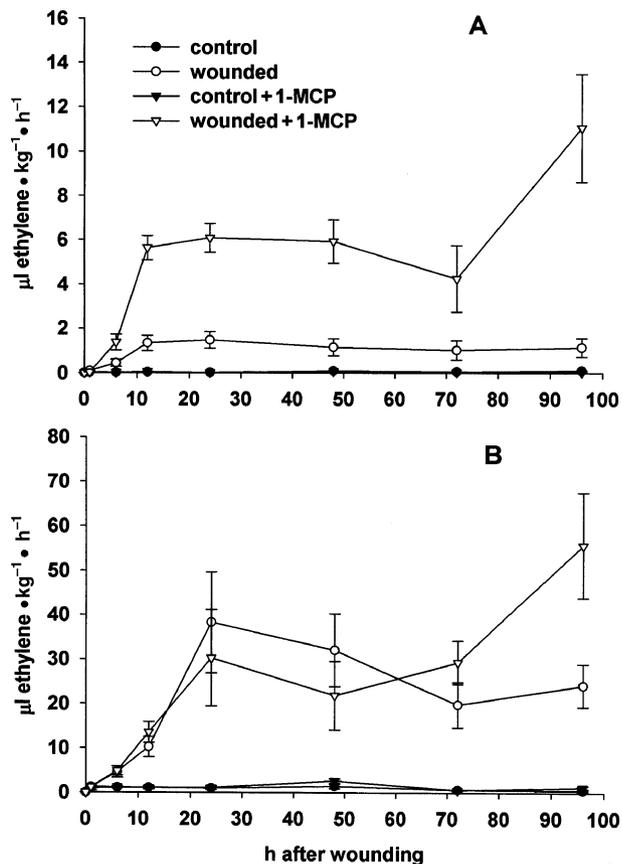


Fig. 2. Ethylene evolution in mature fruit (panel A) and mature leaves (panel B) of 'Valencia' for periods up to 96 h after wounding. Data plotted are the means of eight replicate fruit/time point of a single representative experiment  $\pm$  SE mean.

not essentially change ethylene evolution in leaf blades for the first 48 h after wounding. After this time, ethylene evolution increased in 1-MCP-treated wounded leaf blades, whereas little change occurred in wounded leaves. There was no difference in ethylene evolution between control leaves and 1-MCP treated leaves.

FDF of control fruit or fruit exposed to 1-MCP alone were similar and did not change during the trial (Fig. 3). A large reduction in FDF was measured 96 h after flavedo of mature fruit was mechanically wounded. Exposure of wounded fruit to 1-MCP resulted in a similar reduction in FDF when compared with wounded fruit alone. Fruit drop in both wounding treatments began 72 h after the beginning of the trial and increased at 96 h. Exposure of wounded fruit to 1-MCP resulted in a higher percent fruit drop at 96 h but not 72 h after wounding. Control fruit or fruit treated with 1-MCP alone did not undergo abscission. Leaf drop did not occur in any treatment, despite the large wounding-related increase in ethylene evolution. A gentle pull by hand on leaf blades of any treatment and control or 1-MCP-treated control fruit indicated that these organs remained firmly attached to the parent plant.

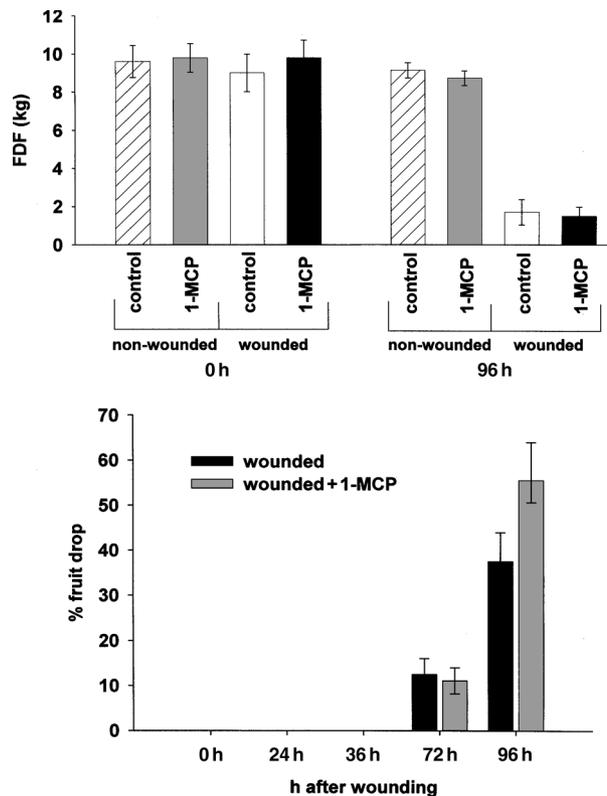


Fig. 3. The effect of wounding and 1-MCP on FDF (top panel) and percent fruit drop (bottom panel) in 'Valencia' orange. Measurements were taken for periods up to 96 h after wounding. Data presented in both panels are the means of a single representative experiment  $\pm$  SE mean.

Inhibiting ethylene biosynthesis with AVG in wounded flavedo of mature fruit markedly reduced ethylene evolution as compared with wounded fruit alone (Table 1). However, the reduction in FDF in both wounding treatments was similar, although percent fruit drop was significantly higher in wounded fruit alone.

Mature fruit flavedo was mechanically wounded at the stem end, equator or blossom end to determine the effect of wounding position on fruit abscission. Reduction in FDF was greater when flavedo was wounded at the stem end than at the equator or blossom end (Fig. 4). Compared

Table 1. AVG reduced wound-induced evolution of ethylene and fruit drop but not wound-induced reduction in FDF. Data are the means 72 h after wounding or initial application of AVG. Means followed by the same letter within a data column are not significantly different ( $P < 0.05$ ).

	FDF (kg)	Ethylene production ( $\text{nl}^{-1} \text{kg}^{-1} \text{h}^{-1}$ )	Percent fruit drop
Control + AVG	9.5 a	25 b	0 c
Wounded	2.2 b	2150 a	46 a
Wounded + AVG	2.5 b	500 b	25 b

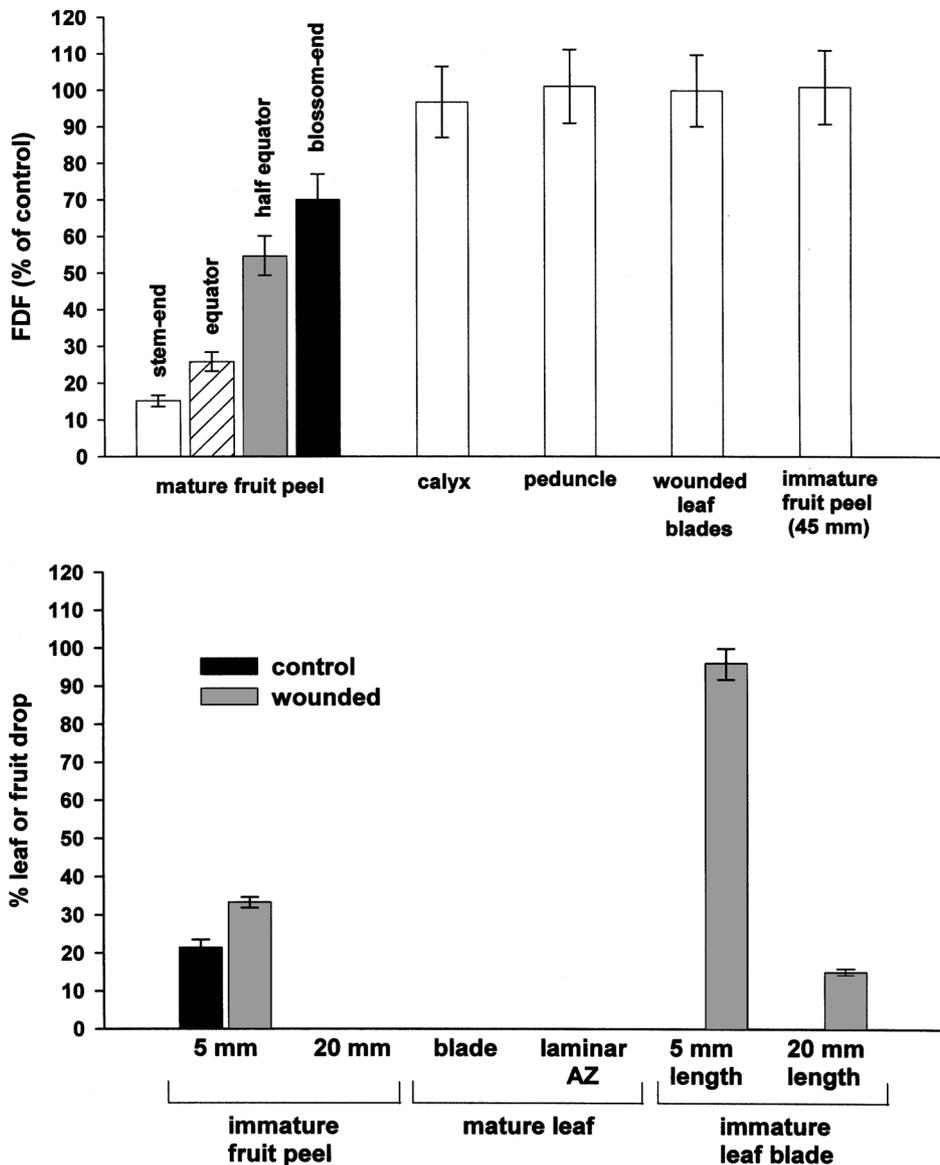


Fig. 4. FDF (top panel) and percent leaf or fruit drop (bottom panel) after wounding various citrus tissues. FDF data are expressed as percent of the control. Measurements were taken 96 h after wounding. Data presented in both panels are the means of one representative experiment  $\pm$  SE mean.

with the relative amount of tissue wounded at the stem end, wounding approximately similar amounts of tissue at the equator of the fruit (half-equator, Fig. 4) resulted in smaller reduction in FDF. Even if twice the amount of tissue was wounded at the equator by removing the same 4-mm wide and 2-mm deep section of flavedo, reduction in FDF was not as great as that of fruit wounded at the stem end. A similar amount of tissue was wounded at the blossom end of the fruit compared with the stem end, but FDF was reduced only 30% when compared with controls. The mature fruit abscission zone is located at the flavedo/albedo interface at the peduncle/fruit attachment point. The calyx is located proximal to the mature fruit flavedo at the peduncle/fruit attachment area. Since wounding the calyx resulted in an apparent reduction in tissue strength around the fruit/peduncle

attachment point, reductions in FDF in wounded calyx were compared with fruit whose calyxes were wounded immediately before FDF readings were taken. Wounding of mature fruit calyx did not result in reduction in FDF. Similarly, FDF of mature fruit was unaffected by wounding of the peduncle. Wounding three leaf blades immediately next to the mature fruit also had no effect on FDF.

Immature fruit differentially responded to wounding. Wounding flavedo of immature fruit that were 12 weeks old and 45 mm in diameter did not reduce FDF (Fig. 4) or cause fruit drop (data not shown). In addition, wounding flavedo of immature fruit approximately 8 weeks old and 20 mm in diameter also did not result in fruit drop. In contrast, wounding flavedo of 2-week-old fruitlets and 5 mm in diameter caused 33% fruitlet

drop. However, natural 'June' drop in unwounded controls approached 21%.

Mechanically wounding mature leaf blades did not result in leaf drop, nor did wounding the laminar abscission zone area perpendicular to the abscission zone fracture plane (Fig. 4). Wounding the petiolar leaf abscission zone in a similar way did not cause leaf drop. Removing an average of 27, 48, or 77% of the total leaf area did not result in leaf drop (data not shown). Immature leaflets responded differentially to mechanical wounding. Recently emerged 1-week-old leaflets measuring approximately 5 mm in length abscised nearly 100% in response to mechanical wounding, but less than 20% leaf drop occurred when 3-week-old leaflets of 20 mm in length were wounded.

## Discussion

Mature fruit abscission was initiated by mechanical wounding of flavedo and the response was associated with ethylene evolution. It is well known that wounding increases ethylene production (Wang et al. 2002). Evensen et al. (1981) demonstrated that wounding mature fruit peel of citrus either mechanically or chemically with an abscission agent resulted in increased ethylene evolution. Although mechanical wounding caused mature fruit ethylene evolution, the inability of the ethylene binding inhibitor 1-MCP to inhibit mature fruit abscission suggests that induction of mature fruit abscission by mechanically wounding flavedo may be independent of ethylene binding. Additional 1-MCP was added 24 h after the initial 1-MCP application at the time of flavedo wounding in an attempt to restore any possible loss of the gaseous inhibitor and control any return of ethylene sensitivity due to the possible synthesis of new binding sites (Blankenship and Dole 2003), but even this treatment failed to halt abscission. The fact that abscission can occur in the absence of ethylene binding has been recently reported. Application of 1-MCP was unable to stop mature fruit abscission in trees exposed to ethephon, an ethylene-releasing agent (Pozo et al. 2004). In this case, treatment with 1-MCP 24 h before or after ethephon application still did not stop abscission. Similar results were obtained when using CMNP, an agent that effectively wounds citrus peel and causes abscission. Abscission in the ethylene perception mutants of *Arabidopsis* still occurs, albeit at a much slower rate (Bleecker and Patterson 1997). Further, research with the delayed floral abscission mutants of *Arabidopsis* has led to the conclusion that ethylene perception is not required to activate abscission, but may act in many cases as an accelerant (Patterson and Bleecker 2004). In our work, we attempted to inhibit ethylene biosynthesis in wounded mature fruit with AVG to determine if ethylene biosynthesis was necessary to accelerate abscission. Although addition of AVG immediately before wounding markedly reduced fruit ethylene evolution, the reduction in FDF was not halted but fruit drop

appeared to be delayed. In previous work, application of AVG 18 h before ethephon or CMNP application to potted calamondin citrus trees was not effective in reducing FDF or fruit drop (Pozo and Burns 2000). Since ethylene evolution was not reduced to control levels, however, further work will be necessary to clarify the role of ethylene either as an abscission accelerant, as a byproduct of the abscission process or as a participant in a parallel pathway of abscission.

Location of the peel wound was an important factor related to the abscission response of mature fruit: the closer the location of the wound to the calyx abscission zone, the larger the reduction in FDF. Wounding other tissues in close proximity to the mature fruit flavedo did not cause mature fruit abscission. These results suggest that an abscission 'signal' and/or associated events with reduced mobility may originate at the site of wounding in mature fruit peel. The signal may not require ethylene binding, as marked reduction in FDF followed by organ separation occurred in the presence of 1-MCP.

Oligogalacturonides (O'Donnell et al. 1996) or other cell wall-related oligosaccharides (Ryan 1987) enzymatically released as a result of decompartmentation in wounded tissue could have a role in the transmission of a wound-related signal that leads to abscission. Oligosaccharide fragments can induce ethylene production, but have also been shown to induce pathogenesis-related gene expression in both an ethylene-dependent and independent fashion (O'Donnell et al. 1996). Reactive oxygen species (ROS) may also play a role in transmission of a wound-related signal leading to abscission and are produced in tissues as a result of wounding (Bolwell and Wojtaszek 1997). ROS can promote membrane degradation, peroxidation and metabolism (Blee 2002), leading to biologically active jasmonates and related products with abscission activity (Hartmond et al. 2000, Burns et al. 2003). The balance of phytohormones such as auxin and abscisic acid may also play a role in the transmission of a wound-related signal or the sensitivity of tissues leading to abscission (Thornburg and Li 1991, Peña-Cortés et al. 1989, Yuan et al. 2001).

Wounding the blade or abscission zone of mature citrus leaves resulted in a substantial amount of ethylene evolution, but abscission was not advanced. Treatment of wounded mature leaves with 1-MCP resulted in little change in ethylene evolution compared to the wounded controls. After 48 h, however, ethylene production in 1-MCP treated wounded leaves increased, suggesting that ethylene can prevent wound ethylene production at this time (negative regulation). In contrast, 1-MCP treated wounded peel had markedly higher ethylene evolution compared with wounded controls throughout the duration of the experiment, suggesting negative regulation of ethylene biosynthesis in wounded peel. Similar autocatalytic and autoinhibitory effects of ethylene on ethylene production in excised citrus leaves, leaf discs and fruit have been reported (Riov and Yang 1982). Our data suggest that the negative feedback mechanism is well pronounced in wounded citrus peel but not in

wounded leaves within the first 72 h after mechanical wounding.

Mature leaves are the main source of carbohydrate for the plant (Harper 1989), but wounding or removing over 75% of the total blade area in this study did not cause abscission. Abscising the damaged leaf may remove the capacity to synthesize carbohydrates. In fact, the remaining leaf and leaves surrounding the injured area can compensate by increasing photosynthetic capacity and efficiency (Syvertsen 1994, Blundell and Peart 2000, Thomson et al. 2003). Source/sink relationships in immature leaves and fruit strongly influence abscission (Ruiz et al. 2001). Immature citrus leaves differentially responded to wounding, depending on their age. Leaves of 1 week old were highly responsive to mechanical wounding and abscised, whereas leaves up to 3 weeks old failed to respond. The reason for this brief window of responsiveness to wounding is not known, but could be related to their contribution to the plants' carbohydrate status at the time of wounding and investment for future carbohydrate gain. The plant may benefit more by removing a very young leaf and flushing a new one rather than expanding a damaged leaf (Harper 1989, Blundell and Peart 2000). Immature fruitlets approximately 2 weeks of age had a weak abscission response to mechanical wounding. Fruitlets at this age undergo a first postanthesis wave of fruit drop ('June' drop) to adjust fruit number with tree metabolite supply (Ruiz et al. 2001), so it is unclear whether the small increase in abscission measured in this study reflects a response to mechanical injury. Wounded immature fruitlets either 8 or 12 weeks of age did not abscise. Since carbohydrate supply is the main factor governing fruitlet drop during this time (Ruiz et al. 2001, Iglesias et al. 2003), these immature organs may have reduced sensitivity or lack sensitivity to other signals.

In conclusion, we have shown that mechanical injury of mature citrus flavedo caused abscission. Although ethylene was produced in wounded mature fruit, ethylene binding was not required for abscission to occur. Mechanical wounding did not cause abscission in mature leaves and young fruit. The ability of an organ to respond is likely governed by its developmental age, potential contribution to carbohydrate accumulation, and its production and sensitivity to an abscission signal(s).

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