

# Physiological changes associated with senescence and abscission in mature citrus fruit induced by 5-chloro-3-methyl-4-nitro-1*H*-pyrazole and ethephon application

Fernando Alferez, Luis Pozo and Jacqueline K. Burns\*

Horticultural Sciences Department, Citrus Research and Education Center, University of Florida, IFAS, 700 Experiment Station Road, Lake Alfred, FL 33850-2299, USA

## Correspondence

\*Corresponding author,  
e-mail: jkbu@ufl.edu

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This research compares effects of the compound 5-chloro-3-methyl-4-nitro-1*H*-pyrazole (CMNP), a plant growth regulator that selectively promotes abscission in mature citrus fruit (*Citrus sinensis*), and the ethylene-releasing agent ethephon (2-chloroethylphosphonic acid). Application of CMNP and ethephon to mature citrus fruit reduced fruit detachment force and changed peel color from green to orange. More total chlorophyll was extracted from flavedo in early season (November) than late season (January), and both compounds caused a similar reduction in chlorophyll. In contrast, total carotenoid content was similar in November and January. Both abscission compounds increased total carotenoids, but induction was greater in January, and CMNP was more effective in both months. Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activity increased after CMNP but not ethephon application. Electrolyte leakage increased 2 h after CMNP treatment, and total protein content was reduced by 50% after 72 h. Ethephon caused only minor changes in electrolyte leakage and total protein content. Inhibition of PLA<sub>2</sub> activity with aristolochic acid did not reduce leakage but inhibited total protein loss and reduced visual peel damage associated with CMNP. Ultrastructural observations indicated decreased number, and length of starch grains 3 h after CMNP treatment. A transient increase in soluble sugars was measured 3 h after CMNP application. Ethephon had little effect on soluble sugar content and changes in starch grains. Collectively, the results indicate that CMNP and ethephon induced color change in peel and advanced mature fruit abscission. However, CMNP but not ethephon promoted other physiological changes associated with senescence.

## Introduction

During development, plants often shed leaves, fruit and flowers independently through a complex and highly regulated process known as abscission. Ethylene is a plant hormone affecting a number of physiological processes such as senescence, fruit ripening and associated color change (Alonso et al. 1995), and abscission

(Bleecker and Kende 2000, Goren 1993). Ethylene is known to accelerate abscission in tissues developmentally competent to respond but may not be required to initiate the process (Patterson 2001, Pozo et al. 2004, Taylor and Whitelaw 2001). Ethylene-insensitive abscission has been reported in *Arabidopsis* (Patterson and Bleecker 2004) and orchid (Van Doorn 2002), although

*Abbreviations* – AT, aristolochic acid; FDF, fruit detachment force; LOX, lipoxygenase; PLA<sub>2</sub>, phospholipase A<sub>2</sub>.

at a slower rate. Thus, abscission may be regulated via ethylene-sensitive and ethylene-insensitive factors and may occur through parallel pathways or a single pathway with multiple inputs.

The selective acceleration and control of abscission remains an important goal of agriculture. Ethephon (2-chloroethylphosphonic acid), an ethylene-releasing agent, has been used to advance abscission. Uptake followed by chemical cleavage to ethylene is thought to be the reason for its action (Warner and Leopold 1969). Unfortunately, lack of organ selectivity and increased phytotoxicity at high temperatures have limited the commercial use of ethephon for fruit loosening in areas where temperatures are above approximately 27°C during harvesting. In Florida, such conditions have prevented adoption of ethephon as an effective abscission agent for two reasons: first, it causes unacceptable leaf abscission when applied at concentrations effective to loosen mature fruit (Burns 2002); and second, high temperatures during the harvest season can result in gummosis and increased efficacy of ethephon resulting in additional undesired leaf loss (Bukovac et al. 1969, Burns 2002).

The compound 5-chloro-3-methyl-4-nitro-1*H*-pyrazole (CMNP) is a pyrazole-derivative that induces abscission selectively in mature citrus fruit when applied to the canopy, with no phytotoxicity at higher temperatures commonly found during the harvest season (Hartmond et al. 2000a, Yuan and Burns 2004). Mature fruit selectivity is particularly important for mechanical harvesting the late-season cultivar 'Valencia' orange. In this cultivar, mature and young developing fruit are found simultaneously on the tree during the majority of its harvest season.

The reason for selectivity of CMNP and its mode of action are unknown. Recently, we demonstrated that CMNP acted as an uncoupler and depleted total cellular adenosine triphosphate (ATP) content, and that efficacy required peel contact (Alferez et al. 2005). Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activity, a lipase involved in lipid signaling (Meijer and Munnik 2003), was markedly increased in fruit peel treated with CMNP. Inhibition of PLA<sub>2</sub> activity with aristolochic acid (AT) arrested fruit abscission advanced by CMNP, suggesting a link between lipid signaling and citrus fruit abscission (Alferez et al. 2005). In general, products of lipid signaling regulate plant responses to several biotic and abiotic stresses (Farmer et al. 2003, Howe and Schilmiller 2002) and promote fruit ripening, senescence (Creelman and Mullet 1997, He et al. 2002) and abscission (Burns et al. 2003, Hartmond et al. 2000b).

CMNP and ethephon application caused ethylene production in mature citrus fruit, and inhibition of

ethylene biosynthesis with compounds such as amino-vinylglycine, and the resulting reduced efficacy demonstrate the role of ethylene as an abscission accelerant (Yuan et al. 2001, 2005). Despite this similarity, our previous work exploring the mode of action of CMNP led us to hypothesize that differences in physiological events leading to mature fruit abscission may exist between this compound and ethephon. In this work, some short- and longer-term physiological consequences of CMNP and ethephon application in mature citrus fruit were measured to give insight into factors that impact abscission and the mode of action of CMNP. We show that both compounds accelerated fruit loosening and peel color change but CMNP and not ethephon promoted other changes associated with senescence, such as lipid signaling activation and membrane disruption.

## Materials and methods

### Plant material and treatment

Seasonal effects on fruit loosening and color and pigment changes were determined in citrus fruit cv. Hamlin (*Citrus sinensis* L. Osbeck) harvested from 15-year-old trees on rough lemon rootstock located at the Citrus Research and Education Center in Lake Alfred, Florida, in early (November 2003) and late season (January 2004). Other physiological measurements were made from fruit harvested at various times from November to January. Three canopy sectors of approximately 3 m<sup>3</sup> each were randomly selected from a group of 10 trees, and treatments applied with a pressurized hand sprayer to run-off. Treatments included 1.5 mM CMNP, 1 mM AT, 1.5 mM CMNP + 1 mM AT, 400 mg l<sup>-1</sup> ethephon (2-chloroethylphosphonic acid) or water alone. Tween 20 (0.15%) was used as adjuvant. The experiment was repeated twice, with at least 30 fruit from each of three canopy sectors selected for analysis. For various times, up to 96 h after application, flavedo (outer pigmented portion of fruit peel) was removed from an area approximately 1 cm below the calyx abscission zone, frozen in liquid nitrogen, and stored at -80°C until needed for physiological analyses. After 5 days, fruit detachment force (FDF) was measured to monitor mature fruit abscission as previously described (Hartmond et al. 2000b).

### Peel color, chlorophyll, and carotenoid extraction and quantification

Peel color of whole fruit was measured by using a Minolta CR-330 colorimeter on three points around the equatorial plane of the fruit. Chlorophyll and

carotenoids were extracted together from 0.1 g flavedo with 20 ml acetone and 0.1 g sodium carbonate at 4°C. A 2-ml aliquot of this extract was used to measure total chlorophyll at 645 and 663 nm (Moran 1982). For carotenoid quantification, the remaining acetone extract was evaporated under vacuum (35°C) to 1 ml, then 20 ml 20% methanolic KOH were added, and the solution was kept in the dark for 1 h at 40°C for saponification. Then, 40 ml distilled water and 2 ml saturated NaCl were added, the solution partitioned three times against 15 ml diethyl ether, and the ether extracts combined, dried under vacuum at 40°C and redissolved in 20 ml n-hexane. Absorbance was measured at 420 nm and the total carotenoid content expressed as mg beta-carotene/g fresh tissue (Eilati et al. 1969).

#### **PLA<sub>2</sub> extraction and analysis, electrolyte leakage and total protein determination**

Total PLA<sub>2</sub> extraction and analysis were performed as described previously (Alferez et al. 2005). Electrolyte leakage measurements were performed as previously described by Lafuente et al. (1991) with some modification. At each time-point, five tissue discs were removed from the fruit peel using a cork borer. Albedo tissue was removed, and the remaining flavedo disks (1 cm × 2 mm) were rinsed, placed in a beaker with deionized water, and gently shaken for 20 min to remove released electrolytes resulting from wounded disk surfaces. Tissue was transferred to test tubes containing 15 ml deionized water, and conductivity was measured after 45 min of continuous shaking using a Thermo Electron Corporation Orion model 105 conductivity meter (Beverly, MA). Samples were then frozen, warmed to room temperature with shaking, and the total conductivity was measured after additional 45 min of shaking. Measurements were made in triplicate, and conductivity resulting from treatment application expressed as percentage of total conductivity. Total protein content was measured by the dye-binding method (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard (Bradford 1976).

#### **Transmission electron microscopy, starch size and soluble sugar determination**

Flavedo from citrus fruit was prepared for transmission electron microscopy as previously described (Burns et al. 1992). Starch grain length in electron microscopy preparations was determined using the ImageJ software package from The National Institute of Health (Advanced Microscopy Techniques, Danvers, MA) for organelle size analysis. Thirty starch grains per treatment were viewed

for analysis. Soluble sugars and starch were quantified as described by Somogy (1952) with some variation. Flavedo was dried at 60°C for 3 days and ground to a powder. Samples (100 mg) were extracted with 80% ethanol, and centrifuged twice for 1 min at 134 g. To recover soluble sugars, we added HCl (1/100 v/v) to the supernatant and boiled for 10 min. Samples were stored at -20°C until measurement. Sample absorbance was measured at 520 nm in a spectrophotometer and compared with a standard curve of increasing glucose concentration. Soluble sugars were calculated and expressed as percentage of control. Extractions were repeated twice, and sugar determinations were made in triplicate.

## **Results**

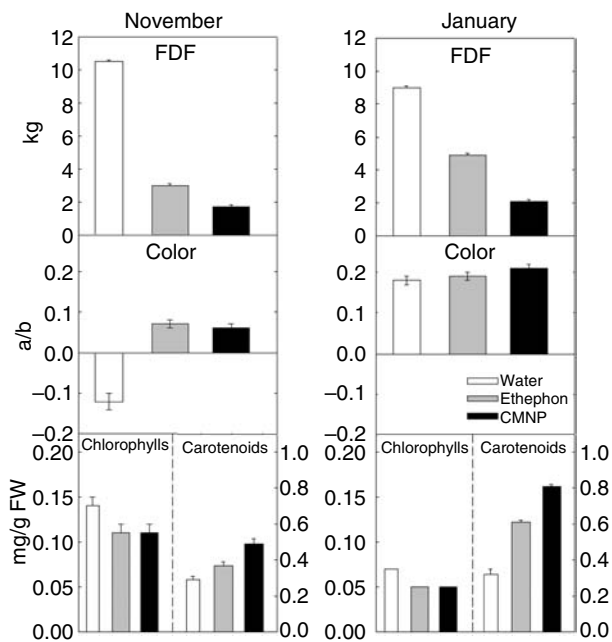
#### **FDF, color change, chlorophyll, and carotenoid content**

CMNP and ethephon markedly reduced FDF five days after application in November and January; however, ethephon was less effective in January (Fig. 1). Both compounds promoted peel color change from green to orange in November as indicated by a shift in a/b ratio from negative to positive but had little effect when fruit were fully orange in color in January. More total chlorophyll was extracted from flavedo in November than January; nevertheless, both abscission compounds decreased chlorophyll content similarly. Total flavedo carotenoid content was essentially the same in November and January, but more carotenoids were extracted in January after ethephon and CMNP treatments. CMNP was more effective than ethephon in promoting carotenoid accumulation.

#### **PLA<sub>2</sub> activity, electrolyte leakage, total protein, and peel damage**

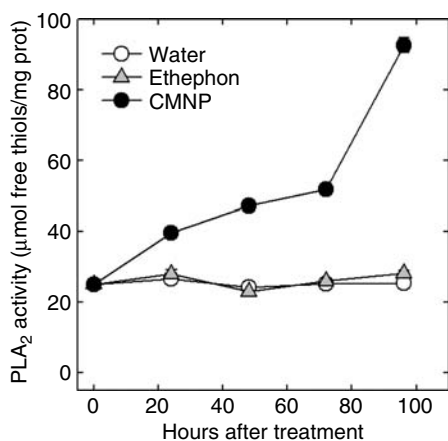
As reported previously (Alferez et al. 2005), PLA<sub>2</sub> activity markedly increased with time after CMNP treatment (Fig. 2). In contrast, ethephon treatment did not change PLA<sub>2</sub> activity as compared with untreated controls.

Increased electrolyte leakage is used to monitor loss of membrane integrity, and hence, indicates cellular damage caused by stress (Lafuente et al. 1991). Electrolyte leakage in CMNP-treated peel remained constant for 30 min, but a sharp increase was measured thereafter (Fig. 3A). AT, a well-known inhibitor of PLA<sub>2</sub> activity (Chandra et al. 1996, Scherer and Arnold 1997), did not reduce CMNP-induced electrolyte leakage. Ethephon did not alter electrolyte leakage during the course of the experiment.



**Fig. 1.** Effect of 1.5 mM 5-chloro-3-methyl-4-nitro-1H-pyrazole (CMNP) and 400 mg l<sup>-1</sup> ethephon on fruit detachment force (FDF), peel color and total chlorophyll and carotenoid content of Hamlin fruit treated at early (November) and late (January) season. Data were collected 5 days after treatment. Vertical lines through bars representing treatment means are SE of 30 fruit. Error bars are not visible when SE less than 0.5% of the mean.

Change in protein content is an early sign of senescence in plant tissues (Hensel et al. 1993). Over a 96-h period, total protein in citrus flavedo declined to less than 50% of controls after CMNP treatment (Fig. 3B).



**Fig. 2.** Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activity in flavedo of mature citrus fruit at times up to 96 h after treatment with water, 1.5 mM 5-chloro-3-methyl-4-nitro-1H-pyrazole (CMNP) or 400 mg l<sup>-1</sup> ethephon. Values are mean ± SE of 3 measurements. Absence of SE indicates that treatment marker larger than SE bars.

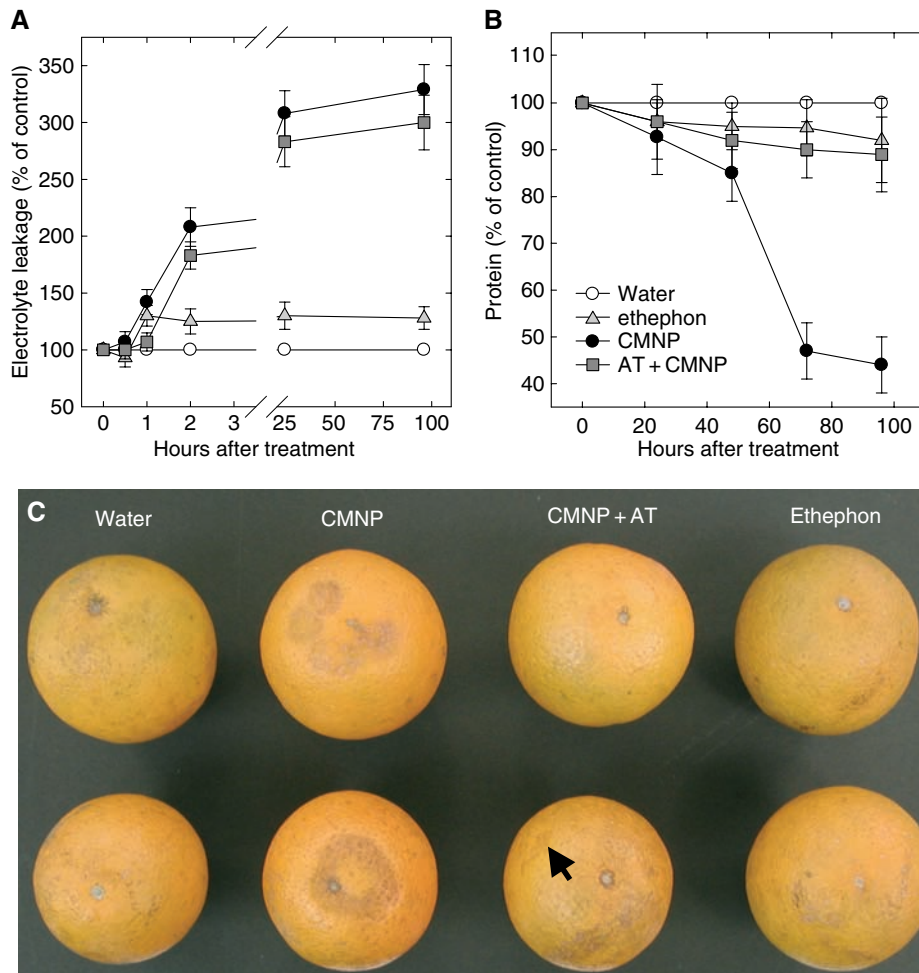
Protein content was not affected after ethephon treatment during this period. When AT was applied, CMNP-induced protein loss was slightly reduced. AT alone had no effect on electrolyte leakage or protein content. Visual symptoms of peel injury appeared at the blossom end of the fruit 48 h after CMNP treatment. Injuries consisted of a brown ring of depressed flavedo tissue in areas in which treatment solution accumulated after run-off (Fig. 3C). AT largely diminished this peel injury when applied in combination with CMNP. Peel injury did not occur after ethephon treatment.

### Starch and soluble sugars

Ultrastructural observation of untreated citrus flavedo showed numerous starch grains and some plastoglobules within the chromoplast, but an extensive internal membrane network was absent (Fig. 4A). After 3 h of CMNP treatment, starch grains diminished in number and size (Figs 4B, D). Ethephon treatment had little effect on starch grain number and size (Fig. 4C). A transient increase in soluble sugars was measured 3 h after CMNP application that later declined below the level of the untreated control (Fig. 4E). Ethephon did not alter soluble sugar content during the course of the experiment.

### Discussion

The compound CMNP is a plant growth regulator that selectively advances abscission in mature citrus fruit. Recent work demonstrated the uncoupling effect of CMNP, and a link between cellular energy shortage, lipid signaling and citrus abscission was suggested (Alferez et al. 2005). Ethephon is an agrochemical used to advance ethylene-related processes such as abscission in cherries and flowering in pineapple (Bukovac et al. 1969, Turnbull et al. 1999). Ethephon uptake into plant tissue results in cleavage of the molecule to yield ethylene, but hydrochloric acid and phosphate are also released (Warner and Leopold 1969). Phosphate may also advance abscission by promoting ethylene biosynthesis (Banno et al. 1993, Burnik-Tiefengraber et al. 1994, Goren et al. 1998). It is well known that ethylene accelerates abscission (Brown 1997), but recent work showed that ethylene was not required to initiate the process in *Arabidopsis thaliana* (Patterson and Bleecker 2004). In this study, a comparison was made between the abscission-accelerating compounds CMNP and ethephon to examine similarities and differences in physiological events preceding organ separation in mature citrus fruit.

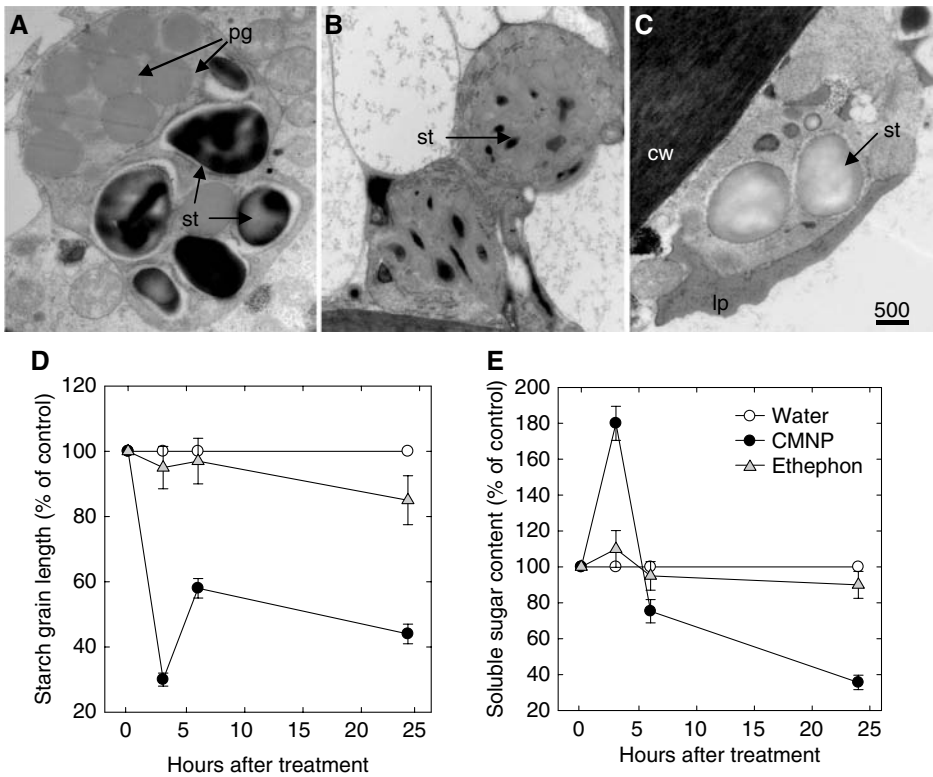


**Fig. 3.** (A) Electrolyte leakage; (B) total protein content; and (C) visual peel damage of mature fruit flavedo associated with 1.5 mM 5-chloro-3-methyl-4-nitro-1*H*-pyrazole and 400 mg l<sup>-1</sup> ethephon application at times up to 96 h after treatment. Images in (C) were taken 5 days after treatment. Arrow shows peel damage in CMNP + AT-treated fruit. Numeric data are mean ± se of three measurements and are expressed as percentage of the control.

CMNP and ethephon reduced FDF, promoted color change in peel, decreased chlorophyll, and increased carotenoid content in flavedo, indicating both compounds accelerated abscission and senescence (Bonora et al. 2000, Buchanan-Wollaston et al. 2003, Yuan et al. 2001). However, CMNP promoted other physiological changes that were minimally affected by ethephon. PLA<sub>2</sub> activity increased in flavedo after CMNP treatment, and recent work showed that inhibiting PLA<sub>2</sub> activity with AT inhibited reduction in FDF and abscission (Alferez et al. 2005). In addition, total protein content was reduced over 50%. Large reductions in total protein content typically occur in senescing organs (Buchanan-Wollaston et al. 2003, Hensel et al. 1993) and may reflect a lack of synthesis or increased protein catabolism in flavedo with reduced ATP content after CMNP treatment (Alferez et al. 2005). Increased electrolyte leakage was an early event induced by CMNP, indicating a loss of membrane semipermeability and onset of senescence (Leverentz et al. 2002, Thompson et al. 1998) caused by the

substituted pyrazole. AT treatment was unable to arrest CMNP-induced electrolytic leakage. This suggests that the loss of membrane integrity caused by CMNP was not promoted by PLA<sub>2</sub> and that other lipolytic enzymes insensitive to AT, reactive oxygen species generated by the uncoupling action of CMNP or other membrane injury caused by CMNP triggered electrolyte leakage. The timing of increased PLA<sub>2</sub> activity (after 6 h of CMNP treatment, Alferez et al. 2005) indicates that electrolyte leakage preceded induction of this phospholipase activity. Interestingly, AT treatment greatly reduced peel damage and prevented total protein loss caused by CMNP, indicating that PLA<sub>2</sub>-related physiological events are associated with changes in protein content and visual symptoms of peel injury.

Previous work demonstrated that CMNP treatment uncoupled energetic membranes and reduced total cellular ATP content (Alferez et al. 2005), resulting in the loss of stored food reserves. CMNP reduced the number and size of starch grains and transiently increased



**Fig. 4.** Ultrastructure (A, B, C), starch grain length (D), and soluble sugar content (E) of flavedo after treatment with 1.5 mM 5-chloro-3-methyl-4-nitro-1*H*-pyrazole (CMNP), 400 mg l<sup>-1</sup> ethephon, and water for time periods up to 24 h (A) Adjuvant control; (B) 1.5 mM CMNP; and (C) 400 mg l<sup>-1</sup> ethephon treatment 3 h after treatment. (D) Starch grain length and (E) soluble sugar content measured at various times up to 24 h after treatment. Numeric data are mean ± SE of three measurements and are expressed as percentage of the control. Bar represents 500 nm. cw, cell wall; lp, lipids; pg, plastoglobules; st, starch granule.

soluble sugars in mature fruit flavedo, indicating that uncoupling triggered starch mobilization. Released soluble sugars are used to support metabolic processes in mature fruit flavedo and have also been shown to accelerate color break (Iglesias et al. 2001). Increased stress and the onset of senescence have been shown to increase PLA<sub>2</sub> and LOX activities and reduce lipid and starch reserves (Chandra et al. 1996, Feussner et al. 1995, Guiamet et al. 1999, May et al. 1998, Melan et al. 1993, Pohnert 2002). Interestingly, starch began to reappear in flavedo cells after 48 h CMNP application (data not shown), indicating that mobilization of stored reserves was a transient event. Detoxification of CMNP before a critical threshold of cellular damage was reached, and/or source-sink readjustments may explain the resynthesis of starch grains in flavedo. Further work will be needed to confirm if starch resynthesis is a physiological consequence of CMNP action.

Abscission of citrus flowers and young fruitlets has been linked to reduction of carbohydrates due to fruitlet-to-fruitlet competition and defoliation (Mehouachi et al. 1995, Ruiz et al. 2001). In this study, even though carbohydrate shortage was transient after CMNP treatment, abscission progressed, suggesting that changes in carbohydrates may be involved in triggering abscission of mature fruit. Although the involvement of carbohydrates in wound and stress signaling has been shown previously

(O'Donnell et al. 1996), further investigation is needed to demonstrate a link between sugar sensing/signaling and abscission.

In conclusion, CMNP and ethephon promoted color change and triggered mature fruit abscission. However, increased PLA<sub>2</sub> activity, electrolyte leakage, loss of total protein content and starch were physiological events unique to CMNP. Current research concluded that abscission may occur via parallel pathways or a single pathway with multiple inputs (Patterson and Bleeker 2004). Our data suggest that either CMNP acts upstream of ethylene (ethephon) or both compounds act independently to accelerate mature citrus fruit abscission.

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