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# Production of Nitric Oxide by the Abscission Agent CMNP and Its Impact on Citrus Fruit Loosening

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**The following study was conducted to determine if the abscission agent CMNP (5-chloro-3-methyl-4-nitro-1H-pyrazole) can produce nitric oxide (NO), if NO promotes fruit abscission, and if alcohol dehydrogenase (ADH) can produce NO. CMNP was applied to run off on one-half of a 'Valencia' tree with 300 ppm CMNP in Apr. 2011. Fruit detachment force (FDF) of treated fruit decreased from 80 N to 10 N. NO increased in treated fruit from undetectable to about 1.2 nM/g fresh weight by 48 h after application and declined to below 0.2 nM/g fresh weight by 120 h. Additional untreated fruit were clipped from a 'Valencia' tree and fruit were dipped in a 2.0 mM solution of sodium nitroprusside, which releases NO after exposure to water. FDF of fruit dipped for 45 min in the solution and held at 25 °C declined similarly as after application of CMNP to field grown trees. FDF of treated fruit held at 10 °C did not decline. CMNP inhibited ADH activity in vitro. Purified ADH in solution with CMNP did not produce $NO_2^-$ as measured by the Griess method, but whether it produces NO directly was not determined. The results indicate that CMNP applied to field grown trees may be converted to NO, which then promotes abscission of sweet orange. The methods used to determine if ADH produced NO were inconclusive in this study.**

Considerable research has been conducted the last few decades to find an abscission agent as an aid to mechanical harvesting to improve fruit removal of sweet oranges. Several abscission agents, such as CMNP, ethephon, coronatine, and methyl jasmonates, have been studied. The abscission agent 5-chloro-3-methyl-4-nitro-1H-pyrazole (CMNP) has been shown to selectively loosen mature 'Valencia' fruit without causing leaf and immature fruit abscission and phytotoxicity (Hartmond et al., 2000a; Yuan and Burns, 2004). Hence, it does not affect yield the following season (Burns et al., 2006). Loosening of sweet oranges by CMNP has been shown to be sensitive to air temperatures that often occur in Florida during winter, especially with air temperatures below 15.6 °C (Alferez et al., 2005; Bensalem et al., 2001; Ebel and Burns., 2008; Kender and Hartmond., 1999; Salyani et al., 2002; Yuan and Burns, 2004).

Previous work on the mode of action of CMNP on citrus has shown a link between lipid signaling and abscission (Alferez et al., 2005). CMNP has also been show to inhibit alcohol dehydrogenase (ADH) allowing acetaldehyde to accumulate that promoted electrolyte leakage from membranes (Alferez et al., 2005). Various substituted pyrazoles have been shown to inhibit ADH (Alferez et al., 2005; Dahlbom et al., 1974), which promotes anoxia related gene expression and senescence in *Arabidopsis* (Burns et al., 2007). Although research has produced valuable insights into the mode of action of CMNP, the full mechanism is still unclear (Burns et al., 2007).

Research on animal systems may provide clues to additional aspects of the mode of action of CMNP in promoting citrus abscission. Eleven nitro-pyrazoles were shown to increase NO levels in cultured rabbit lacrimal gland cells (Xuan and Chiou, 2003). Sodium nitroprusside (SNP), an NO generator , was found to induce programmed cell death in cultured sweet orange cells at only 10 µM (Saviani et al., 2002). The mechanism of NO generation by CMNP in biological systems is unknown. Aldehyde dehydrogenase (ALDH) has been shown to reduce nitroglycerin and produce NO (Berreta et al., 2008a; Mayer and Beretta, 2008). It is assumed that the mechanism of action of producing NO from GTN via ALDH and from CMNP via ADH might be similar since both enzymes contain sulphydryl groups from cysteine in their active sites.

The objective of this study was to test the hypotheses that CMNP produces NO via ADH, that NO induces abscission of mature citrus fruit, and to determine if loosening by NO is temperature sensitive.

## Material and Methods

**Plant material.** Fully mature 'Valencia' trees located at a commercial grove near Immokalee, FL and the experimental groves at the Southwest Florida Research and Education Center were used. The trees were maintained using standard commercial fertilization, irrigation, and pest control practices.

**In vivo NO generation by CMNP.** CMNP (ASI-100 17 EC, 17.2% w/w) was applied at 300 ppm with 0.1% (w/v) Activator-90 as an adjuvant to leaf runoff to selected branches. Ten fruit were sampled daily for 5 d. Production of $NO_2^-$ in citrus flavedo samples was determined using the Griess method (Ding et al., 1988). Flavedo tissue was removed from the surface of fruit, weighed, and ground in liquid nitrogen. Fifty milliliters of glacial acetic acid (pH 3.6) were added to each sample, homogenized, and centrifuged at 15,000 rpm for 30 min. The supernatant was removed and incubated with 1% sulfanilamide and 0.1% N-1-napthylethylenediamine dihydrochloride at room temperature for 10 min. Absorbance was measured at 550 nm with a spectrophotometer (BioSpec-mini, Shimadzu Corp., Tokyo, Japan). Although the method measures $NO_2^-$, this compound is readily converted to NO via enzymatic and non-enzymatic pathways

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and NO is readily converted to nitrite and nitrate ions in the presence of oxygen (Neill et al., 2003). The presence of NO has been routinely assessed by determining formation of nitrite and nitrate ions (Neill et al., 2003).

**Effect of NO and temperature on fruit loosening.** Stems of 10 'Valencia' fruit were clipped from a field-grown tree, brought into the laboratory, and fruit were dipped in 2 mM SNP for 15, 30, 45, or 60 min. The fruit were held at 25 or 10 °C in growth chambers and humidity was maintained by covering fruit trays with a plastic sheet and wetted paper towels on the bottom of the trays. Four days after treatment, fruit detachment force (FDF) was determined using a pull-force gauge (Force One Digital Force Gauge; Wagner Instruments, Greenwich, CT) using a standard procedure (Pozo et al., 2004). FDF of fruit removed from the tree was also determined on day 4 to demonstrate that fruit removal did not have a significant impact on abscission independent of CMNP treatment. We found that after 4 d, fruit held at 25 °C had an FDF of 64.3 N and fruit from the tree had a FDF of 63.6 N.

**Role of ADH in producing NO from CMNP.** CMNP at 0, 0.05, 0.25, 0.5, 0.75, 1.25, or 1.5 mM was added to a solution of pure ADH (Sigma-Aldrich, Saint Louis, MO), 50 mM glycelglycine buffer, 1 mM $ZnSO_4$, 100 mM ethanol, and 2.5 mM $NAD^+$. The activity of ADH was monitored spectrophotometrically at $A_{340}$ for 60 s according to the method described by MacDonald and Rees (1983). Activity of ADH was expressed as nmol $NAD^+$/min/unit enzyme unit using NADH extinction coefficient of 6.2 m/M.

**Statistical analysis.** Differences among treatments was determined using a *t*-test at $\alpha = 0.05$ (SAS Institute, 2009).

## Results and Discussion

**In vivo NO generation by CMNP.** Fruit treated with CMNP generated NO with a peak of over 1.2 nM/g FW occurring 48 h after treatment (Fig. 1). The rapid rise in NO levels from 0 to 48 h after application corresponded to a decline in FDF from 80 N to 17 N, after which FDF declined further to a low of about 5 N by 72 h after application. The rate and level in decline of FDF as shown here are typical for sweet orange fruit treated with CMNP in field studies (Yuan and Burns, 2004). FDF appeared to increase again at 120 h after application, which is a phenomenon that has been reported previously with CMNP and appears to indicate a reversal in fruit loosening after the effects of CMNP wear off. NO declined rapidly to levels near the controls by 120 h after application.

**Effect of NO and temperature on fruit loosening.** Fruit treated with SNP for various time periods demonstrated a decline in FDF compared to the controls (Fig. 2). Fruit treated with SNP for 45 min demonstrated the maximum decline in FDF from 62 N for untreated controls to about 4 N. SNP was applied at 2.0 mM, which is slightly below the maximum concentration used in field studies, and the amount of decline in FDF was similar to that of field grown trees (Ebel et al., 2009). Fruit held at 10 °C did not demonstrate a decline in FDF at all SNP exposure times. FDF for fruit not treated with SNP (0 min) was higher (97 N) for fruit held at 10 °C than fruit held at 25 °C (62 N) indicating that temperature had an effect on FDF independent of treatment. The higher FDF at lower temperatures is typical for sweet orange (Yuan and Burns, 2004).

NO has inconsistent effects on plants at different concentrations. In general, NO has growth promoting effects at low concentrations and promotes senescence at higher concentrations (Beligni and Lamattina, 1999). NO delayed senescence at 0.1 µM in sunflower (Selcukcam and Cavahir; 2008), at 0.25 µM in various fruits and vegetables (Leshem et al., 1998), at 10 µM in cultured sweet orange cells (Saviani et al., 2002), and at 50 µM in *Arabidopsis* (Neill et al., 2000). NO promoted senescence at 400 µM in sunflower (Selcukcam and Cavahir, 2008) and at 500 µM in soybean (Delledonne et al., 1998, 2001). The effect of NO on senescence is further complicated by tissue sensitivity, which is a function of tissue age as shown in strawberries where endogenous NO declines as the fruit matures (Leshem and Pinchasov, 2002; Leshem et al.,1998). Furthermore, although high concentrations of NO can induce senescence, application of low NO can reverse senescence (Mishina et al., 2007). The peak level of NO found

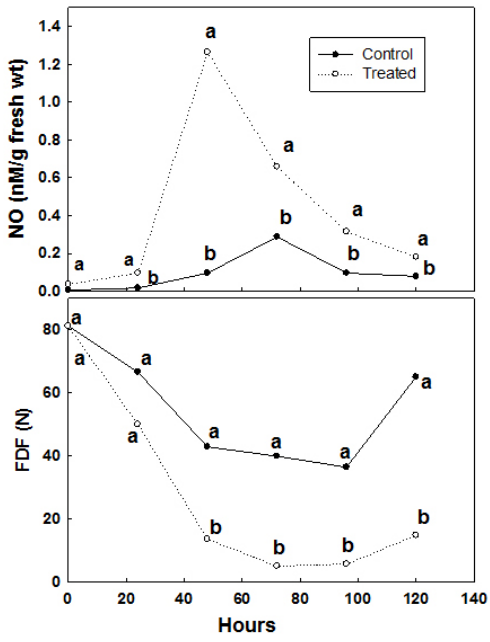


Fig 1. NO concentration of flavedo tissues and FDF of 'Valencia' sweet orange after treatment with the abscission agent CMNP (2.0 mM). Means within each sampling time followed by different letters indicate significant difference at $P < 0.05$.

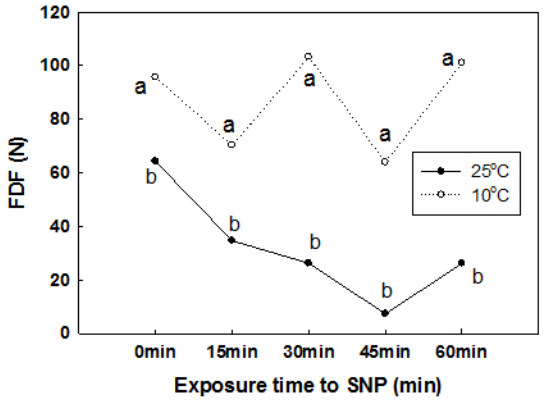


Fig 2. Effect of 2.0 mM exposure time with the NO generating compound sodium nitro prusside (SNP) on FDF of 'Valencia' sweet orange fruit held at two different temperatures. Different letters within each exposure time indicate significant differences among temperature treatments at $P < 0.05$.

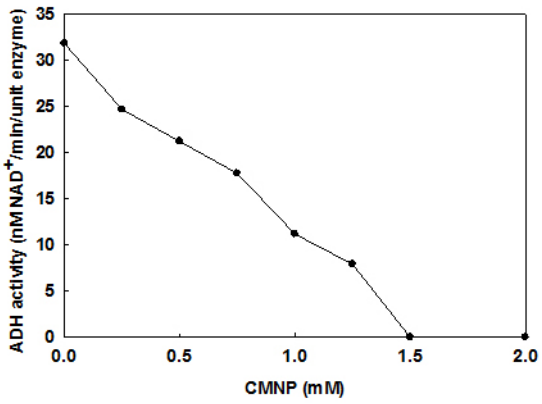


Fig 3. Inhibition of in vitro alcohol dehydrogenase (ADH) activity by the abscission agent CMNP.

in the current study of mature sweet oranges is below the level that induced cell death in sweet orange cell cultures (Saviani et al., 2002), which is likely a function of tissue age.

**Role of ADH in producing NO from CMNP.** ADH activity declined with increasing CMNP concentration (Fig. 3), a result that confirms a previous report (Burns et al., 2005). ADH activity was completely inhibited at 2.0 mM CMNP which is a little below the maximum CMNP that has been used in field studies. Pyrazole-substituted compounds have been shown to covalently bond to $NAD^+$ and form a ternary complex with ADH (Li and Theorell, 1969). Substitution of the pyrazole ring at 3 and 5 positions makes it a reversible and competitive inhibitor (Li and Theorell, 1969).

The Griess method used in this study did not detect any $NO_2^-$ in solutions of CMNP and ADH. These results indicate that if any NO was formed, it was not converted to $NO_2^-$ in solution. Researchers used the Griess method as a diagnostic test to demonstrate previous presence of NO in tissues/solutions since NO reacts rapidly with oxygen to produce $NO_2^-$ and $NO_3^-$, which are stable and non-volatile decomposition products of NO in aqueous solutions (Neill et al., 2003). One reason for not finding $NO_2^-$ in pure solution could be that in presence of oxygen the content of $NO_3^-$ ions in solution is always greater than $NO_2^-$. Nitrite ions quickly convert into nitrate under oxygenated environments (Neill et al., 2003). Also, NO is produced from $NO_2^-$ via many enzymatic and non-enzymatic pathways in plants. Enzymatic conversion of $NO_2^-$ to NO involves cytosolic nitrate reductase (Dean and Harper, 1988), PM-NR/Ni:NOR (plasma membrane bound NR/nitrite: NO oxidoreductase) (Stohr et al., 2001), mitochondrial nitrite: NO reductase activity under anoxic conditions (Planchet et al., 2005) and xanthine oxidase/dehydrogenase under hypoxic conditions (Millar et al., 1998). In citrus, it has been shown that nitrite reduction is mainly a non-enzymatic process which involves reduction of $NO_2^-$ by ascorbic acid (Bar-Akiva and Sternbaum, 1966). Currently, we are testing production of NO directly from CMNP using a different assay (Murphy and Noack, 1994).

In summary, we demonstrated that NO is produced in sweet orange flavedo tissues when treated with CMNP and that NO can cause abscission. We also demonstrated that low temperature inhibited abscission at low temperature and that this may be at least one step in the abscission process inhibited by low temperatures. It appears that CMNP does not produce $NO_2^-$ via ADH, but more work needs to be performed to determine if CMNP produces NO directly from ADH.

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