

Citrus abscission and *Arabidopsis* plant decline in response to 5-chloro-3-methyl-4-nitro-1*H*-pyrazole are mediated by lipid signalling

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ABSTRACT

The compound 5-chloro-3-methyl-4-nitro-1*H*-pyrazole (CMNP) is a pyrazole-derivative that induces abscission selectively in mature citrus (*Citrus sinensis*) fruit when applied to the canopy and has herbicidal activity on plants when applied to roots. Despite the favourable efficacy of this compound, the mode of action remains unknown. To gain information about the mode of action of CMNP, the effect of application to mature citrus fruit and *Arabidopsis thaliana* roots was explored. Peel contact was essential for mature fruit abscission in citrus, whereas root drenching was essential for symptom development and plant decline in *Arabidopsis*. CMNP was identified as an uncoupler in isolated soybean (*Glycine max*) mitochondria and pea (*Pisum sativum*) chloroplasts and an inhibitor of alcohol dehydrogenase in citrus peel, but not an inhibitor of protoporphyrinogen IX oxidase. CMNP treatment reduced ATP content in citrus peel and *Arabidopsis* leaves. Phospholipase A₂ (PLA₂) and lipoxygenase (LOX) activities, and lipid hydroperoxide (LPO) levels increased in flavedo of citrus fruit peel and leaves of *Arabidopsis* plants treated with CMNP. An inhibitor of PLA₂ activity, aristolochic acid (AT), reduced CMNP-induced increases in PLA₂ and LOX activities and LPO levels in citrus flavedo and *Arabidopsis* leaves and greatly reduced abscission in citrus and delayed symptoms of plant decline in *Arabidopsis*. However, AT treatment failed to halt the reduction in ATP content. Reduction in ATP content preceded the increase in PLA₂ and LOX activities, LPO content and the biological response. The results indicate a link between lipid signalling, abscission in citrus and herbicidal damage in *Arabidopsis*.

Key-words: alcohol dehydrogenase; ATP; lipid hydroperoxide; lipoxygenase; membrane breakdown; phospholipase A₂; senescence; uncoupler.

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INTRODUCTION

Abscission in plants is a highly co-ordinated developmental process that results in shedding of organs such as leaves, flowers and fruits. Abscission is often considered a stress response in plants, as it can be triggered by environmental and pathogen-related challenges (Eyal *et al.* 1993; Taylor & Whitelaw 2001; Roberts, Elliot & Gonzalez-Carranza 2002). Induction of genes involved in secondary metabolism, the PR response, cell wall modification, hormonal metabolism and signal transduction have been described during the abscission process in citrus as well as other plants (Taylor & Whitelaw 2001; Burns 2002; Kostenyuk, Zón & Burns 2002; Ruperti *et al.* 2002; Wu & Burns 2003, 2004). Thus, once abscission has been initiated, the suite of genes that are induced in the abscission zone as abscission progresses appears to be similar among a variety of plant species. However, the cellular events that prepare organs for abscission and initiate downstream abscission events have not been well defined.

There is some indication that the lipid-signalling pathway is operative during abscission. Genes encoding lipid-signalling pathway components, such as those involved in jasmonic acid biosynthesis, were up-regulated in the abscission zone in response to stress (Kubigsteltig, Laudert & Weiler 1999) and during anther dehiscence (Sanders *et al.* 2000), suggesting a role for this pathway in the abscission process. Furthermore, methyl jasmonate (Ueda, Miyamoto & Hashimoto 1996; Hartmond *et al.* 2000) and a biological analog coronatine (Burns *et al.* 2003a) were shown to promote fruit loosening, suggesting that bioactive octadecanoid compounds generated through phospholipid signalling can promote abscission. Interestingly, the mature fruit-specific abscission compound 5-chloro-3-methyl-4-nitro-1*H*-pyrazole (CMNP) was found to induce expression of genes with high similarity to allene oxide synthase and 12-oxophytodienoate reductase in mature fruit abscission zones (Burns 2002). CMNP causes significant reduction in fruit detachment force (FDF) of mature fruit selectively when applied to the canopy, with little or no phytotoxicity to foliage or immature fruit. Despite its favourable efficacy

as a selective abscission agent for citrus, the mode of action of CMNP remains unknown.

Pyrazoles used in agriculture are generally herbicidal compounds that target components of energetic machineries in plant cells (Duke, Dayan & Rimando 2000). Efficient uptake is primarily through the root system. CMNP is a nitro-substituted pyrazole containing chlorine (Fig. 1). Nitro-substituted organic compounds are known to have uncoupling activity. A number of structural features of CMNP suggest that it may behave as an uncoupler of oxidative phosphorylation in biological systems. CMNP is weakly acidic and lipophilic; common features identified in uncouplers (Hanstein 1976). The nitro group and chlorine atom are electron-drawing groups, creating a partial positive charge on N1. Under these conditions, N1 can accept or donate protons readily (Chopineaux-Courtois *et al.* 1999), suggesting that CMNP may be capable of carrying protons across energetic membranes and dissipating the electrochemical gradient that drives ATP synthesis.

Under certain stress conditions, such as anoxia, decreased ATP availability can trigger membrane breakdown (Rawlyer *et al.* 1999, 2002). Membrane breakdown can occur via action of lipid acyl hydrolase and/or various phospholipases, and this is considered to be a first step in the generation of lipid-derived messengers (Wang *et al.* 2002). In our own preliminary investigation, several patatin-related genes with reported phospholipase A₂ (PLA₂) activity (Senda *et al.* 1996; Holk *et al.* 2002) and other lipid-signalling pathway-related genes were induced in the vegetative portion of *Arabidopsis* plants showing symptoms of plant decline in response to root applications of CMNP (Alferez & Burns 2003). Factors such as hyperosmotic stress (Meijer *et al.* 2001), wounding (Lee *et al.* 1997), drought (Benhassaine-Kesri *et al.* 2002) and pathogen attack (Laxalt & Munnik 2002) can trigger lipid-based signalling that ultimately lead to responses such as senescence and programmed cell death.

Signal transduction in the lipid-based pathway begins with receptors located in the plasma membrane that receive a signal(s) and transmit information via effectors that convert membrane lipids into signalling molecules

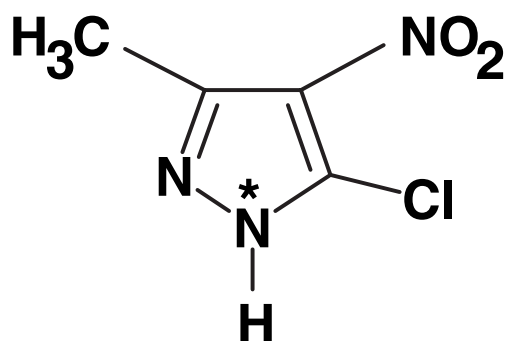


Figure 1. Structure of 5-chloro-3-methyl-4-nitro-1H-pyrazole. Asterisk indicates N1.

(Meijer & Munnik 2003). These effectors can be phospholipases or lipid acyl hydrolases that hydrolyse membrane phospholipids. Further metabolism of these fatty acids results in production of key components of the lipid-signalling pathway with downstream signalling roles (Meijer & Munnik 2003). Linoleic and linolenic acids are major constituents of polyunsaturated fats in plants and are common substrates for plant lipoxygenases (LOX). LOX are dioxygenases that add oxygen to linoleic and linolenic acids to produce highly reactive lipid hydroperoxides (LPOs); LPOs stand at the branch point of several competing metabolic pathways that form a wide variety of final response molecules in plants.

In this study, we investigated effects of the pyrazole derivative CMNP on cell energetics and on initial phases of the lipid-signalling cascade in citrus fruit and *Arabidopsis* to gain information about mode of action and plant response. Phospholipase A₂ (PLA₂) and LOX activities, and LPO content of CMNP-treated tissues provide evidence for the role of the lipid-based signalling pathway during abscission in mature citrus fruit and plant decline in *Arabidopsis*.

MATERIALS AND METHODS

Reagents

CMNP (5-chloro-3-methyl-4-nitro-1H-pyrazole) was isolated from an agrochemical formulation from Abbott Laboratories (Abbott Park, IL, USA) following a procedure previously described (Burns *et al.* 2003b). All other compounds were obtained from Sigma Chemical (St. Louis, MO, USA) unless otherwise stated.

Plant material and treatments

Soybean (*Glycine max* [L.] Merr. cv FFR 665) seedlings were greenhouse-grown in flats under a natural photoperiod with supplemental lighting. Seeds were immersed in water and bubbled with air for 2 h to improve uniformity of germination time. Cotyledons were harvested between 6 and 8 d after planting and used for further work. English pea (*Pisum sativum* L. cv. Laxton's Progress 9) was germinated and cotyledons from 9- to 14-day-old-plants were used.

Mature citrus fruit cv. 'Valencia' (*Citrus sinensis* L. Osbeck) from 15-year-old trees were used. Three canopy sectors of approximately 3 m³ each were randomly selected from a group of 10 trees and treatments applied with a pressurized hand sprayer to run-off. Treatments were 1.5 mM CMNP, 1 mM aristolochic acid (AT), 1.5 mM CMNP + 1 mM AT, or water alone. The experiment was repeated twice, with at least 30 fruit from each of three canopy sectors selected for analysis. After 3, 6, 24, 48, 72 and 96 h of application, fruit detachment force (FDF) in kilograms was measured using a digital force gauge ('Force Five'; Wagner Instruments, Greenwich, CT, USA) as previously described (Hartmond *et al.* 2000). The 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 analysis of PLA_2 and LOX activities. For whole fruit or leaf applications, mature fruit or leaves were briefly submerged into a solution of 1.5 mM CMNP. Targeted applications to calyx, pedicel, or flavedo were accomplished using a cotton swab saturated with a CMNP solution. Each experiment contained 10 replicates and was repeated twice.

The entire above-ground vegetative portion of adult plants of *Arabidopsis thaliana* was used. Seeds were first stratified by imbibing at 4°C for 48 h and then transferred to flats containing soil (Metro Mix 5000; Scotts, Milwaukee, WI, USA). For PLA_2 , LOX, LPO, and ATP determination, plants were reared in growth chambers for 2 weeks at 20°C (10 h light at $300\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ and 14 h dark) and then transferred to 26°C under incandescent lamps ($300\ \mu\text{mol m}^{-2}\text{ s}^{-1}$) with a 14 h light/10 h dark cycle for 1.5 weeks before treatment. Plants were treated by drenching soil to saturation with test solutions containing 1.5 mM CMNP, 50 μM AT, 1.5 mM CMNP + 50 μM AT, or water alone. Individual plants were removed from the plastic flats and immersed in beakers containing 50 mL test solutions without contacting the leaves or plant 'crown'. Tissues for PLA_2 , LOX, LPO, and ATP analysis were harvested for various times up to 16 h after treatment. All treatments contained Tween 20 (0.01%) as adjuvant.

Mitochondrial and chloroplast uncoupling assays

Mitochondria were isolated and purified from soybean cotyledons according to the method of Umbach & Siedow (1993), based on the continuous Percoll gradient protocol method (Day, Neuberger & Douce 1985), except that 0.4 M sucrose, 2% bovine serum albumin (BSA), and 40 mM ascorbate were used in the grinding buffer and 0.2% BSA was used in the wash buffer.

Mitochondrial respiratory activity and its response to CMNP and other compounds were monitored as NADH-supported oxygen uptake using a Clark-type oxygen electrode. A 2.0 mL volume of reaction buffer (10 mM KCl, 5 mM MgCl_2 , 10 mM K phosphate, pH 7.2, 0.3 M mannitol, 0.1% BSA) was used with 0.1–0.15 mg mitochondrial protein. NADH was supplied at a final concentration of 1 mM. Respiratory state 3 to state 4 transitions were induced by addition of 30 μM ADP. FCCP was used at a final concentration of 5 μM , shown by preliminary experiments to cause complete uncoupling. Myxothiazol was used at 6 μM to inhibit the cytochrome oxidase pathway. Under these assay conditions, SHAM-sensitive respiratory activity was undetectable so SHAM was not routinely included in the assays. For each mitochondrial isolation experiment, CMNP was added up to 2 mM final concentration. DNP and oligomycin in ethanol were used at final concentrations of 10 μM and 1 $\mu\text{g mL}^{-1}$, respectively. The mitochondria were well-coupled, displaying respiratory control ratios greater than 2.0 for all isolations.

Chloroplasts were isolated and prepared for OE17 import studies essentially as described (Cline *et al.* 1993; Summer & Cline 1999). OE17 radiolabelled precursor was prepared by *in vitro* translation in the presence of [^3H]leucine in a wheat germ system. Chloroplasts (20 μg protein) were incubated in the presence of 5 mM MgCl_2 and 50 μL radiolabelled OE17 under $150\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ white fluorescent lighting. To determine the effect of CMNP on transport of OE17 into the thylakoid lumen, isolated chloroplasts were preincubated for 10 min with 0–2 mM CMNP. Nigericin (0.5 μM) + valinomycin (1 μM) was used as a positive control. The import assay was then run for 10 min and terminated by transfer to an ice bath. Chloroplasts were recovered by centrifugation at 3200 g for 8 min. Chloroplast proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose and visualized by exposing the blot to film.

ADH extraction and assay

To test ADH activity in citrus flavedo, increasing CMNP concentrations (from 0 to 2 mM) + 0.01% Tween[®] 20 adjuvant were applied to fruit in the field. Samples were taken after 24 h of treatment. At least 30 fruit were used in each treatment. Fruit were washed with a mild soap solution and rinsed three times with distilled water to remove any CMNP adhering to the outer fruit surface. For each treatment, 3 g flavedo were ground to a powder in liquid nitrogen, dissolved in 10 mL extraction buffer [1 M Tris HCl (pH 7.5), 0.5 M ethylenediaminetetraacetic acid (EDTA), 100 mM phenylmethylsulphonyl fluoride (PMSF), 5% polyvinylpyrrolidone (PVPP), and 2 mM dithiothreitol (DTT)], homogenized and centrifuged at 8900 g for 6 min. Supernatant was centrifuged at 50 000 g for 35 min. A Sephadex column (PD-10, Amersham Biosciences, Piscataway, NJ, USA) was used to recover protein with elution buffer [10 mM HEPES/KOH (pH 7.0), 2 mM DTT and 1 mM MgCl_2]. To assay ADH activity *in vitro*, extracts from untreated controls were incubated with 0.5 mM CMNP, 4-nitropyrazole or pyrazole in 50 mM glycyglycine buffer, 2.5 mM NAD^+ , 100 mM ethanol and 1 mM ZnSO_4 . ADH activity was measured by monitoring the $A_{340\text{nm}}$ for 5 min as described by MacDonald & ap Rees (1983). The reaction rates were determined using the NADH extinction coefficient of $6.2\ \text{mM}^{-1}$. Activities were expressed as $\text{nmol NAD}^+\ \text{min}^{-1}\ \text{mg}^{-1}\ \text{prot}$.

PLA_2 , LOX and LPO extraction and assay

For each treatment, 4 g adult *Arabidopsis* leaves or 1 g flavedo were immersed in liquid nitrogen and ground to a powder. The frozen powder was dissolved in 8 mL of extraction buffer [1 mM EDTA, 100 mM Tris-HCl (pH 7.5), 2% PVPP, and sorbitol 0.15 M] and centrifuged at 8900 g for 15 min. Supernatant proteins were precipitated overnight using ammonium sulphate to saturation. After centrifugation, the pellet was washed three times and re-suspended

in 80 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM CaCl₂, 4 mM Triton® X-100, 30% glycerol and 1 mg mL⁻¹ BSA for total PLA₂ analysis or in 100 mM Tris-HCl (pH 7.4) for LOX analysis. The procedure was carried out at 4 °C.

Total PLA₂ activity (patatin and secretory PLA₂) in citrus flavedo and *Arabidopsis* was assayed using a cPLA₂ kit from Cayman Chemical Co (Ann Arbor, MI, USA). The appropriate sample volume was incubated in the presence of arachidonoyl Thio-PC as substrate for 60 min to ensure complete hydrolysis of the arachidonoyl thioester bond. Released free thiols were detected by the addition of DTNB/EGTA (5,5'-dithiobis[2-dinitrobenzoic acid] Ellman's reagent) and measuring the change in A_{414nm} at the end of the incubation period. The reaction rate was determined using the DTNB extinction coefficient of 10.66 mM⁻¹. Activities were expressed as μmol free thiols min⁻¹ mg⁻¹ prot. Commercial bee venom PLA₂ was used as a standard positive control, and spiking samples with increasing concentrations of the standard assessed reaction linearity.

LOX activity was assayed using a commercial lipoxigenase inhibitor screening assay kit from Cayman Chemical. An appropriate sample volume was incubated for 5 min with linoleic acid as substrate. Chromogen supplied by the manufacturer was added to stop enzyme catalysis and develop the reaction. LOX activity was measured by monitoring the change in A_{500nm} for 20 min. The reaction rate was determined using the chromogen extinction coefficient of 9.47 mM⁻¹. 15-LOX from soybean was used as a positive control, and spiking samples with increasing amounts of 15-LOX ensured reaction linearity. LOX activity was expressed as μmol hydroperoxide min⁻¹ mg⁻¹ prot.

Protein was determined by the dye-binding method (Bio-Rad Laboratories, Hercules, CA, USA) using BSA as standard.

To test AT effect on PLA₂ activity *in vitro*, the appropriate volume of PLA₂ standard, or citrus and *Arabidopsis* enzymatic extracts were incubated with 12.5, 25, 50 or 100 μM AT in the presence of arachidonoyl Thio-PC as described above. The effect of AT on LOX activity was determined by incubating the appropriate volume of LOX standard for 5 min with 12.5, 25, 50 or 100 μM AT and linoleic acid as substrate. For these assays, citrus and *Arabidopsis* extracts selected were those with the highest specific activity during time-course experiments (*Arabidopsis*, 3 h after CMNP treatment; citrus, 24 h after CMNP treatment).

LPO content in citrus and *Arabidopsis* was extracted and measured using a lipid hydroperoxide assay kit from Cayman Chemical Co. The entire procedure was carried out at 4 °C. Briefly, tissue was homogenized with 2 mL H₂O and centrifuged at 17 500 g for 5 min. The supernatant was combined with an equal volume of methanolic extractant for deproteination and extraction. LPO was eluted in deoxygenated chloroform and collected by centrifugation at 1400 g for 5 min. The resulting chloroform extracts were stored at -80 °C and utilized for assay within 72 h of collection. Sample LPOs were reacted with ferrous ions in a

direct redox reaction. The resulting ferric ions were detected using thiocyanate ions as the chromogen. Five hundred microlitres extract was mixed with 450 μL of a mixture of chloroform : methanol (2 : 1 v/v). After stirring, 50 μL of chromogen (mixture of equal volumes of 4.5 mM FeSO₄ in 0.2 M HCl and a 3% methanolic solution of NH₄SCN) was added and stirred. The A_{490nm} was determined as described above. Sample LPO content was compared against a standard curve plotted with serial dilutions of a 50 μM ethanolic solution of 13-hydroperoxy octadecadienoic acid. The LPO concentration was expressed as nmol per gram fresh weight. The experiment was repeated four times in citrus and *Arabidopsis* with at least four replicates.

ATP extraction and measurement

ATP extraction and measurement was carried out as previously reported (Rawlyer *et al.* 1999). Briefly, ATP was extracted by homogenizing 1 g fresh weight with 12 mL of chilled 5% trichloro acetic acid (TCA). After centrifugation for 6 min at 17 500 g and 4 °C, the supernatant was neutralized and diluted with 0.1 M Tris acetate buffer (pH 7.75) so that the final sample concentration of TCA was reduced to less than 0.1%. Following extraction, ATP content was determined using a commercially available luciferin-luciferase system from Promega (Madison, WI, USA). The intensity of emitted light was directly proportional to sample ATP content and was measured using a luminometer (Optocomp I; MGM Instruments, Hamden, CT, USA). The experiment was repeated at least three times for citrus and *Arabidopsis*, with three replicates each.

Carfentrazone effects in citrus and *Arabidopsis*

The effect of 300 μM carfentrazone (CFZ) and 1.5 mM CMNP on FDF was assayed on potted 5-year-old Hamlin citrus trees. Four trees were placed under a 10 h day/14 h night (1000 μmol m⁻² s⁻¹) or a 24 h dark regime for 8 d in growth chambers held at 25 °C. Ten fruit and three replicates per treatment were used. Fruit drop was monitored daily. FDF was measured 8 d after treatment. If all fruit from a treatment abscised before FDF was measured, the fruit weight was considered as the final FDF on day 8. To determine the effect of CMNP on protoporphyrinogen IX oxidase (Protox) activity, 1 g leaf tissue from 3-week-old *Arabidopsis* plants were extracted as described (Lee & Duke 1994). Protogen IX substrate was prepared (Jacobs & Jacobs 1982; Lee & Duke 1994) with modifications. Ten millilitres of Protogen IX stock solution (0.5 mM Protoporphyrin IX; Frontier Scientific, Logan, UT, USA) in 20% ethanol containing 10 mM KOH were reduced to Protogen IX by using 1.3 g of mercury sodium under a continuous N₂ stream. The solution was adjusted to pH 8.0 by addition of 500 mM HEPES (pH 7.5) and 25 mM EDTA, filtered and 2 mM DTT was added. Protox was assayed as described (Sherman *et al.* 1991; Lee & Duke 1994). CFZ or CMNP was added to a final concentration of 0, 5, 10, 50, 100 or

200 μM CFZ in dim light at room temperature. Fluorescence was measured using a Bio-Rad Versafluor Fluorometer (Hercules, CA, USA) with excitation and emission wavelengths set at 395 and 620 nm, respectively. Reaction rates did not vary over a 5-min period. Heat-inactivated extract (15 min, 80 °C) confirmed autoxidation of Protogen IX was undetectable. Treatments for Protogen assays were duplicated and extractions and activity measurements were triplicated. Data are expressed as means \pm SEM.

RESULTS

CMNP induced mature fruit abscission when applied to the peel

Application of CMNP to various tissues of citrus demonstrated that peel contact was necessary to induce mature fruit abscission (Table 1). When abaxial and adaxial surfaces of one or two leaves adjacent to a mature fruit were dipped in a solution of 1.5 mM CMNP, no reduction in FDF occurred. Similarly, application of CMNP to the pedicel located 1 cm above the mature fruit abscission zone, or application directly to the fruit calyx that overlies the mature fruit abscission zone did not reduce FDF. Significant reduction in FDF was achieved only when CMNP was applied to mature fruit peel. Further, efficacy increased when CMNP application was made to a larger surface area of the fruit. However, a targeted treatment of a 1-cm-banded peel application 1 cm below the calyx reduced FDF to that of whole fruit application, suggesting that proximity of CMNP application relative to abscission zone of the mature fruit is an important factor in the response of fruit to this compound.

CMNP has uncoupling activity and inhibits alcohol dehydrogenase

The ability of CMNP to uncouple energetic membranes was tested in pea chloroplasts and soybean cotyledon mitochondria. In chloroplasts, the ability to transport the 17 kDa subunit of the PSII oxygen evolution complex (OE17) into the thylakoid lumen is dependent upon the

ΔpH gradient. OE17 transport is a two-step process: the stroma-targeting domain is removed upon import into the stroma, followed by removal of the lumen-targeting domain upon transport into the thylakoid lumen (Summer & Cline 1999). Thus, uncoupling activity of CMNP can be assessed by following the size reduction of radiolabelled OE17 in isolated chloroplasts. CMNP inhibited the transport of OE17 from the stroma to the thylakoid lumen in a concentration-dependent manner (Fig. 2a). OE17 transport was partially inhibited when 375 μM CMNP was included in the incubation medium, whereas no transport occurred when 2 mM CMNP was used. Effective concentrations of CMNP were higher than the 1.0 μM valinomycin/0.5 μM nigericin combination.

To assess the effect of CMNP on NADH-supported mitochondrial respiratory activity, a state 4 rate first was established by allowing ADP depletion and a concurrent decrease in respiratory rate due to the presence of a large proton gradient (Fig. 2b, trace 3). An uncoupler under state 4 conditions will relieve this gradient, resulting in a respiratory rate increase. Accordingly, addition of 5 μM FCCP resulted in a three- to four-fold respiratory rate increase (Fig. 2b, trace 3; Fig. 2c). CMNP was found to have uncoupling effects like FCCP, but the effective concentrations were higher. At 10 μM CMNP, uncoupling was essentially undetectable (Fig. 2c). As CMNP concentrations increased, uncoupling occurred, beginning at 50 μM and becoming maximal at 1.0 mM. The CMNP effective concentration range was similar to that of 2,4-dinitrophenol (DNP), although DNP was effective at lower concentrations than CMNP. Uncoupling with DNP was detectable at 10 μM , causing rate increases of 21 and 26% for two different mitochondrial preparations, and was maximal at 500 μM (data not shown). As another check for uncoupling activity, CMNP was tested for its ability to relieve oligomycin inhibition. Oligomycin, by inhibiting the mitochondrial ATPase, prevented formation of a state 3 rate transition upon addition of ADP (Fig. 2b; compare traces 1 and 3). Like FCCP (Fig. 2b, trace 1), CMNP was able to relieve oligomycin inhibition and stimulate the rate of oxygen consumption (Fig. 2b, trace 2).

CMNP had some inhibitory effect on respiratory rates in that although maximum uncoupled rates were high in the presence of CMNP, they were lower than those achieved with FCCP (Fig. 2c). For two separate mitochondrial isolations, CMNP added after FCCP decreased the oxygen uptake rate 15 and 16% for 500 μM CMNP, 19 and 25% for 1 mM CMNP, and 31 and 41% for 2 mM CMNP. Similar inhibition was seen when succinate was used as a substrate. In addition, 0.5 and 1.0 mM DNP also inhibited uncoupled (i.e. in the presence of FCCP) respiration (data not shown). Therefore, the secondary inhibitory effect of CMNP on respiration appears to be non-specific and may simply result from the presence of a relatively large amount of uncoupler in the mitochondrial membrane.

Pyrazole and some pyrazole derivatives are known ADH inhibitors (Dahlbom *et al.* 1974). CMNP inhibited ADH activity isolated from untreated citrus flavedo in a concen-

Table 1. Effect of CMNP application to various tissues of citrus on fruit detachment force (FDF, in kg) measured 4 d after application

Control	10.0 \pm 0.3
One leaf	10.2 \pm 0.2
Two leaves	10.1 \pm 0.3
Pedicel	9.8 \pm 0.2
Calyx	10.0 \pm 0.4
1/4 fruit	7.8 \pm 1.1
1/2 fruit	4.5 \pm 0.7
3/4 fruit	2.1 \pm 0.3
Whole fruit	2.2 \pm 0.5
1 cm band below calyx	2.4 \pm 0.3

Data are means \pm SEM from three experiments.

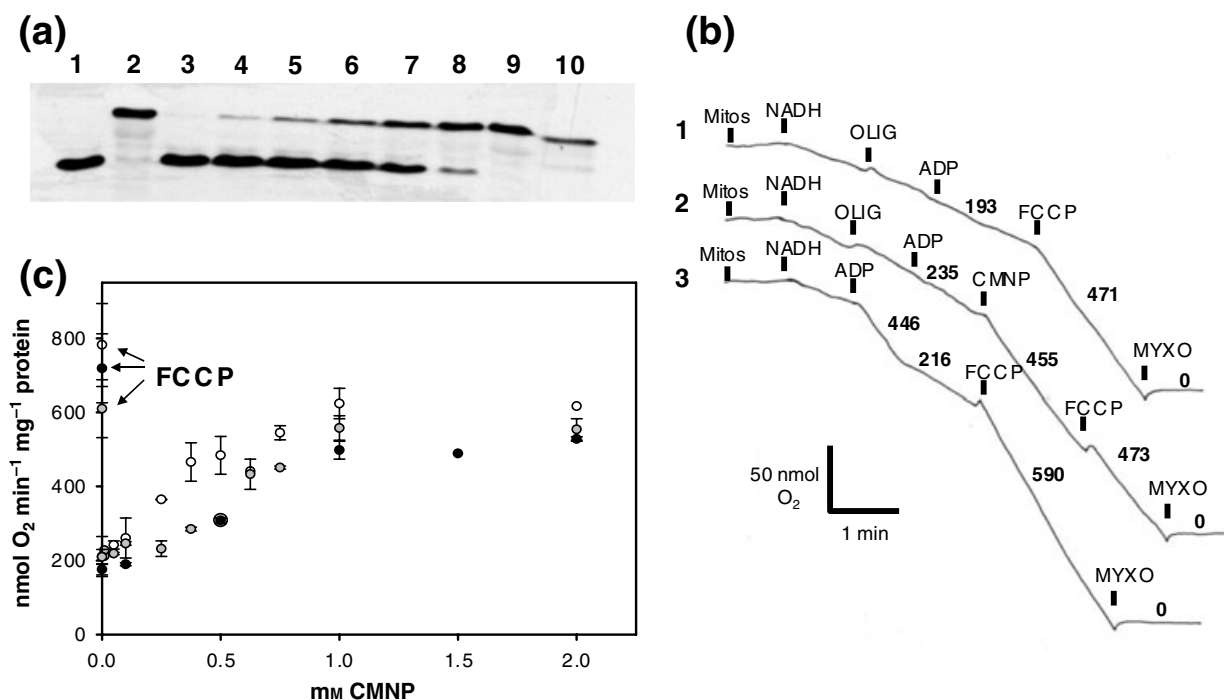


Figure 2. CMNP is an uncoupler. (a) Effect of CMNP on transport of OE17 in isolated chloroplasts of pea cotyledons. Chloroplasts (20 μg protein) were added to the reaction and incubated in the presence of 0–2 mM CMNP. Lane 1, control; lane 2, valinomycin + nigericin; lane 3, 0.25 mM CMNP; lane 4, 0.375 mM CMNP; lane 5, 0.5 mM CMNP; lane 6, 0.625 mM CMNP; lane 7, 0.75 mM CMNP; lane 8, 1.0 mM CMNP; lane 9, 2.0 mM CMNP; and lane 10, labelled OE17 translational product. (b) Representative traces of O_2 consumption by isolated soybean cotyledon mitochondria showing the effects of oligomycin, CMNP, and FCCP, but not ADP, stimulated activity after oligomycin addition. Mitochondria (0.145 mg) were added to the oxygen electrode cuvette and respiration was initiated by the addition of 1 mM NADH. Final concentrations for other additions made to the assays were: 1 $\mu\text{g mL}^{-1}$ oligomycin, 30 μM ADP, 5 μM FCCP, and 1 mM CMNP. OLIG, oligomycin; MYXO, myxothiazol. Numbers associated with the traces are respiration rates ($\mu\text{mol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ mitochondrial protein), and (c) effect of CMNP on mitochondrial state 4 respiratory rates. The figure shows results from three separate mitochondrial isolations. All assays were with 1 mM NADH as substrate. FCCP (5 μM) or CMNP were added when a state 4 rate had been established after addition of 30 μM ADP. The rates shown at 0 mM CMNP are the state 4 rates, or, if designated, the rates in the presence of FCCP. Isolation 1 (grey circles); Isolation 2 (black circles); Isolation 3 (white circles); number of replicates per concentration were 1 (0.01, 0.625, 2.0 mM), 2 (0.05, 0.625 mM), 3 (0.375, 0.75 mM), 4 (0.10, 0.50, 1.0 mM). Error bars show standard deviation.

tration-dependent manner (Fig. 3a). A concentration of 1 mM reduced flavedo ADH activity below 20% of the control. Pyrazole was the most effective inhibitor when compared with CMNP and 4-nitropyrazole (4-NP) at a similar concentration (Fig. 3a, inset). To determine whether canopy applications of CMNP were influencing flavedo ADH activities, 1.5 mM was applied and mature fruit harvested at 0, 24 and 48 h after application. Fruit were washed with a mild soap solution and rinsed three times to remove CMNP that may have adhered to fruit surfaces. After washing, flavedo was excised and extracted. Flavedo ADH activity markedly decreased 24 h after CMNP application and remained low thereafter (Fig. 3b).

CMNP increased PLA_2 and LOX activities and LPO content

PLA_2 and LOX activities were determined in extracts of citrus flavedo treated with 1.5 mM CMNP alone or in combination with 1 mM AT, a specific inhibitor of PLA_2 activity (Chandra, Heinstejn & Low 1996; Scherer & Arnold 1997),

during a 96-h-time course. PLA_2 activity remained constant in flavedo of fruit treated with water alone, and no increase in PLA_2 activity was observed during the first 6 h after CMNP treatment (Fig. 4a). Thereafter, PLA_2 activity progressively increased to more than four-fold after 96 h. The combination treatment of AT + CMNP greatly reduced PLA_2 activity. Similar trends were observed with LOX. LOX activity in CMNP-treated flavedo was essentially comparable to water-treated controls during the first 24 h after treatment (Fig. 4b). After 48 h of CMNP treatment, a two-fold increase in LOX activity was observed, and after 96 h, LOX activity increased approximately four-fold. Concomitantly, LPO content also increased in flavedo of CMNP-treated fruit after 48 h of treatment and remained at that level until the end of the experiment (Fig. 4c). The combination treatment of AT + CMNP counteracted this effect.

PLA_2 and LOX activities were also determined in extracts of *Arabidopsis* mature leaves treated with 1.5 mM CMNP as a root drench. PLA_2 activity remained constant in leaves of plants treated with water alone, but after 2 h of

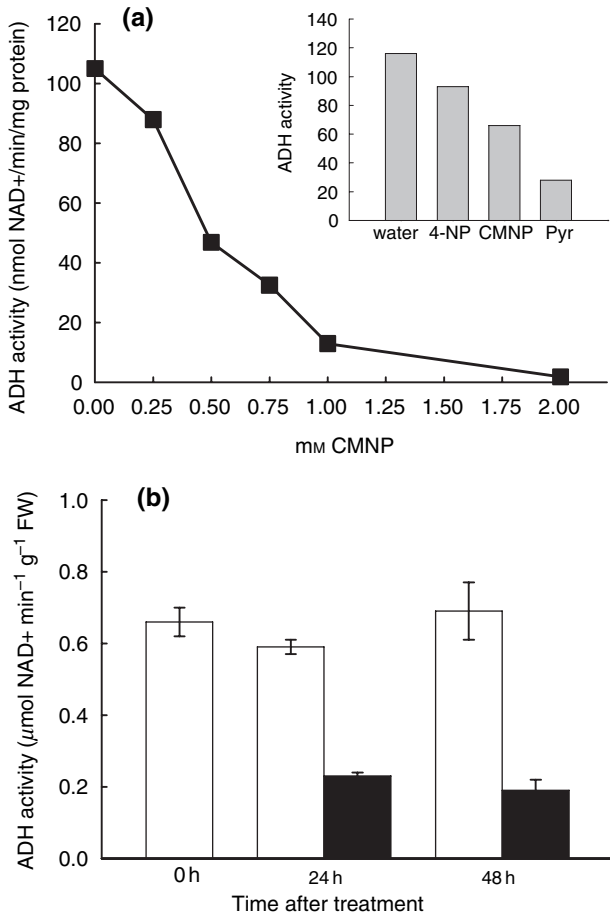


Figure 3. CMNP inhibits ADH activity. (a) Effect of increasing concentrations of CMNP (0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75 and 2 mM) or water added to extracted ADH activity from citrus flavedo. Inset panel shows CMNP inhibition of ADH as compared to other pyrazoles (4-NP and pyrazole). Concentrations used were 0.5 mM. (b) Effect of 1.5 mM CMNP applied to the tree canopy on ADH activity of citrus flavedo 24 and 48 h after treatment. White bars, control; black bars, CMNP.

root exposure to CMNP, PLA₂ activity markedly increased (Fig. 5a). After 3 h, PLA₂ activity decreased gradually during the 16-h-time course. Application of CMNP + AT to *Arabidopsis* roots prevented the increase in PLA₂ activity. LOX activity was low but variable in control and AT-alone treatments throughout the course of the experiment (Fig. 5b). A five-fold increase in LOX activity was measured 2 h after CMNP treatment, followed by a decrease to control levels after 16 h. When AT was combined with CMNP applications, LOX activity was reduced. LPO formation was enhanced by CMNP in mature leaf tissue (Fig. 5c). Although some variation in LPO content occurred during the first 2 h, no clear increase was observed until 3 h after CMNP application. Thereafter, LPO declined to control levels. AT prevented LPO accumulation.

We assessed specificity of AT and sensitivity of PLA₂ and LOX activities to AT *in vitro*. PLA₂ activities isolated from untreated *Arabidopsis* leaves and in commercial bee

venom standard were completely inhibited at 15 μM AT, and more than 80% reduction was observed in extracts of untreated flavedo. AT had no effect on citrus or *Arabidopsis* LOX activity, or in commercial soybean LOX standard (data not shown).

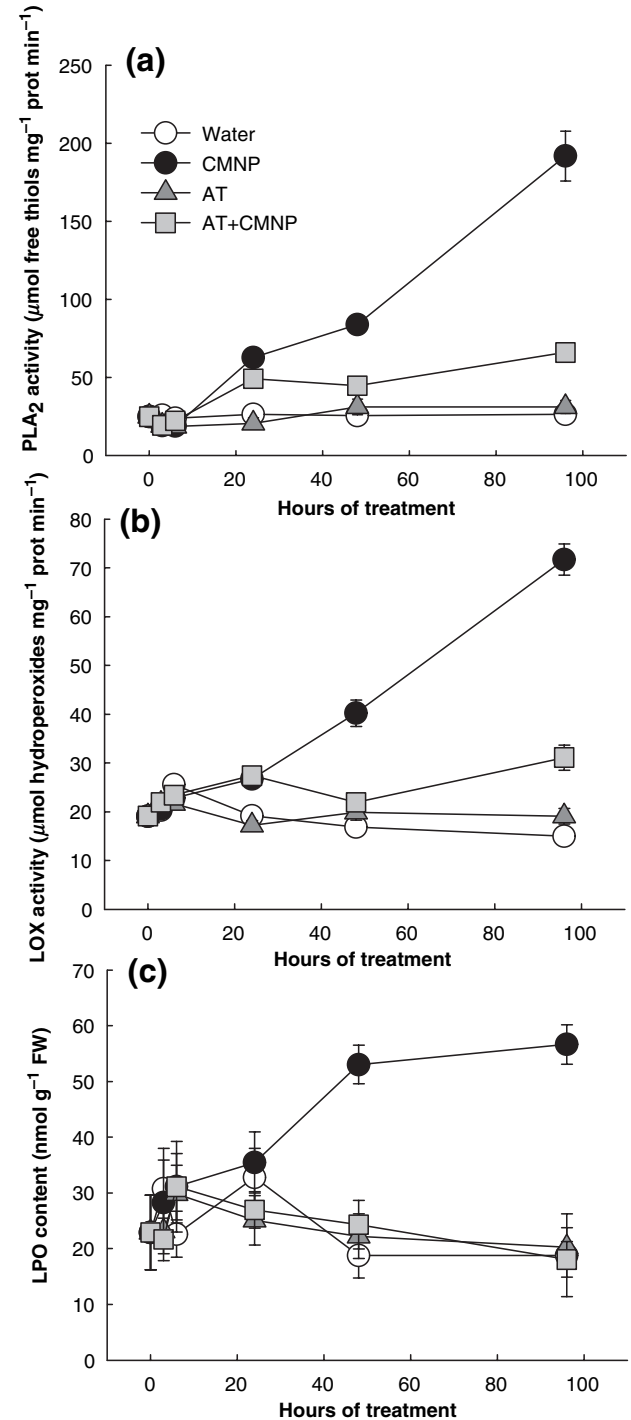


Figure 4. CMNP increases PLA₂ and LOX activities and LPO content in citrus. (a) PLA₂ activity; (b) LOX activity; and (c) LPO content in citrus flavedo over a 96-h period in response to 1.5 mM CMNP, 1.5 mM CMNP + 1 mM AT, 1 mM AT alone or water. Data shown are means ± SEM from four experiments.

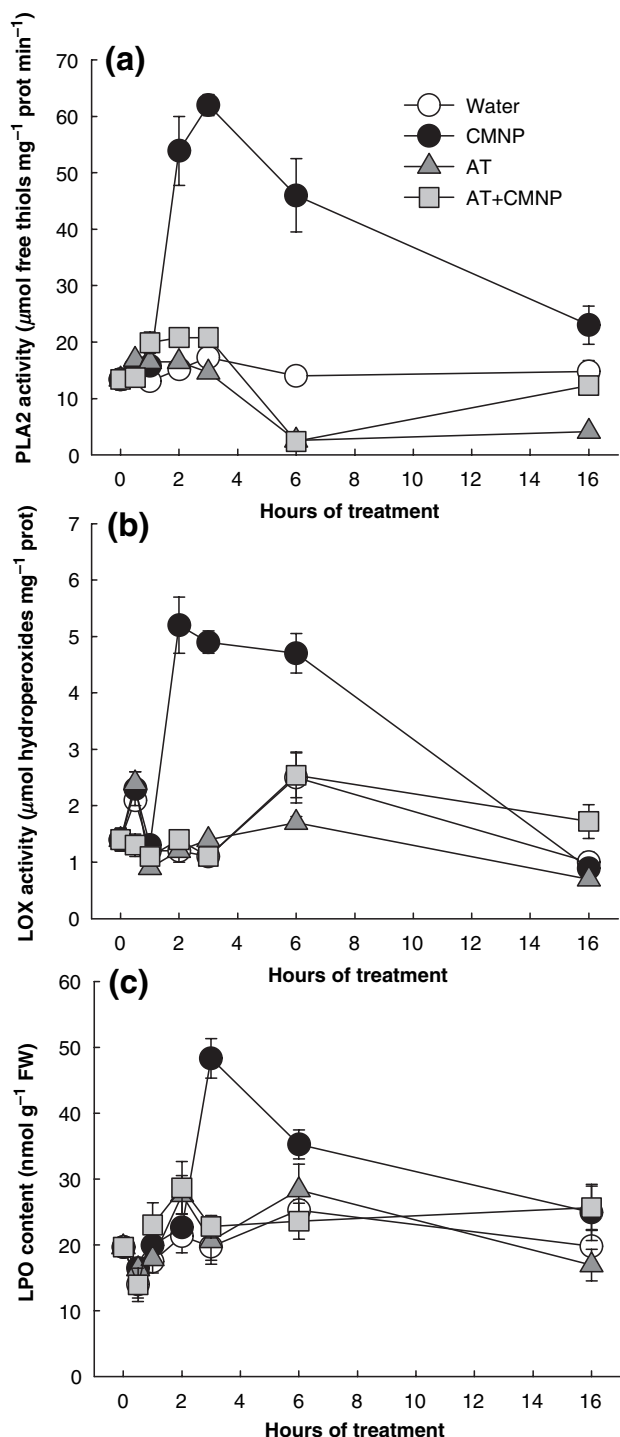


Figure 5. CMNP increases PLA₂ and LOX activities and LPO content in *Arabidopsis*. (a) PLA₂ activity; (b) LOX activity; and (c) LPO content in *Arabidopsis* adult leaves over a 16-h period in response to 1.5 mM CMNP, 1.5 mM CMNP + 50 µM AT, 50 µM AT or water. Data shown are means ± SEM from four experiments.

CMNP decreased total cellular ATP content

A reduction in ATP content after CMNP treatment is expected as a consequence of CMNP-induced uncoupling. To test this, total ATP content was measured in citrus fla-

vedo and *Arabidopsis* leaves treated with CMNP alone or in combination with AT. In citrus flavedo treated with water alone, ATP levels were maintained between 65.0 and 80.0 nmol g⁻¹ FW throughout the 96 h experiment. ATP content declined 10% 3 h after CMNP treatment; after 6 h, a 33% reduction in ATP content was measured (Fig. 6a). Including AT with the CMNP treatment did not prevent reduction in ATP. Reduced ATP content was maintained during the first 24 h after treatment with both CMNP alone or CMNP combined with AT. After this time, ATP levels increased. In *Arabidopsis*, ATP content of mature leaves from control plants was between 26.8 and 32.8 nmol g⁻¹ FW throughout the experiment. ATP content declined to approximately 80% of the controls 0.5 h after application of CMNP (Fig. 6b). Thereafter, ATP content fell to 65, 52 and 43% of control values after 3, 6 and 16 h, respectively. As in citrus flavedo, AT failed to prevent the decline in ATP content.

Plant response and symptom development

The response of citrus and *Arabidopsis* to CMNP differed, likely as a result of differences in method of application. In citrus fruit, no significant reduction in FDF was observed during the first 24 h after treatment (Fig. 7a). After 48 h, FDF was markedly reduced. AT lowered efficacy of CMNP. During the first 24 h after AT + CMNP treatment, no reduction in FDF was observed, and after 48 and 96 h, FDF was reduced only 21% and 33%, respectively. In *Arabidopsis*, leaf margins of adult plants treated with CMNP began to visibly wilt 6 h after root application (Fig. 7b). Wilting progressed with time and areas of marginal wilting followed by necrosis continued and after 24 h, plants were completely wilted. The combination treatment of CMNP + AT delayed plant decline, and plants looked no different than control or AT treatments for 12 h of continuous application. After this time, CMNP + AT plants began to wilt and decline.

CMNP-induced reduction in FDF is light independent

This work demonstrates the uncoupling effect of CMNP. However, some pyrazole-derivatives are also inhibitors of Protox (Scalla & Matringe 1994). Furthermore, chlorosis and desiccation symptoms in *Arabidopsis* treated with CMNP are similar to those described with Protox inhibitors. Protox inhibition results in the accumulation of protoporphyrin IX. In the presence of light, protoporphyrin IX produces reactive oxygen species, and the resulting peroxidation of lipids is associated with the herbicidal action of these compounds. To test whether the abscission action of CMNP is a result of Protox inhibition, potted citrus trees containing mature citrus fruit were treated with either CMNP or a well-known Protox inhibitor, carfentrazone (CFZ; Dayan & Duke 1997) and placed under a 10 h day/14 h night regime (1000 µmol m⁻² s⁻¹) or a 24 h dark regime for 8 d. CMNP reduced FDF from an average of 9.5 to 0.25 kg at 8 d after treatment, irrespective of light condi-

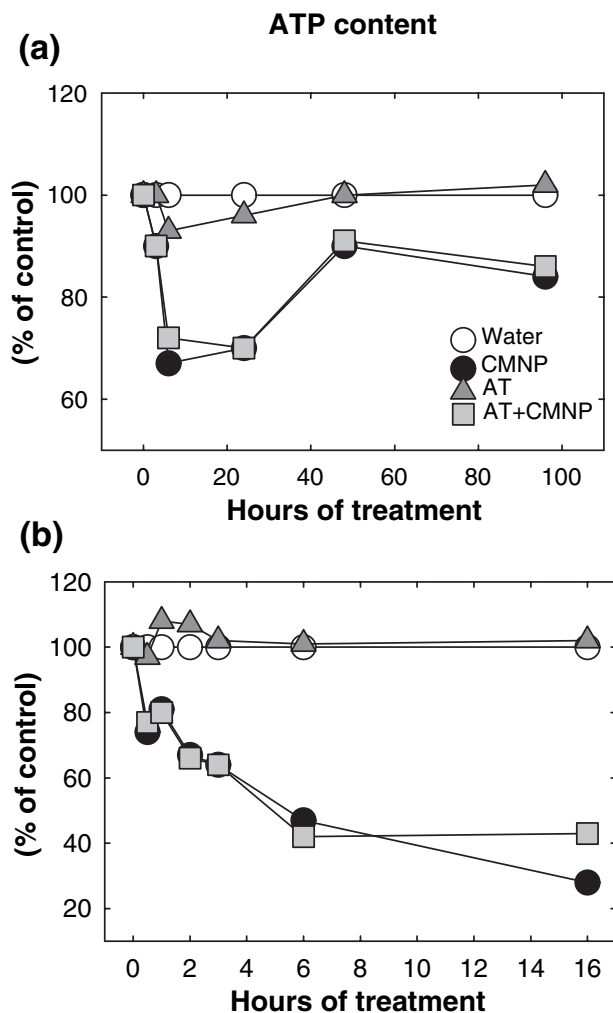


Figure 6. Effect of CMNP alone or in combination with AT on ATP content in citrus flavedo and *Arabidopsis* adult leaves. (a) Citrus fruit were treated over a 96-h period with 1.5 mM CMNP, 1.5 mM CMNP + 1 mM AT, 1 mM AT or water. (b) *Arabidopsis* plants were treated over a 16-h period with 1.5 mM CMNP, 1.5 mM CMNP + 50 μ M AT, 50 μ M AT or water. Data shown are means \pm SEM from four experiments. ATP content expressed as percentage of control.

tions (Fig. 8a). In contrast, CFZ reduced FDF in trees kept in the day/night light regime but not the dark. Although fruit drop was not as striking with CFZ compared with CMNP treatment, it followed similar trends with respect to light and dark conditions. The effect of CMNP root drench on Protox activity in *Arabidopsis* leaf extracts *in vitro* was also tested. CFZ caused 60% inhibition of Protox activity, and 200 μ M virtually eliminated activity (Fig. 8b). Increasing concentrations of CMNP up to 200 μ M had no effect on Protox activity.

Reduction in total cellular ATP content is an early response to CMNP

A timescale was constructed to depict relative changes in ATP content, PLA₂ activity, LOX activity and LPO content

after application of CMNP in citrus and *Arabidopsis*. In citrus, ATP content declined 3 h after CMNP treatment, followed by an increase in PLA₂, LOX activity and LPO content after 24 h (Fig. 9a). Reduction in FDF and fruit drop was observed 48 and 96 h after CMNP application, respectively. In *Arabidopsis*, reduction in ATP occurred as soon as 0.5 h after CMNP treatment, followed by increased PLA₂ and LOX after 2 h, and LPO content after 3 h (Fig. 9b). These results demonstrate that decline in total cellular ATP content preceded the increase in PLA₂ and the biological response in citrus and *Arabidopsis*.

DISCUSSION

CMNP is a pyrazole derivative that specifically promotes abscission in mature citrus fruit but not in immature fruit, leaves, or flowers (Burns 2002). Mature fruit flavedo appeared to be the tissue-mediating CMNP action, as application only to this tissue led to a reduction in FDF. Other citrus tissues were not visually affected by CMNP when applied in a wide range of concentrations, suggesting that the material is rapidly detoxified, or that uptake and/or transport of CMNP is limited. The compound does not cause abscission or visual symptoms of decline when applied to canopies of other plants tested to date. When applied as a root drench to many plants including citrus, however, CMNP has an herbicidal effect. Unfortunately, no radioactive tracer is available to study uptake and metabolism. To gain insight into mode of action and biological activity, we studied changes in cell energetics, and assessed the contribution of lipid signalling to the biological response via changes in PLA₂ and LOX activities, and LPO content after application of CMNP.

A number of structural features of CMNP suggested that this compound might have uncoupling activity in biological systems. In this study, three lines of evidence demonstrated that CMNP acted as an uncoupler. First, uncoupling activity of CMNP was assessed by following the import of OE17 in isolated chloroplasts of pea (Cline *et al.* 1993; Summer & Cline 1999). CMNP inhibited transport of OE17 from the stroma to the thylakoid lumen in a concentration-dependent manner, demonstrating that CMNP dissipated Δ pH. Second, CMNP, like the classic uncouplers FCCP and DNP, dissipated the proton gradient generated during state 4 respiration in soybean mitochondria. It is interesting to note that the low-molecular weight uncoupler DNP also promoted abscission when applied to citrus fruit. However, FCCP had no effect, possibly as a result of poor uptake due to its larger molecular size (data not shown). Third, treatment of citrus fruit or whole *Arabidopsis* plants with CMNP resulted in a rapid decline in ATP level. Decline in total cellular ATP content may also be due to other events besides lack of synthesis via mitochondrial and chloroplast uncoupling. For example, electrochemical potential in plasma membrane and tonoplast may be uncoupled by CMNP, resulting in activation of ATPases to restore the potential (Zharona & Vinogradov 2004). Increased ATPase activity would contribute to depletion of ATP content.

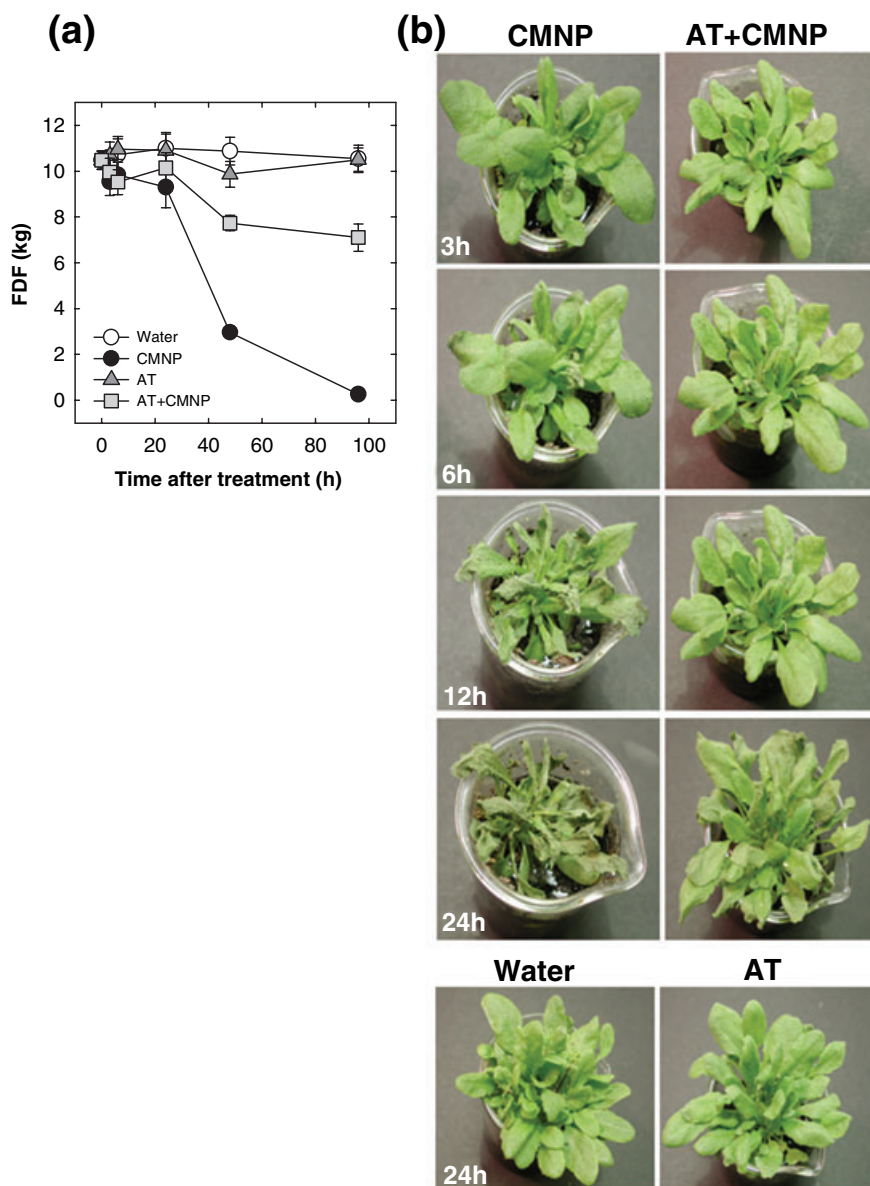


Figure 7. Biological effect of CMNP and AT on citrus and *Arabidopsis*. (a) Fruit detachment force (FDF) of mature citrus fruit over a 96-h period in response to 1.5 mM CMNP, 1 mM AT, 1.5 mM CMNP + 1 mM AT, or water alone. Data shown are means \pm SEM from three experiments. (b) *Arabidopsis* adult plants under continuous root exposure for periods up to 24 h to water, 1.5 mM CMNP, or 1.5 mM CMNP + 50 μ M AT. Images are 3, 6, 12, and 24 h after CMNP and AT + CMNP treatments. Water and AT-alone treated plants are shown after 24 h treatment for comparison.

The parent compound of CMNP, pyrazole, and many substituted pyrazoles such as 4-methyl-pyrazole, are well-known alcohol dehydrogenase (ADH) inhibitors (Dahlbom *et al.* 1974; Massantini *et al.* 1995). Inhibition of ADH would reduce the NAD-linked production of ATP in various pathways, such as glycolysis. Our data showed a marked decrease in ADH activity in citrus flavedo after CMNP treatment. Thus, CMNP may alter both membrane-linked and substrate-level phosphorylations, resulting in depletion of total ATP content. Although the main determinant of energy balance must ultimately be the rate of ATP synthesis rather than the absolute ATP level (Tadege, Braendle & Kuhlemeier 1998), reduced total cellular ATP levels indicate enhanced ATP consumption and/or reduced synthesis.

Treatments that induce anoxia-like symptoms and reduce ATP content, such as application of uncouplers combined with substrate-level phosphorylation inhibitors, have been

shown to reduce cellular energy supply; and without capability to restore membrane integrity, lipid hydrolysis and membrane breakdown occur (Rawlyer *et al.* 1999, 2002). The timing of CMNP-induced events indicated that reduction in total cellular ATP content preceded the increase in PLA₂ and LOX activities and LPO content and the biological response in citrus and *Arabidopsis*. This suggests that change in cellular energy status may trigger physiological events including production of lipid-derived signals that lead to abscission and plant decline. The generation of membrane breakdown products is considered to be a first step in the lipid signalling pathway (Wang *et al.* 2002; Meijer & Munnik 2003). Lipid degradation is generally promoted under stress conditions (Blée & Joyard 1996), and the involvement of PLA₂ and LOX activities in response to several stresses has been well documented (Melan *et al.* 1993; Chandra *et al.* 1996; Pohnert 2002). The

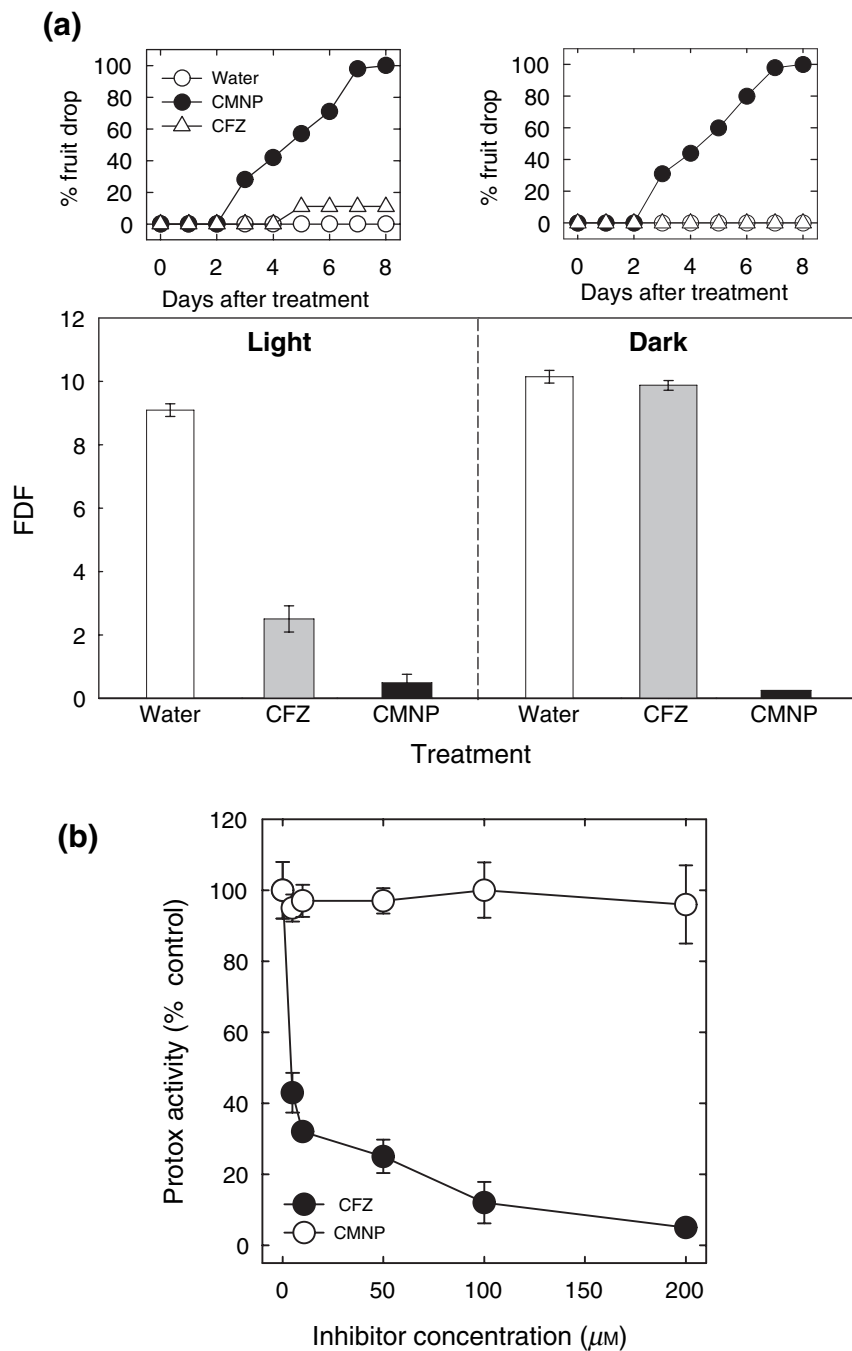


Figure 8. Effect of CFZ and CMNP on citrus FDF and *Arabidopsis* Protox activity. (a) Percentage of fruit drop over a 8-d period (upper panels) and FDF at day 8 (lower panels) of mature citrus fruit in response to water, 1.5 mM CMNP or 300 μM CFZ. Potted trees were held under light or dark conditions during the experiment. FDF data are expressed as means \pm SE from 30 fruit per treatment. (b) Protox activity (% of control) of *Arabidopsis* chloroplast extracts in response to 0, 5, 10, 50 and 100 μM CFZ or CMNP. Data are means \pm SEM from three different experiments.

products of membrane breakdown by PLA₂, linoleic and linolenic acids, can also contribute to uncoupling either by dissipating the electrochemical gradient themselves or by promoting backflow of protons by impairing the structural integrity of energetic membranes (Hanstein 1976; Macri *et al.* 1991; Petrusa *et al.* 1992; Braidot *et al.* 1993).

On the other hand, data presented in this work do not allow us to rule out alternative possibilities that may explain membrane breakdown by CMNP action. For example, generation of reactive oxygen species and subsequent lipid peroxidation could be earlier events that cause membrane disorganization and promote membrane breakdown.

The reduction in total ATP content could simply be a consequence of CMNP (or DNP) treatment with minimal impact on the biological response. No matter whether CMNP initiated abscission or plant decline through uncoupling followed by a reduction in ATP content, or by a direct effect on membranes through mechanisms such as ROS generation; inhibiting PLA₂ activity with AT reduced LOX activity and LPO content, and arrested the biological response. AT did not prevent the loss of ATP after CMNP application, however, suggesting that the generation of lipid signals through PLA₂ activity was a downstream event. The central role of membrane breakdown was also

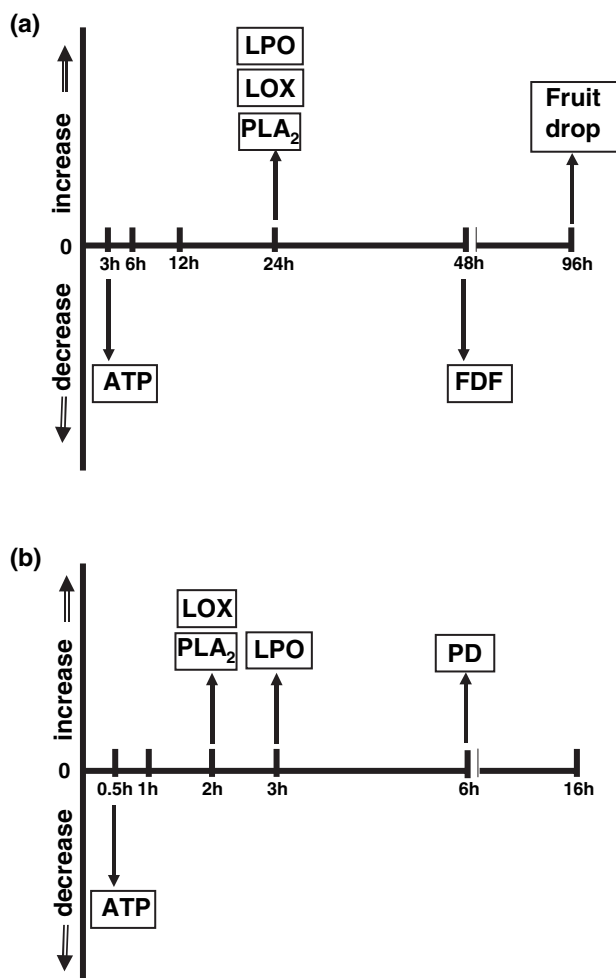


Figure 9. Timing of physiological changes and biological response to CMNP over 96 h in citrus (a) and 16 h in *Arabidopsis* (b). FDF, fruit detachment force; PD, plant decline.

demonstrated by the acceleration of mature fruit abscission by carfentrazone. The herbicidal action of Protoporphyrin IX accumulation (Wakabayashi & Boger 2004). The fact that 1 mM AT inhibited CMNP-induced reduction in FDF by 70% suggests that lipid signalling was a major contributor to mechanisms leading to citrus mature fruit abscission. Trials using 2 mM AT did not reduce FDF greater than that of 1 mM AT (data not shown). Lack of complete inhibition at higher AT concentration suggests that a lipid signalling independent pathway(s) may be contributing to the overall reduction in FDF (about 30% total reduction in FDF). Further work will be necessary to confirm this mechanism and determine its identity.

The role of PLA₂ in auxin-induced processes such as cell elongation, cell wall acidification, and gravitropism has been reported (Yi, Park & Lee 1996; Holk *et al.* 2002; Scherer 2002; Lee *et al.* 2003). Although the inhibitory effect of elevated auxin content on abscission is well documented (Roberts *et al.* 2002), cross-talk between auxin and ethylene is known to regulate the process. In particular,

auxin content is reduced and ethylene production increased as abscission is advanced (Taylor & Whitelaw 2001). Another role of PLA₂ may be to induce H⁺-pumping that promotes wall changes in preparation for cell elongation during the activation stage of abscission (Patterson 2001).

In conclusion, CMNP application resulted in reduction of total cellular ATP content followed by activation of the lipid-signalling pathway and abscission and plant decline in citrus and *Arabidopsis*, respectively. It is likely that the method of CMNP application, its subsequent distribution in the plant, and efficiency of detoxification governed the response. The partial recovery in ATP content in citrus fruit may have occurred because of inefficient uptake followed by removal of topically applied CMNP by detoxification mechanisms and restoration of the ability to synthesize ATP and repair membranes damaged by CMNP application. The fact that carfentrazone also promoted citrus mature fruit loosening suggests that membrane disruption is an event common to Protoporphyrin IX inhibitors and CMNP that can accelerate abscission. Root drench application of CMNP in *Arabidopsis* resulted in rapid distribution throughout the plant. Under this pressure, we postulate that the rate of detoxification was insufficient to prevent uncoupling and membrane damage. Inhibiting PLA₂ activity greatly reduced efficacy of CMNP and mature fruit abscission; however, herbicidal symptoms and plant death were only delayed. This could have been due to the difference in uptake and distribution of CMNP and AT. Nevertheless, the results indicate a link between lipid signalling, abscission in citrus and herbicidal damage in *Arabidopsis*.

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