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# ORIGINAL ARTICLE

## VOC-mediated within-plant communications and nonvolatile systemic signals upregulate pyrethrin biosynthesis in wounded seedlings of *Chrysanthemum cinerariaefolium*

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Natural pyrethrins biosynthesized by *Chrysanthemum cinerariaefolium* exhibit insecticidal activity, thereby acting as a defense system against herbivores. Here, we study the effects of mechanical wounding and wound-induced volatile organic compounds (VOCs) on pyrethrin biosynthesis in the seedlings of *C. cinerariaefolium*. Mechanical wounding resulted in enhanced content of pyrethrins I and II in the seedlings. Wrapping the intact leaves of the wounded seedlings prevented the increase in pyrethrin I but not in pyrethrin II, suggesting that the wound-induced VOCs are involved in the regulation of pyrethrin I biosynthesis, whereas nonvolatile systemic signals play a more important role than the wound-induced VOCs in the regulation of pyrethrin II biosynthesis.

**Keywords:** pyrethrins; *Chrysanthemum cinerariaefolium*; biosynthesis; wounding; volatile organic compounds

## Introduction

Plants make use of multiple defense systems to protect themselves against herbivores. For example, many plants produce secondary metabolites that exhibit repellent, antifeeding, and insecticidal activity as direct defense measures (Karban and Baldwin 1997). Plants also employ volatile organic compounds (VOCs) as indirect defense measures. The herbivore-induced VOCs not only attract natural enemies (Dicke et al. 1990; Turlings et al. 1990; Pieterse and Dicke 2007; Arimura et al. 2009) but also mediate within plant (Heil and Karban 2010) and plant–plant (Arimura et al. 2009) communications. In both instances, the VOCs reinforce intact leaves before herbivore attacks in terms of rapid defense gene expression for the accumulation of defense measures (Matsui 2006; Arimura et al. 2009). Notably, in damaged plants, systemic signals further add to the factors upregulating the defense mechanisms. Upon herbivory, early electrical signals spread from the damaged to intact regions (Maffei et al. 2007), triggering a series of biochemical events. At present, little is known about whether the systemic signals and herbivore-induced VOCs either concertedly, or differentially, work to enhance the defense mechanisms. To address this question, we employed *Chrysanthemum cinerariaefolium* which produces insecticide pyrethrins.

Pyrethrins consist of six compounds resulting from the esterification of acid and alcohol moieties that are biosynthesized via the 2-$C$-methyl-D-erythrytol 4-phosphate (MEP) and oxylipin pathways, respectively (Figure 1; Matsuda et al. 2005). The six compounds are classified into groups I and II according to the structure of the acid moiety, with group I possessing 2-methyl-1-propenyl moiety and group II (*E*)-3-methoxy-2-methyl-3-oxoprop-1-enyl moiety. Of the six compounds, pyrethrins I and II are produced in the largest amount in the respective groups. Although pyrethrins are commercially extracted from the flowers of *C. cinerariaefolium*, they are also present in the leaves, playing a direct defense role against herbivores. To elucidate how the induced VOCs regulate pyrethrin biosynthesis in *C. cinerariaefolium*, we recently investigated the VOCs emitted by young seedlings in response to wounding and their effect on pyrethrin biosynthesis. The wound-induced VOCs upregulated the biosynthesis in a dose-dependent fashion only when mixed together as a specific blend that mimics the wound-induced VOCs (Kikuta et al. unpublished). Given the results, questions are raised as to whether pyrethrin biosynthesis is wound inducible, and if so, how the wound-induced VOCs and nonvolatile systemic signals influence pyrethrin biosynthesis in the damaged plants. Hence, we investigated the effects of mechanical wounding on the pyrethrin content in young seedlings of *C. cinerariaefolium*. In addition, we examined whether wrapping the intact leaves of wounded seedlings prevents the increase of pyrethrins. Here, we report for the first time that wound-induced systemic and VOC signals differentially regulate the biosynthesis of pyrethrins I and II in damaged seedlings.
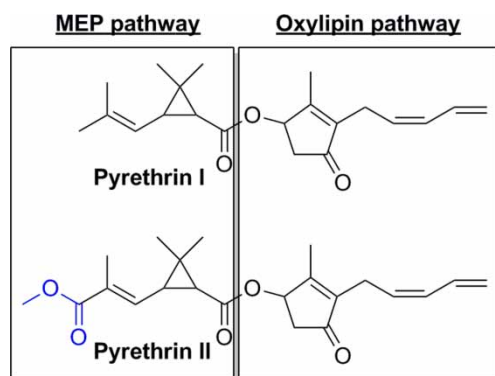
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Figure 1. Pyrethrin I and II biosynthesized by *Chrysanthemum cinerariaefolium*. Structural differences between two pyrethrins are indicated in blue.

## Materials and methods

### 1. Plants

*Chrysanthemum cinerariaefolium* was grown in commercially available soil in a climate-controlled chamber under constant illumination at 25°C for approximately one month.

### 2. Mechanical damage

Scissors were used to cut-off half the end of all but the top two youngest leaves of *C. cinerariaefolium* seedlings. To examine the effects of receiving the wound-induced VOCs on the pyrethrin biosynthesis, the two intact leaves were wrapped with a plastic bag while the others were mechanically damaged by the scissors. After this treatment, the plants were placed in a 3 liter glass container for 24 h under constant illumination at 25°C.

### 3. HPLC analysis of pyrethrins

After several treatments, either the entire plant or the upper two leaves of the *C. cinerariaefolium* seedlings (50–250 mg FW) were frozen in liquid nitrogen and ground using a mortar and pestle. The leaves were then homogenized with 4 ml of acetone containing 500 ng/ml allethrin in $CH_3CN$ solution (10 μl) as an internal standard. The homogenate was filtered and an aqueous solution (500 μl) containing 1.25% $NH_4Cl$ and 2.5% phosphoric acid was added to the filtrate. The mixture was cooled in an ice bath, and the resulting precipitate was removed by centrifugation at $800 \times g$ for 15 min at 4°C. The supernatant was supplemented with 10% NaCl aqueous solution (1 ml), and the mixture was extracted with hexane $(2 \times 1$ ml). The organic layer was dried over anhydrous $MgSO_4$ and evaporated *in vacuo*. The residue was dissolved in $CH_3CN$ (1 ml) and then passed through a Sep-Pak® C18 cartridge (Nihon Waters, Tokyo, Japan), and the cartridge was washed with $CH_3CN$ (4 ml). The $CH_3CN$ solution was evaporated to dryness, and the resultant residue was dissolved in 100 μl $CH_3CN$ for HPLC analysis. HPLC was carried out using a VP10A system (Shimadzu Corp, Kyoto, Japan) equipped with a Cadenza CD-C18 column $(100 \times 4.6$ mm i.d., Imtakt Co., Kyoto, Japan) with a $CH_3CN–H_2O$ mixture (65:35) at a flow rate of 1 ml/min. Pyrethrins were monitored at 230 nm using a Shimadzu SPD-10AV VP ultraviolet detector.

## Results and discussion

To examine if pyrethrins are wound inducible, we mechanically damaged all but the two youngest leaves (Figure 2) of the *C. cinerariaefolium* seedlings and quantified pyrethrins I and II in the intact leaves 24 h after wounding. It was found that the pyrethrin content in the intact leaves, whether type I or II, was significantly enhanced in response to wounding (pyrethrin I, $741.2 \pm 62.7$ ng/mg fresh leaf; pyrethrin II, $269.5 \pm 21.4$ ng/mg fresh leaf) compared with the control (pyrethrin I, $395.4 \pm 42.9$ ng/mg fresh leaf; pyrethrin II, $154.4 \pm 12.1$ ng/mg fresh leaf) (Figure 3). It is conceivable that the increase in pyrethrins is the result of the (1) nonvolatile systemic signals, (2) airborne VOC signals, or (3) both.

Therefore, we wrapped the top two youngest leaves with a plastic bag (Figure 2) to examine if the wound-induced VOCs contribute to the enhanced pyrethrins by preventing the access of VOCs. Having confirmed that the wrapping itself had no effect on the pyrethrin content (pyrethrin I, $465.3 \pm 66.4$ ng/mg fresh leaf; pyrethrin II, $131.0 \pm 9.9$ ng/mg fresh leaf) (Figure 3), we added mechanical damage to the seedlings with the wrapped intact leaves. Under this condition, pyrethrin I in the wrapped leaves was not significantly increased in response to wounding $(514.1 \pm 54.9$ ng/mg fresh leaf) (Figure 3A). In contrast, wrapping failed to suppress the wound-induced increase of pyrethrin II $(282.8 \pm 48.9$ ng/mg fresh leaf) (Figure 3B). One direct interpretation of this result is that the wound-induced VOCs play a more important role than the systemic signals in regulating the biosynthesis of pyrethrin I, whereas the reverse is the case for pyrethrin II. The acid moiety of pyrethrin I is oxidized and then esterified by methyltransferase, yielding the acid moiety of pyrethrin II (Figure 1). Hence, the pathway from pyrethrin I to pyrethrin II may be critically regulated by the systemic signals.
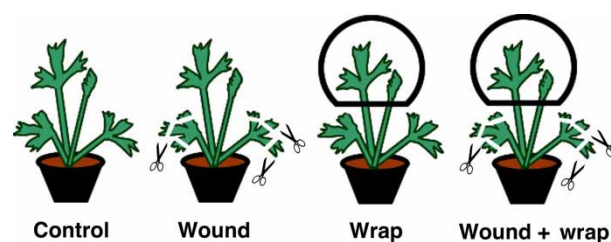


Figure 2. Schematic representation of the experiments conducted in this study. To add mechanical damage, all but the two youngest leaves were cut with scissors ('Wound' treatment). To prevent the access of wound-induced VOCs, the intact leaves were wrapped with a plastic bag ('Wrap' treatment).
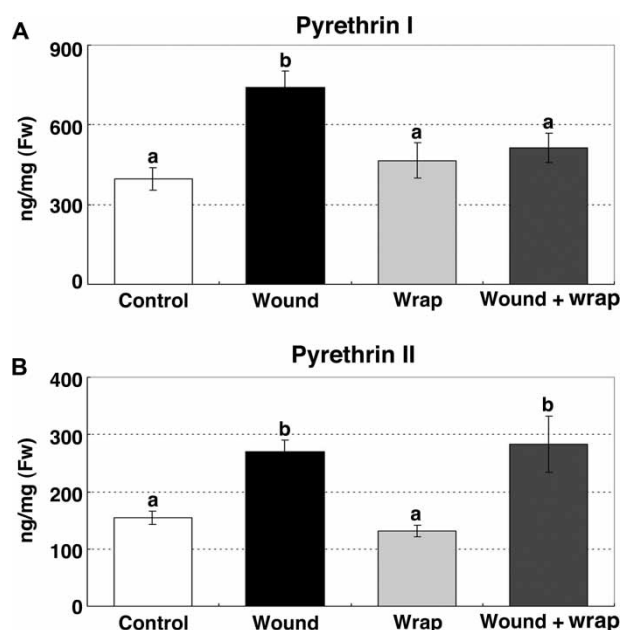
Figure 3. The effects of wounding and wrapping on the biosynthesis of pyrethrin I (A) and II (B). All but the two youngest leaves of wrapped and unwrapped seedlings were mechanically damaged with scissors (see Figure 2), and then pyrethrin I and II content in the two intact leaves was quantified by HPLC. Each bar graph indicates the mean $\pm$ standard error of the mean ($n = 4 - 7$). Different letters are used to indicate that the data compared were statistically significant at $p < 0.05$ (one-way ANOVA, Tukey's test).

Although more experiments are necessary to investigate the relevant gene expression, the present results added to our understanding of the mechanism of pyrethrin biosynthesis.

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