Arabidopsis synchronizes jasmonate-mediated defense with insect circadian behavior

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Diverse life forms have evolved internal clocks enabling them to monitor time and thereby anticipate the daily environmental changes caused by Earth's rotation. The plant circadian clock regulates expression of about one-third of the *Arabidopsis* genome, yet the physiological relevance of this regulation is not fully understood. Here we show that the circadian clock, acting with hormone signals, provides selective advantage to plants through anticipation of and enhanced defense against herbivory. We found that cabbage loopers (*Trichoplusia ni*) display rhythmic feeding behavior that is sustained under constant conditions, and plants entrained in light/dark cycles coincident with the entrainment of the *T. ni* suffer only moderate tissue loss due to herbivory. In contrast, plants entrained out-of-phase relative to the insects are significantly more susceptible to attack. The in-phase entrainment advantage is lost in plants with arrhythmic clocks or deficient in jasmonate hormone; thus, both the circadian clock and jasmonates are required. Circadian jasmonate accumulation occurs in a phase pattern consistent with preparation for the onset of peak circadian insect feeding behavior, providing evidence for the underlying mechanism of clock-enhanced herbivory resistance. Furthermore, we find that salicylate, a hormone involved in biotrophic defense that often acts antagonistically to jasmonates, accumulates in opposite phase to jasmonates. Our results demonstrate that the plant circadian clock provides a strong physiological advantage by performing a critical role in *Arabidopsis* defense.

circadian rhythm | diurnal rhythm | plant–herbivore interaction | jasmonic acid | salicylic acid

In the battle between plant host and herbivore, plants appear to be disadvantaged due to their relative immobility because once herbivorous insects attack, plants cannot escape by relocating. However, it is well-documented that plants can detect attack by insect herbivores and, in response, activate defense responses (1). These defense responses include accumulation of proteins and compounds that are toxic to and/or act to deter feeding of herbivores, thereby reducing herbivore performance and increasing plant resistance. Jasmonate hormones are critical for plant herbivore defense (1–3) and for the regulation of both herbivore and wound-responsive gene expression (4).

Remarkably, many genes regulated in expression by wounding also have strong circadian regulation. Over 40% of genes whose expression is induced by wounding have peak circadian expression at subjective dusk, and over 80% of genes down-regulated by wounding have peak expression at subjective dawn (5). However, it remained to be determined whether this circadian regulation of wound-inducible genes enables plants to anticipate herbivore attack through a cyclical activation of defense response. Here we demonstrate that *Arabidopsis* plants that are entrained such that their subjective day is in-phase with *Trichoplusia ni* subjective day have increased resistance to herbivory. In contrast, when the plant subjective day is the insect subjective night, plant resistance is largely lost and *T. ni* performance is greatly increased. *T. ni* is shown to display circadian-controlled feeding behavior, peaking during late day. Circadian clock and active jasmonate functions are required for this phase-dependent enhanced resistance. Finally, we show that both jasmonate and salicylate hormone levels are circadian-regulated and thus reveal an underlying mechanism for clock-mediated plant defense.

Results

Clock Coentrainment Increases Plant Resistance and Decreases Herbivore Performance. To test whether the plant endogenous clock enhances defense against insect pests, we compared herbivory on *Arabidopsis* plants entrained either in-phase or out-of-phase with the cabbage looper *T. ni*. Sequential 12-h light/12-h dark cycles were used for clock coentrainment of both organisms. To confer opposite and out-of-phase entrainment, a second set of plants was subjected to light/dark cycles that were offset by 12 h. To observe only circadian- and not diurnal-regulated behavior, *T. ni* loopers and both in-phase and out-of-phase entrained plants were then subjected to constant dark for 24 h before coincubation (Fig. 1*A*). After allowing *T. ni* loopers to feed freely on the plants for 72 h, plants entrained in-phase with the loopers had visibly less tissue damage (Fig. 1*B*) and significantly more tissue area remaining (Fig. 1*C*) than plants entrained out-of-phase with the insects (Fig. 1 *B* and *C*). Furthermore, *T. ni* that fed on the in-phase entrained plants failed to gain as much weight as those that fed on the out-of-phase entrained plants, with an average final weight of 1.6 mg and 5.16 mg, respectively (Fig. 1 *D* and *E*). Similar results were obtained when coincubation occurred under constant light (Fig. S1*A*): Plants coentrained with *T. ni* have enhanced resistance relative to plants entrained out-of-phase with *T. ni* (Fig. S1*B*), and *T. ni* that fed on out-of-phase plants perform better, as monitored by final weight (Fig. S1 *C* and *D*). Therefore, when *Arabidopsis* and *T. ni* experience a common circadian regime, *Arabidopsis* is less susceptible to insect herbivory.

***T. ni* Feeding Behavior Is Circadian-Regulated.** The results in Fig. 1 suggest that the feeding behavior of *T. ni* may be circadian-regulated. To test this idea, *T. ni* loopers were entrained in 12-h light/dark cycles for 3 d and then allowed to feed freely for 72 h on insect diet media either in diurnal 12-h light/dark cycles or under constant dark. Under diurnal conditions, feeding activity, measured as food weight loss every 4 h, was highly cyclical. Food loss was greater during the light periods, with maxima occurring at dusk (Fig. 2*A*, hours 12, 36, and 60) and minima at dawn (Fig. 2*A*, hours 24, 48, and 72), indicating that *T. ni* prefer to feed during the day with peak activity just before dusk. This rhythmic

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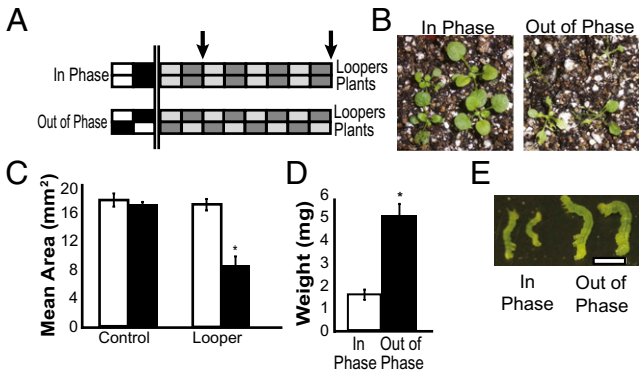

Fig. 1. *Arabidopsis* is more resistant to herbivory when entrained in-phase rather than out-of-phase with *T. ni* looper entrainment. (*A*) Light/dark cycle entrainment scheme and experimental protocol. Rectangles symbolize 12-h periods of light (open), darkness (filled), darkness representing subjective day (light gray), and darkness representing subjective night (dark gray). The two arrows represent timing of *T. ni* looper addition and looper removal, respectively. Double vertical bars symbolize the shift from light/dark cycles to constant darkness. (*B*) Photographs of representative plant tissue remaining from plants entrained in-phase and out-of-phase with looper entrainment after 72 h of plant–*T. ni* coincubation. (*C*) Area of plant tissue remaining from plants entrained in-phase (white bars) and out-of-phase (filled bars) with *T. ni* entrainment after 72 h of incubation without (control) or with *T. ni* loopers (looper). Mean area ± SE; $n = 6$; $^*P < 0.0002$; two-tailed paired *t* test. (*D*) Looper wet weights. Mean ± SE; $n = 15$; $^*P < 0.05$; two-tailed paired *t* test. (*E*) Representative loopers at 72 h postcoincubation. (Scale bar, 0.5 mm.)

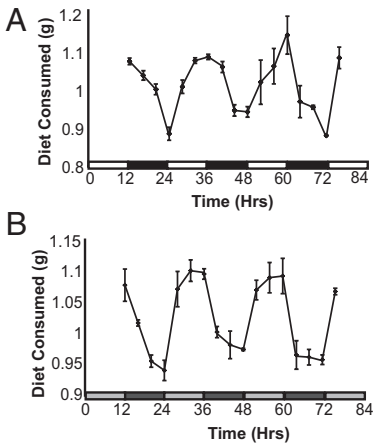

Fig. 2. *T. ni* feeding is circadian-regulated, with enhanced eating during subjective day. *T. ni* entrained in 12-h light/dark cycles were provided food under (*A*) light/dark or (*B*) constant dark conditions. Diet weight lost as a result of looper feeding after each 4-h interval is graphed. Graphed values were determined by calculating the difference of diet weight before and after incubation with loopers and, to account for evaporative weight loss, subtracting the weight difference of comparable diet samples before and after incubation without loopers for the same 4-h interval under similar conditions. Fresh diet was given every 4 h. Mean ± SE; $n = 3$.

feeding behavior is circadian-controlled, because *T. ni* shifted from diurnal to constant dark conditions maintain the cyclic feeding behavior, with food loss most prominent during subjective day and maxima and minima of food loss occurring at subjective dusk and subjective dawn, respectively (Fig. 2*B*). This cyclical *T. ni* feeding behavior also continued for 36 h in constant light but thereafter became arrhythmic (Fig. S2); under these conditions, constant light negatively affected *T. ni* viability, which may account for the apparent decline in behavior rhythmicity. The results shown in Fig. 2 and Fig. S2 indicate that *T. ni* likely have a circadian clock that governs feeding behavior. This is consistent with studies demonstrating that other insects display circadian behavior and that *Drosophila* feeding is circadian-controlled (6–8).

Circadian Clock Function Is Required for in-Phase–Dependent Enhanced Herbivore Defense. The enhanced plant resistance to *T. ni* attack when plants and *T. ni* experience the same light/dark entrainment (Fig. 1) suggests that the plants time their defense response to coincide with the rhythmic *T. ni* feeding behavior (Fig. 2). To investigate whether the plant circadian clock is required for this behavior, the arrhythmic clock mutant *lux2* (9) and the arrhythmic transgenic line *CCA1-OX* (10) were entrained in- and out-of-phase with *T. ni* and examined for resistance under constant dark conditions. In contrast to wild-type (Fig. 1 *B* and *C*), plants with defective clock function showed no significant differences in plant tissue loss (Fig. 3 *A* and *B*) between in- and out-of-phase entrainment regimes. Final *T. ni* weight was also largely unaffected by the entrainment conditions (Fig. 3 *C* and *D*). A similar loss of coentrainment benefit to the clock-defective plants occurs under constant light conditions (Fig. S3). These data demonstrate that the *Arabidopsis* circadian clock is essential for enhanced plant defense against *T. ni* herbivory when entrainment is synchronized.

Jasmonates Are Required for in-Phase–Dependent Enhanced Resistance. Because *T. ni* resistance in *Arabidopsis* requires a functional jasmonate pathway to regulate defense responses (2, 3, 11), we next examined whether the mutants *aos* and *jar1*, which fail to accumulate active jasmonates (12, 13), also fail to show enhanced *T. ni* resistance when entrained in-phase with the herbivore. Unlike wild-type (Fig. 1) or *gl-1* (Fig. 4 *A* and *B*), which is the genetic background for *aos*, entrainment regimes of *aos* and *jar1* have no significant effect on plant resistance (Fig. 4 *A* and *B*) or *T. ni* performance (Fig. 4 *C* and *D*) when *T. ni* were allowed to feed on plants under constant dark conditions. Similar results were obtained under constant light (Fig. S4). Therefore, a functional jasmonate pathway is required for *Arabidopsis* to demonstrate an advantage in *T. ni* cabbage looper defense through circadian entrainment.

Accumulation of Jamonates and Salicylates Is Circadian-Regulated. Finally, we directly measured levels of jasmonates that accumulate in wild-type plants entrained in 12-h light/dark cycles followed by constant darkness. Plant jasmonates show peak accumulation in the middle of the subjective day (Fig. 4*E*), a few hours before peak expression of many jasmonate-regulated genes (14) and maximal *T. ni* feeding behavior (Fig. 2). The circadian pattern for jasmonate accumulation continues even in plants coincubated with *T. ni* (Fig. S5); however, the levels of jasmonates are 20–35% elevated in the plants subjected to herbivory (Fig. S5), suggesting that the clock- and herbivory-induced jasmonate accumulation induction are additive. Furthermore, we found that salicylates, which often act antagonistically to jasmonates (15), show opposite accumulation phasing relative to jasmonates, with peaks in the middle of the subjective night (Fig. 4*E*). This cyclical accumulation of salicylates may underlie the enhanced resistance of *Arabidopsis* to biotrophic bacteria when infection occurs in the early morning as opposed to the evening (16).

Discussion

The results presented here indicate that the *Arabidopsis* circadian clock has strong physiological relevance in plant defense against herbivory. *Arabidopsis* has elevated wound-responsive gene expression (5), elevated jasmonates (Fig. 4*E*), and enhanced defense response (Fig. 1) timed to be coincident with the circadian-regulated feeding behavior of the *T. ni* herbivore (Fig. 2). Clock function is required for the in-phase–dependent

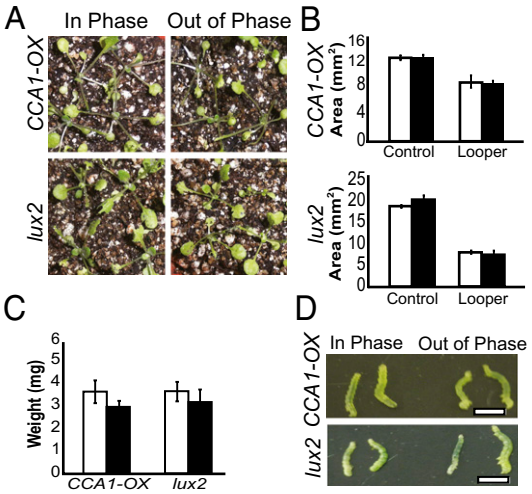

Fig. 3. Arrhythmic *Arabidopsis* plants lack enhanced herbivory resistance when entrained in-phase with *T. ni* loopers. (*A*) Photographs of representative plant tissue remaining from *CCA1-OX* and *lux2* entrained in-phase and out-of-phase with *T. ni* entrainment after 72 h of plant–*T. ni* coincubation. (*B*) Area of plant tissue remaining from plants entrained in-phase (white bars) and out-of-phase (filled bars) with *T. ni* entrainment after 72 h of incubation without (control) or with *T. ni* (looper). Mean area ± SE; $n = 6$; $P < 0.8$; two-tailed paired *t* test. (*C*) Wet weights of *T. ni* fed on in-phase (open bars) or out-of-phase (filled bars) plants. Mean ± SE; $n = 15$; $P < 0.05$; two-tailed paired *t* test. (*D*) Representative *T. ni* loopers at 72 h postcoincubation. (Scale bars, 0.5 mm.)

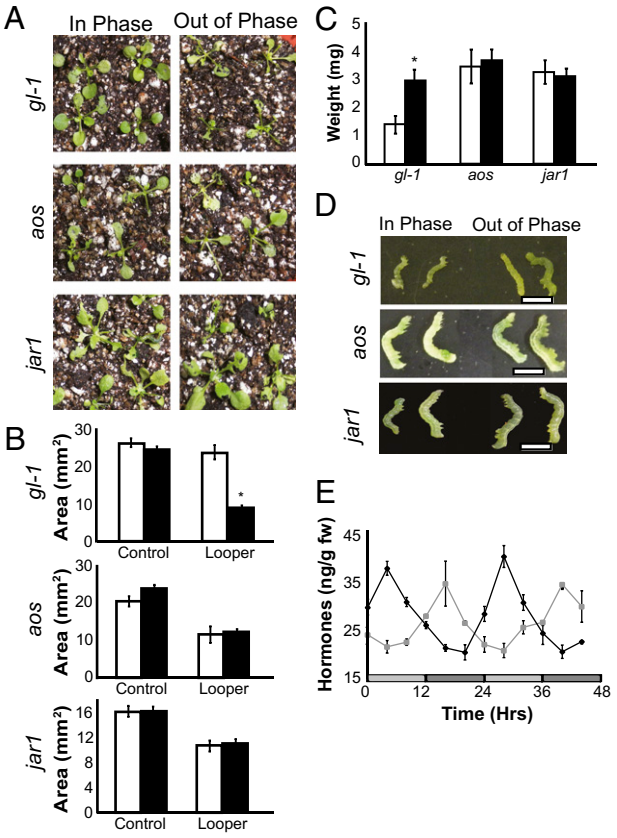

Fig. 4. Jasmonates are required for enhanced herbivory resistance when entrained in-phase with *T. ni* loopers, and jasmonates and salicylate accumulation patterns show circadian rhythms with opposite phasing. (*A*) Photographs of representative plant tissue remaining from *gl-1*, *aos*, and *jar1* entrained in-phase and out-of-phase with *T. ni* entrainment after 72 h of plant–*T. ni* coincubation. (*B*) Area of plant tissue remaining from plants entrained in-phase (white bars) and out-of-phase (filled bars) with *T. ni* entrainment after 72 h of incubation without (control) or with *T. ni* (looper). Mean area ± SE; $n = 6$; $*P < 0.0002$; two-tailed paired *t* test. (*C*) Wet weights of *T. ni* fed on in-phase (open bars) or out-of-phase (filled bars) plants. Mean ± SE; $n = 15$; $*P < 0.05$; two-tailed paired *t* test. (*D*) Representative *T. ni* loopers at 72 h postcoincubation. (Scale bars, 0.5 mm.) (*E*) Jasmonate and salicylate accumulation patterns are circadian-regulated with opposite phasing. Jasmonates peak in the middle of subjective day (black line) and salicylates (gray line) peak in the middle of subjective night. Mean ± SE; $n = 3$. fw, fresh weight.

enhanced resistance (Fig. 3), and the in-phase resistance continues under both constant darkness (Fig. 1) and constant light (Fig. S1) conditions, evidence that the enhanced defense is mediated by the plant circadian clock.

We demonstrate the circadian accumulation of jasmonates with peak levels at midsubjective day (Fig. 4*E*). As jasmonate accumulation is responsible for inducing expression of diverse genes important in defense responses, this clock-dependent hormone accumulation regulation provides an underlying regulatory mechanism for the anticipatory herbivore defense. Included in the large set of genes identified to be circadian-regulated (17) are *AOS* and *OPR3*, genes encoding enzymes essential for jasmonate biosynthesis.

Our results suggest the possibility that the clock-controlled jasmonate fluctuations lead to circadian accumulation of defense metabolites. Indeed, diverse metabolites accumulate with daily rhythms in plants grown under light/dark (diurnal) cycles (18–21). Whereas accumulation of some of these metabolites may be in response to differential abiotic stimuli, such as light, it is possible that accumulation of others may be under the control of the endogenous circadian clock. For example, herbivore-induced volatiles in corn (18, 19, 21) and kidney bean plants (20) are differentially emitted in the day versus night. In addition, recent metabolomic analysis of *Nicotiana attenuata* has found that diverse metabolites accumulate rhythmically under diurnal conditions, including enhanced jasmonate hormone accumulation in roots during the night (19).

Cabbage loopers (*T. ni*) feed primarily during subjective day (Fig. 2), correlating with the timing of clock-regulated accumulation of jasmonates in *Arabidopsis* (Fig. 4*E*). Furthermore, the clock gates herbivore-induced jasmonate accumulation; insect-infested plants maintain the rhythmic accumulation of hormone but at elevated levels. The plant clock is therefore advantageous in this specific herbivore relationship, whereas the insect clock-controlled behavior is disadvantageous. However, the relationship is likely much more complex in the natural environment, with the contribution of many more abiotic and biotic factors. Indeed, there are many examples of nocturnal feeding insects (22), and at least some insects alter their behavior in response to plant signals (18).

We also find that salicylates, the hormone critical for biotrophic pathogen resistance in plants, accumulate with a circadian pattern (Fig. 4*E*). Remarkably, salicylates, which are often found to act antagonistically with jasmonates (15), accumulate in opposite phase as jasmonates, with peak salicylates accumulating at midsubjective night (Fig. 4*E*). Clock-controlled salicylate accumulation may be the basis for enhanced *Arabidopsis* resistance to *Hyaloperonospora arabidopsis*, a biotrophic pathogen, when infection occurs at dawn rather than at dusk (16), because salicylates mediate biotrophic pathogen resistance (15). These data demonstrate that the plant clock coordinately regulates hormonal fluctuations in tune with distinct pathogen behaviors. The plant defense response may have evolved this phase relationship because of the antagonistic relationship between jasmonate- and salicylate-regulated defenses (15) and/or because jasmonates can negatively affect the cell cycle and vegetative growth (23). In summary, in the daily herbivory battle between *T. ni* and

Arabidopsis, evolution of the circadian clock gives the advantage to the plant.

Materials and Methods

Plant Materials. All *Arabidopsis* genotypes have the Col-0 genetic background except for *aos*, which is in the *gl-1* genetic background. Seed sources: Col-0 (Lehle Seeds); *aos* (S6149; *Arabidopsis* Biological Resource Center); *jar1* (Katayoon Dehesh, University of California, Davis, CA); and *lux2* and *CCA1-OX* (Stacey Harmer, University of California, Davis, CA). Seeds were surface-sterilized with 70% ethanol for 5 min and 95% ethanol for 15 min followed by three washes with sterilized water. Sterilized seeds were cold-treated at 4 °C for 4 d before being sown on growth medium containing half-strength Murashige and Skoog (24) medium and 30 g/L sucrose and solidified with 12 g/L type E agar (A4675; Sigma) (pH 5.7). After 1 wk, seedlings were transferred to soil, with 16 plants per pot, and grown for 2 more weeks on soil (Sunshine MVP soil; Sun Gro Horticulture) at 22 °C under 12-h light/dark cycles (140 $\mu E \cdot m^{-2} \cdot s^{-1}$) (E = einstein, a unit defined as one mole of photons). At 3 wk of age, when plants were still in a vegetative stage without inflorescence development, plants were moved to constant conditions, as specified in each experiment. Following *T. ni* feeding experiments, remaining plant tissue was imaged and quantified using ImageJ software (National Institutes of Health). Two-tailed paired Student's *t* tests were performed and *P* values of 0.05 were used as cutoff values for showing significance.

***T. ni* Handling.** *T. ni* eggs (Benzon Research) were hatched at 25 °C, incubated with pinto bean diet under 12-h light/dark cycles for 3 d, and then moved to constant conditions for 24 h as specified in each experiment. Therefore, 4-d-old *T. ni* were used at the initiation of every experiment. When necessary, *T. ni* were gently moved onto plates or plants using a fine brush. Visibly similar-size *T. ni* loopers were placed with plants for coincubation experiments. The experiments were stopped after 72 h of coincubation, and *T. ni* weights were determined. Two-tailed paired Student's *t* tests were performed and *P* values of 0.05 were used as cutoff values for showing significance. For feeding behavior studies, 20 *T. ni* loopers per plate were incubated with ~5 g of food. Every 4 h the diet was weighed and a new 5-g aliquot was substituted. Weight loss of artificial diet incubated without *T. ni* was subtracted from the diet weight loss after *T. ni* feeding to account for evaporation loss.

Pinto Bean Artificial Diet. Homemade diet feed for *T. ni* was generated by adding 25 g agar (Sigma-Aldrich) to 900 mL boiling water, then adding 18 g of organic pinto beans (Whole Foods). The mixture was blended on high for 1.5 min with a 12-speed blender (Oster) with an additional 90 mL water. Additional ingredients were blended in at high speed for an additional 1.5 min: 3.3 g vitamin diet fortification (MP Biomedicals), 42 g casein (Sigma-Aldrich), 42 g sucrose (Sigma-Aldrich), 36 g wheat germ (MP Biomedicals), 12 g Wesson's salt mix (MP Biomedicals), 6 g Alfacel (nonnutritive bulk; MP Biomedicals), 1.66 g methyl paraben in 18 mL 95% ethanol (Bioreagent; Sigma-Aldrich), 1.66 g sorbic acid (Sigma-Aldrich), 5 g ascorbic acid (BioXtra; Sigma-Aldrich), and 0.16 g streptomycin sulfate (Sigma-Aldrich). The mixture was poured into dishes to harden and stored at 4 °C wrapped in cellophane. Fresh media were used within 3 wk.

Phytohormone Measurements. *Arabidopsis* Col-0, grown at 22 °C under 12-h light/dark cycles, were transferred to constant darkness at the five-leaf stage for 24 h. For clock-regulated jasmonate and salicylate quantification, leaves from about eight plants were harvested every 4 h for 48 h. For comparison of jasmonate accumulation levels in *T. ni*-infested plants relative to non-infested plants, one set of plants was exposed to *T. ni* loopers and the others were left as controls. Leaves were harvested every 6 h for 24 h. For all samples, leaves were weighed and flash-frozen. Jasmonates and salicylates were extracted as described (25, 26). The produced methyl ester volatiles were captured on HaySep-Q (Grace Davison Discovery Sciences) columns by vapor-phase extraction. The trapped metabolites were then eluted with 150 μL dichloromethane and analyzed by GC-MS using a Hewlett Packard 6890 series gas chromatograph coupled to an Agilent Technologies 5973 network mass selective detector operated in electronic ionization mode. One microliter of sample was injected in splitless mode at 250 °C and separated using a Restek Rtx-35MS column (30 mm × 0.25 mm × 0.1 mm) held at 40 °C for 1 min after injection and then at increasing temperatures programmed to ramp at 15 °C/min to 250 °C (10 min), with helium as the carrier gas (constant flow rate 0.7 mL/min). Measurements were carried out in selected ion-monitoring mode with retention times and M^+ *m/z* ions as follows: methyl jasmonate (JA-ME) (*trans* 11.98 min, *cis* 12.28 min, 224) and methyl salicylate (SA-ME) (*trans* 7.81, *cis* 8.12, 120).

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