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PLANT–PLANT SIGNALING: APPLICATION OF *trans*- OR *cis*-METHYL JASMONATE EQUIVALENT TO SAGEBRUSH RELEASES DOES NOT ELICIT DIRECT DEFENSES IN NATIVE TOBACCO

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Abstract—*Nicotiana attenuata* plants growing in close proximity to damaged sagebrush (*Artemisia tridentata* ssp. *tridentata*) suffer less herbivory than plants near undamaged sagebrush. Sagebrush constitutively releases methyl jasmonate (MeJA), a compound that when applied directly to *N. attenuata*, elicits herbivore resistance and the direct defense traits [protease inhibitors (PIs), nicotine]. Damage increases the release of volatile MeJA, primarily in the cis epimer, suggesting that *cis*-MeJA may mediate this apparent interplant signaling. We characterized sagebrush's MeJA plume before and after damage in nature and in the laboratory, and compared the activity of *trans*- and *cis*-MeJA in inducing PIs, nicotine, and *Manduca sexta* resistance in *N. attenuata*. We used both lanolin applications and aqueous sprays that mimic natural exposures, and we determined the amount of volatilized MeJA required to elicit a nicotine response in open-grown plants. Wounding rapidly and transiently increased *cis*-MeJA emissions from damaged parts (but not systemically), and the released plume did not rapidly dissipate in nature. *cis*-MeJA was not consistently more active than *trans*-MeJA, and the order of exposure (*trans*- then *cis*-) did not influence activity. We conclude that volatile MeJA, either *trans*- or *cis*-, when applied at

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levels consistent with those released by sagebrush does not elicit direct defenses in *N. attenuata*.

Key Words—*Nicotiana attenuata*, *Artemisia tridentata* ssp. *tridentata*, *cis*-MeJA, *trans*-MeJA, interplant communication, induced defenses, protease inhibitor, nicotine, *Manduca sexta*.

INTRODUCTION

Interplant communication *via* airborne signals has received much attention recently (Karban and Baldwin, 1997; Dicke and Bruin, 2001; Dicke et al., 2003). While some studies have examined only interactions within laboratory bell jars (Farmer and Ryan, 1990; Shulaev et al., 1997; Preston et al., 1999; Arimura et al., 2000a,b), two studies provide evidence for communication in natural populations. In populations of alder (*Alnus glutinosa*), herbivory experienced by undamaged alders was inversely related to their distance from an artificially damaged tree, and subsequent laboratory experiments demonstrated that leaves collected from undamaged trees growing closer to the damaged tree were less desirable to herbivores for both consumption and oviposition compared to leaves taken from more distant trees (Dolch and Tscharntke, 2000). Either airborne or soilborne signals may be responsible for the observed change in resistance. In a second study, wild tobacco (*Nicotiana attenuata*) plants growing adjacent to damaged sagebrush (*Artemisia tridentata* ssp. *tridentata*) plants suffered less herbivory than tobacco plants located near undamaged sagebrush, an interaction that appears to be mediated by an airborne signal (Karban et al., 2000). The signal(s) mediating this interaction has not yet been identified.

Sagebrush is an aromatic plant that releases a complex blend of volatiles (Muller et al., 1966; Kelsey et al., 1978; Personius et al., 1987; Preston et al., 2001). Included in this volatile blend is the methyl ester of a ubiquitous plant hormone, jasmonic acid (JA). While a number of volatiles are released at greater levels after damage than methyl jasmonate (MeJA; Preston et al., 2001), MeJA is a likely candidate for the signal mediating the interplant communication because of its activity in eliciting herbivore resistance in *N. attenuata* (Baldwin, 1998) and protease inhibitors (PIs) in tomato (Farmer and Ryan, 1990). Exposure of *N. attenuata* to volatile MeJA differentially regulates several genes, some believed responsible for orchestrating complex metabolic shifts after herbivore attack (Hermsmeier et al., 2001). Moreover, these changes in transcript accumulation are likely linked to many of the well-studied JA-induced responses in *N. attenuata*, including changes in levels of nicotine, phenolics, flavonoids, phenolic putrescine conjugates, PIs, PPOs, diterpene sugar esters, and volatile releases of monoterpenes, sesquiterpenes, C_6 alcohols, and aldehydes (Baldwin et al., 1998; Halitschke et al., 2000; Kahl et al., 2000; Keinänen et al., 2001; van Dam et al., 2001).

Two fundamental predictions for an effective airborne signal are that (1) the signal must be closely associated with damage, with quantitative or qualitative changes providing information, and (2) it must reach the recipient at physiologically active levels (Firn and Jones, 1995). The MeJA released by damaged sagebrush exhibits both quantitative and qualitative changes as the amount of MeJA released increases, due primarily to an increase in the amount of a single epimer, *cis*-MeJA (Karban et al., 2000). This epimeric change is interesting as the cis orientation is thermodynamically less stable, rapidly epimerizing to the more stable trans configuration, until an equilibrium trans–cis ratio of approximately 93:7 is attained (Beale and Ward, 1998), but it is thought to be the biologically more active of the two naturally occurring epimers of MeJA (Beale and Ward, 1998; Sarkar and Ghorai, 1999). Alternatively, the activity of the airborne signal may not be due to the absolute amounts of *cis*- and *trans*-MeJA released after damage, but perhaps the change in the predominate epimer. *Nicotiana sylvestris*, a tobacco species native to Central America, exhibits an immunological "memory" of previous MeJA exposures that is readily seen as a more rapid increase in nicotine concentrations of previously exposed but uninduced plants compared to uninduced plants without previous MeJA exposures (Baldwin and Schmelz, 1996). This immunological memory suggests that those plants that experience low levels of *trans*-MeJA (i.e. from an undamaged sagebrush plant) may become sensitized and respond rapidly when exposed to *cis*-MeJA released from a damaged sagebrush plant. Similarly, corn (*Zea mays*) seedlings exposed to green leaf volatiles from herbivore-attacked neighboring plants respond to damage with greater JA and volatile sesquiterpene levels than seedlings without prior exposure (Engelberth et al., 2004).

Sagebrush's MeJA emission has only been characterized from excised branches in bell jars (Farmer and Ryan, 1990), and from undamaged sagebrush plants and for 1–2 hr immediately following damage to plants growing in the field (Karban et al., 2000). However, the longevity of the increased cis emission after damage, whether the release is systemic (whole plant) or localized to damaged tissues, and the distance over which the signal is detectable are unknown. Other volatile organic compounds (VOCs) released from damaged plants are known to exhibit temporal and spatial variability (Paré and Tumlinson, 1999; Halitschke et al., 2000). Immediately after damage, VOC releases occur local to the damaged area and consist primarily of "green leaf" volatiles (mainly C_6 aldehydes, alcohols, and acetates) that are followed by a delayed (2–24 hr) systemic release of other compounds. The release of these VOCs, which are triggered by herbivore-specific elicitors, can function as indirect defenses by attracting predators and parasitoids to feeding herbivores (Röse et al., 1996; De Moraes et al., 1998, 2001; Turlings et al., 1998; Paré and Tumlinson, 1999; Keßler and Baldwin, 2001).

Here, we characterize both the emission of MeJA and the response of *N. attenuata* to exposures of *trans*- and *cis*-MeJA in ecologically relevant quantities. We quantify the amount and trans–cis ratio of MeJA released by *Artemisia*

tridentata ssp. *tridentata* by trapping volatiles from natural populations in the Great Basin Desert of southwestern Utah before and after damage. After damage, we determine: (1) the kinetic of the release; (2) if the release is local to the damaged tissue or also from undamaged leaves; and (3) the distance over which the MeJA release is detectable. We determine the concentration of JA and MeJA in sagebrush leaves to determine the size of the endogenous pools for MeJA emission. Second, we compare the activity of *cis*- and *trans*-MeJA in laboratory experiments using two methods of delivering the MeJA to the plant: in lanolin applications and aqueous sprays. Lanolin application quantitatively delivers MeJA to a defined area of leaf. Aqueous sprays deliver MeJA as an aerosol over the plant canopy, thereby simulating volatile exposure. We test the activity of both trans and cis epimer by measuring two known JA-induced defense metabolites, namely nicotine and protease inhibitors (PIs), as well as monitor the short-term growth of an herbivore, *Manduca sexta*, that commonly attacks *N. attenuata* in nature (Keßler and Baldwin, 2001) and against which MeJA applications elicit resistance in the laboratory (van Dam et al., 2000). We tested the epimers individually and in a sequence mimicking the exposures that occur when *N. attenuata* grows immediately adjacent to an undamaged sagebrush that is subsequently damaged.

METHODS AND MATERIALS

Volatile Collection. Volatile collection and analysis followed methods described in Karban et al. (2000) and Preston et al. (2001). Briefly, sagebrush volatiles were collected by pulling air through traps containing activated charcoal (150-mg ORBO traps; Supelco, Bellefonte, PA) for 2–8 hr, depending on the experiment. The flow rate of air through the traps was 450–500 ml/min (measured by a mass flow meter: Aalborg Instruments, Orangeburg, NY). With the exception of the experiment that determined the distance over which MeJA is detected from sagebrush, branches were enclosed in 3.75-l transparent plastic conical containers, with an open bottom (22-cm i.d.) and top (7-cm i.d.) such that the volume of the headspace was equal for all trappings. For determining the distance over which MeJA is detectable, no sampling containers were used so as not to disturb the dispersion of volatiles from the sagebrush. All traps were stored at -20°C before being transported to the laboratory on dry ice where they were again stored at -20°C until analysis. After trapping and prior to elution, 710 ng of the internal standard, a triple ^{13}C-labeled MeJA (MW = 227) with a trans–cis ratio of 96:4, was added to each trap. Traps were eluted with 4 ml of dichloromethane (CH_2Cl_2), gently dried, reconstituted in 100 μl CH_2Cl_2, and transferred to a 150-μl glass insert of a 1.5-ml crimp-top glass vial. Samples were analyzed by GC-MS for MeJA content under the following conditions: fused silica-column (30 m $\times$ 0.25 mm) with a 0.25-μm DB-5 stationary phase held at 60°C for 4 min after injection (250°C), increased at 10°C/min to 200°C, followed by 20°C/min ramp to 300°C

for 7 min with He carrier gas maintained at 1 ml/min. Eluting compounds were detected by a Varian Saturn 2000 MS-MS ion trap (150°C) in electron-impact-ionization mode scanning masses 223-227. The trans and cis epimers eluted at 17.45 and 17.55 min, respectively (Preston et al., 2001). Since the flow rates were equal for all trappings, the results are presented as ng/hr.

While *A. tridentata* has several subspecies, all *A. tridentata* plants used in the experiments were *A. tridentata* ssp. *tridentata*. All collections were from naturally occurring sagebrush populations located in the Great Basin Desert of southwestern Utah [locations: township and range coordinates (section): kinetic T41S R18W (11); local vs. systemic T43S R18W (36); distance T43S R17W (6)]. Within each experiment, care was taken to choose plants of equal size and vigor.

The kinetic of the MeJA release was characterized by trapping the volatiles released by sagebrush in 2-hr intervals after a single damage event. Volatiles from eight sagebrush plants were first trapped from 8:30–10:30 A.M. to determine constitutive MeJA release. Each plant was then damaged by manually clipping the leaves contained within the enclosures with scissors (approximately 1–3 g of leaf material, 20% of the amount of total sagebrush leaf tissue enclosed was removed). Trappings were performed from 0–2, 2–4, 4–6, and 24–26 hr after damage.

To determine whether the MeJA release occurred from damaged leaves or systemically from undamaged leaves on damaged plants, four sagebrush plants located directly adjacent to each other and along a roadside were selected. Each plant was approximately 40–70 cm tall and 40–50 cm wide, and two to three other sagebrush plants on either side bordered the four plants. Two trapping containers were placed on opposite sides of each sagebrush plant to sample the headspace of the damaged portion separately from the undamaged portion of the plant. Branches were enclosed in trapping containers that remained on the plants for the entire experiment. One container from each plant was haphazardly assigned to either a damage treatment (local) or left undamaged (systemic), and volatile emissions were trapped for 4 hr first from the undamaged leaves of both branches before the damage treatment to determine if MeJA release was equivalent. Leaves in the local enclosure were then damaged by clipping with scissors and volatiles from all branches were trapped for 4 hr. Volatile emissions were again trapped 20 hr after damage for 4 hr to determine if there was a delayed increase in MeJA emissions from the systemic branches.

Naturally occurring tobacco plants neighboring damaged sagebrush suffered less herbivore damage as compared to tobacco neighboring undamaged sagebrush, but only when the plants were located within 10 cm of each other (Karban, 2001). To determine the distance over which the MeJA release is detectable, we trapped airborne MeJA emitting from single point source in the field (air temperature 36.6°C; soil temperature 50.5°C and a slight breeze). Four cotton wicks were impregnated with 500 μl MeJA each and positioned 20 cm above the ground. Volatile traps were positioned 10, 30, and 140 cm downwind from the cotton

swabs, and the volatilized MeJA was trapped for 5 hr. Of the MeJA placed onto the wick, approximately 19.8% was trapped at 10 cm, 7.3% at 30 cm, and 0.8% at 140 cm after 5 hr (Figure 2, inset). This exponential decay is consistent with the proposed model for dispersion of volatile compounds after release (Firn and Jones, 1995) and with the bioassay data; herbivore resistance decreases rapidly within a short distance from a damaged sagebrush canopy (Karban, 2001). Accordingly, we trapped volatiles at three distances from damaged sagebrush to determine the decay of the MeJA signal: directly within the sagebrush canopy, 20 cm and 40 cm from the sagebrush canopy. Traps were situated along a linear transect from the sagebrush out into a dirt road, such that no other sagebrush plants were located near any of the traps. Volatiles from eight undamaged sagebrush plants were collected for 8 hr, beginning at 11 A.M. The next day at the same time, the plants were damaged by clipping leaves along the entire canopy, removing approximately 5–8 g of leaf material, and volatiles were trapped for 8 hr. Plants were redamaged after 4 hr. For this experiment, the sagebrush branches were not placed within trapping containers, which would have disturbed the volatile dispersion.

To estimate the maximum MeJA volatile release from sagebrush plants, excised leaves (8.5–23 g) from 10 replicate 2-year old sagebrush plants were each placed into 4-l plastic chambers fitted with an activated charcoal trap containing 997 ng of internal standard positioned directly above each pile. Volatiles released by the material were trapped for 2 hr, and the release rate was calculated as ng/g/hr.

JA Analysis. Undamaged leaves from six sagebrush plants were collected separately in 2-ml microcentrifuge tubes, placed onto dry ice and stored at -20°C. The tissues were transported to the laboratory on dry ice and stored at -20°C until analyzed for JA content. JA was measured by GC-MS with [1,2-^{13}C] JA as an internal standard (Baldwin et al., 1997). Briefly, 436 ng of internal standard were added to approximately 0.15 g of leaf material and extracted first with 1.25 ml of extraction buffer, homogenized for 90 sec at 6.5 m/sec using the FastPrep® homogenizer (FP120; Q·Biogene, Heidelberg, Germany), centrifuged for 8 min at 13,000 rpm, and the supernatant transferred into a 4-ml glass vial. The leaf material was extracted again by adding 1.0 ml extraction buffer, homogenized as above, and centrifuged for 10 min at 13,000 rpm. JA contents of the pooled supernatants were analyzed by GC-MS after the clean-up procedures described in Schittko et al. (2000).

MeJA Analysis. To determine the size of the endogenous MeJA pools in undamaged sagebrush leaves, 3 replicates of approximately 0.2 mg sagebrush leaves were collected from a natural sagebrush population, and stored at -20°C, and extracted by soaking in 5 ml of CH_2Cl_2 for 5 min. Prior to adding CH_2Cl_2, each replicate received 1.42 μg of internal standard. The solvent was transferred to a clean 20-ml glass vial and gently dried. The extracted materials were reconstituted

in 100 μl CH_2Cl_2 and transferred into a 150-μl glass insert of a 1.5-ml crimp-top vial and analyzed by GC/MS as described earlier.

Plant Growth. Nicotiana attenuata [Torr. ex Wats. (synonymous with *Nicotiana torreyana* Nelson and Macbr.)] (Solanaceae) seeds, originally from bulk collections made from several plants growing in natural populations in Utah and inbred for three to seven generations in the glasshouse, germinated and grew for 14–17 days in a peat soil–perlite mixture soaked in 1:50 dilution of liquid smoke (House of Herbs, Passaic, NJ). Seedlings were transplanted and grown in either 250-ml or 2-l pots filled with a peat soil–perlite (approximately 3:1) mix of soil with 2 g (small pots) or 7 g (large pots) 14-14-14 N-P-K Osmocote slow-release fertilizer beads (Scotts Deutschland, Nordhorn, Germany) and 200 mg (small pots) and 1 g (large pots) Micromax micronutrient (Scotts Deutschland).

Herbivore Bioassay. M. sexta (Lepidoptera: Sphingidae) eggs were obtained from Carolina Biological Supply Company (Burlington, NC). Eggs were placed into rectangular polystyrene food containers (200-ml) with a clear lid (Neupack Verpackungen, Hamburg, Germany), lined with moist filter paper and maintained at 28°C, 65% relative humidity (RH), and a 16:8 hr light:dark photoperiod. Under these conditions, the eggs hatched 2–3 days after arrival. Larvae were used immediately after hatching and received no food prior to receiving the experimental leaf material. For the herbivore bioassays, one neonate larva was placed onto each treated leaf in separate bioassay containers.

Nicotine Analysis. Harvested tissues were flash frozen, lyophilized, ground to a fine powder, and 10-mg aliquots were used for nicotine extraction. Nicotine in the extracts was analyzed by high pressure liquid chromatography following methods described in Keinänen et al. (2001).

Protease Inhibitor Assay. Harvested plant tissue was flash frozen, lyophilized, ground to a fine powder, and 10–25-mg aliquots were extracted for PI activity. Each sample was analyzed for trypsin protease activity and protein content according to van Dam et al. (2001), except that the amount of extraction buffer was adjusted to maintain an equal ratio of plant material–extraction buffer.

MeJA Lanolin Treatments. Six days after transplanting into 250-ml pots, 10 plants were haphazardly assigned to each of three treatment groups: control, *trans*-MeJA [(original trans–cis ratio, 92:8; in lanolin, 100:0), Aldrich, Gillingham, Dorset, UK], and *cis*-MeJA [(original trans–cis ratio, 5:95; in lanolin, $63.34 \pm 0.99 : 36.66 \pm 0.99$), purified cis produced as described in Preston et al. (2001)]. Each MeJA epimer was applied to plants in concentrations of 0.5, 2.5, 10, and 25 μg per 20 μl of lanolin. Controls consisted of 20 μl lanolin. Ten plants were haphazardly assigned to each treatment, and the lanolin was applied in a thin strip across the leaf surface perpendicular to the mid-rib on the first fully expanded leaf. The amount of epimerization of trans and cis in the lanolin was estimated by measuring the MeJA in the headspace above each lanolin treatment. Lanolin,

containing either trans or cis, was aliquoted onto glass Petri dishes in 20 μl drops. Petri dishes were enclosed in transparent plastic 18.5-l chambers (Rubbermaid; Wooster, OH) each covered with a piece of UV transparent Plexiglas (UV-T). A trap was inserted alongside each Petri dish, and the volatile MeJA was trapped for 6 hr, and the volatiles were analyzed by GC/MS for the epimeric ratio. Due to the epimerization of the *trans*- and *cis*-MeJA in lanolin, the actual amounts of each epimer applied within each treatment were control, 0; 0.5 μg trans, 0.5 trans; 0.5 μg cis, 0.315 μg trans and 0.185 μg cis; 2.5 μg trans, 2.5 μg trans; 2.5 μg cis, 1.575 μg trans and 0.925 μg cis; 10 μg trans, 10 μg trans; 10 μg cis, 6.3 μg trans and 3.7 μg cis; 25 μg trans, 25 μg trans; and 25 μg cis, 15.75 μg trans, and 9.25 μg cis.

Two days after treatment applications, the treated leaf from each plant was harvested. Immediately after harvesting, leaves were divided in half parallel to the mid-rib. One leaf half was used in the herbivore bioassay and the other half was flash frozen in liquid N_2, lyophilized, ground to a fine powder, and analyzed for PI activity. PI activity is significantly induced 2 days after an initial MeJA treatment (van Dam et al., 2001). Four days after initial treatment, larvae were weighed, mortality was noted, and the leaf was replaced with the next youngest leaf from the same plant as the first leaf. The remaining rosette leaf of each plant was excised at the shoot–root interface and flash frozen in liquid N_2, lyophilized, finely ground, and analyzed for nicotine content. Nicotine concentrations are known to attain maximum values 4 days after MeJA treatment or wounding (Baldwin et al., 1998). Two days later, larvae were weighed, and mortality was recorded.

Aqueous MeJA Sprays. Three days after transplanting in 2-l soil pots, 7 plants were haphazardly assigned to each of the following treatments: control, trans low, trans high, cis low, cis high, trans–cis low, and trans–cis high. Controls received only sprays of water. Low MeJA treatments received 1 μg, while high MeJA treatments received 5 μg. The *trans*-MeJA treatments were produced by weighing the MeJA into a glass vial and adding sufficient water to produce the desired concentrations. The *cis*-MeJA is stored as a stock solution, with *cis* diluted in N-hexane. Dilutions were produced by measuring out the necessary volume of stock solution, allowing the solvent to evaporate completely, and adding the appropriate volume of water. Since MeJA is not soluble in water, all solutions were vigorously shaken before treating each plant. Treatments were delivered in five sprays of a glass perfume spray bottle, totaling 0.75 ml. To determine the amount of epimerization that occurs in the cis treatment during this procedure, 10 ml of the cis solutions were sprayed into glass scintillation vials and extracted twice with 10 ml CH_2Cl_2. The pooled solvent layer was gently dried, and the MeJA was reconstituted in 100 μl of CH_2Cl_2 and analyzed by GC-MS. After spraying, the trans–cis ratio in the trans treatments were 97.62 $\pm$ 0.5: 2.38 $\pm$ 0.5 and in the cis treatments, 36.9 $\pm$ 2.5: 63.1 $\pm$ 2.5. Treatments were applied every day for 7 days. In the last two treatments, plants received sprays of *trans*-MeJA each day for 5 days followed by

2 days of *cis*-MeJA. Over the 7 days of the experiment, the cumulative amount of MeJA, considering trans–cis epimeric ratios of 98:2 in the trans treatments and 37:63 in the cis treatments, received by each treatment was control, 0; trans low, 6.86 μg trans and 0.14 μg cis; cis low, 2.59 μg trans and 4.41 μg cis; trans–cis low, 5.64 μg trans and 1.36 μg cis; trans high, 34.3 μg trans and 0.7 μg cis; cis high, 12.95 μg trans and 22.05 μg cis; trans–cis high, 28.2 μg trans and 6.8 μg cis.

Seven days after the first treatment application, the first fully expanded leaf was excised at the petiole from each rosette, a technique that elicits only a minimal nicotine response (Baldwin et al., 1998). The leaf was bisected along the mid-rib. One half of each leaf was used in an herbivore bioassay. The other half was divided into two, perpendicular to the cut edge. The top half was analyzed for PI content, and the bottom half was analyzed for nicotine content. After 2 days, the next youngest leaf was similarly harvested. Larvae from the the first treatment leaf were weighed, mortality was noted, and then placed onto one-half of the leaf from the next harvest. Again, the top section of half of the leaf was analyzed for PIs and the bottom half for nicotine. Two days later, larvae were weighed and further mortality was noted. During the experiment, one plant in each control, cis low, and trans high group became diseased and were excluded from the analyses.

Statistical Analysis. One-way and two-way ANOVAs were used to analyze main effects and Fisher's PLSD post hoc tests were used to detect significant differences between groups when the original ANOVA was significant. Analyses were performed with the STATVIEW 5.0 statistical package (SAS Institute, Gary, NC).

RESULTS

JA and MeJA Leaf Concentrations. The concentrations of JA and MeJA are 3 and 4 orders of magnitude greater in excised leaves than the amount of MeJA emitted from undamaged sagebrush plants (Table 1). Sagebrush has

TABLE 1. MEAN ($\pm$1 SEM) AMOUNTS OF JA AND MEJA MEASURED WITHIN AND RELEASED FROM *Artemisia tridentata* SSP. *tridentata* LEAVES. CONSTITUTIVE AND DAMAGE-INDUCED MEJA RELEASE RATES FROM INTACT FIELD GROWN PLANTS ARE FROM KARBAN ET AL. (2000). N.D., NOT DETERMINED

Collection	*trans*	*cis*
Endogenous JA pools (μg g^{-1})	9.95 $\pm$ 1.40	n.d.
Endogenous MeJA pools (μg g^{-1})	19.08 $\pm$ 0.03	76.96 $\pm$ 0.77
Maximum release rates from detached leaves (ng g^{-1} h^{-1})	154.91 $\pm$ 28.94	975.01 $\pm$ 376.69
Constitutive release rates from undamaged plants (ng g^{-1} h^{-1})	21.9 $\pm$ 8.4	3.65 $\pm$ 0.73
Damage-induced release rates (ng g^{-1} h^{-1})	35.35 $\pm$ 9.79	34.43 $\pm$ 11.26

extraordinarily large endogenous JA pools (9.95 $\pm$ 1.40 μg/g) that are nearly 1000$\times$'s larger than that of *N. attenuata* (28.88 $\pm$ 5.17 ng/g; Schittko et al., 2000) and apparently has the ability to methylate and store large quantities, nearly 100 μg/g leaf material. Additionally, each hour 1.17% of this pool can be volatilized. Clearly, sagebrush is capable of releasing quantities of MeJA upon damage that would be physiologically active for *N. attenuata* plants growing nearby.

Characterizing MeJA Emissions

Kinetic of MeJA Emission After Damage. The total amount of MeJA released between the different trapping intervals was significantly different (Figure 1A; one-way ANOVA $F = 4.232$, $df = 4, 32$, $P = 0.007$). When compared to MeJA released by undamaged sagebrush, damage increased both the amounts of *trans*- and *cis*-MeJA released by sagebrush at 0–2 hr (P's = 0.003 and 0.005, respectively) and 2–4 hr (P's = 0.014 and 0.018, respectively) immediately after damage. After 4 hr, the damaged sagebrush's release of MeJA, either total, trans or cis, was no longer significantly different from undamaged levels (P's $\geq$ 0.08). Furthermore, there does not appear to be a delayed MeJA release, with the amount of MeJA detected 24–26 hr after damage being no different from that released from undamaged tissues (P's = 0.83 and 0.99 for trans and cis, respectively). We conclude that the maximum release of MeJA occurs within the first 4 hr following damage.

Local And Systemic Releases of MeJA After Damage. There was a difference in the quantities of MeJA released by the local and systemically located leaves after damage (Figure 1B; $F = 3.252$, $df = 5, 17$, $P = 0.031$). Damage caused an immediate increase in the total amount of MeJA released ($P = 0.010$), as well as in the trans or cis individually ($P = 0.012$ and 0.010, respectively) in the local treatment. The MeJA emissions from the systemically located leaves, either as total, trans or cis, remained similar to levels of MeJA released when the sagebrush was undamaged at both trapping intervals following damage (P's $\geq$ 0.65). At 20–24 hr after damage, MeJA emissions by local leaves were not significantly different than the MeJA levels of the same leaves before damage ($P = 0.84$). The increase in MeJA release after damage is clearly localized to the damaged leaves.

Distance from Sagebrush over Which MeJA is Detectable. A two-way ANOVA revealed a difference in the amounts of MeJA emitted by the undamaged and damaged sagebrush plants (Figure 1C; $F = 4.260$, $df = 1, 31$, $P = 0.048$), but no significant difference in the amount of MeJA trapped from the different distances to the sagebrush plants ($F = 0.444$, $df = 2, 31$, $P = 0.65$) or interaction between damage and distance ($F = 1.054$, $df = 2, 31$, $P = 0.75$). Moreover, the amount of trans and cis was not significantly different at each distance from either the undamaged sagebrush ($F = 0.542$, $df = 2, 12$, $P = 0.59$ and $F = 2.021$, $df = 2, 12$,

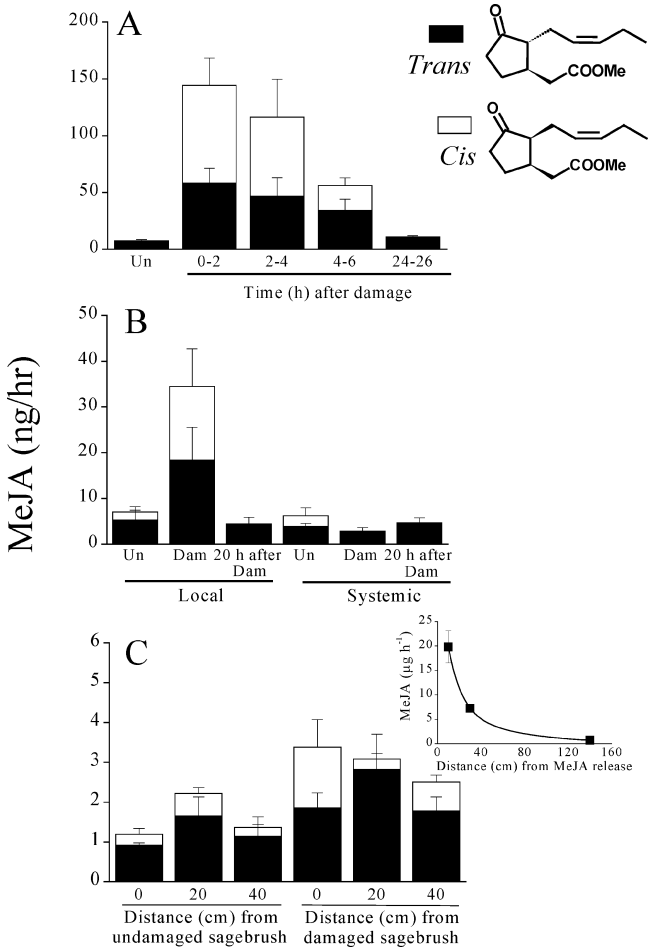

FIG. 1. Mean (+1 SEM) *trans* (black bar) or *cis* (white bar) MeJA trapped: A: from sagebrush branches before damage (Un) or 0–2, 2–4, 4–6, or 24–26 hr after a single mechanical damage; B: from branches that had either directly been damaged (local) or branches that were located on the same plant but were not themselves damaged (systemic); and C: at different distances from (0, 20, and 40 cm from the sagebrush canopy) to undamaged or damaged sagebrush plants. Inset: Amount of MeJA (mean +1 SEM, $N = 4$) trapped at three distances (10, 30, and 140 cm) downwind of a cotton wick impregnated with 500 μl MeJA. Branches in A and B were enclosed in 3.75-l plastic containers with air inlets at the bottom and outlets at the top. Sagebrush plants in C were not enclosed and located along a roadside such that there were no additional sagebrush plants were within 2 m of the traps. Volatile emissions were collected for 2 hr in A, 4 hr in C, 6 hr in C, and 5 hr in C (inset).

$P = 0.18$, respectively) or damaged sagebrush ($F = 0.995$, $df = 2, 19$, $P = 0.39$ and $F = 2.117$, $df = 2, 19$, $P = 0.15$, respectively). The amount of MeJA from undamaged and damaged sagebrush did not decline over a distance of at least 40 cm. While the amounts of MeJA trapped by our collection were low compared with the other trapping data (likely due to the lack of volatile collection chambers), we were able to detect MeJA at all distances. Two explanations may account for this. First, rather than assuming a diffusion-based exponential decay (i.e., Figure 1C inset), the volatile plume released by the sagebrush may remain intact as it is transported from the canopy. Second, the canopy is not a single point-source of volatile release, but rather a large surface of volatile release and the traps 20 and 40 cm from the canopy were sampling this continually produced cloud of MeJA.

Activity of cis- *and* trans-*MeJA in Lanolin Applications*

PI-Inducing Activity. Concentrations of PIs were different among the MeJA treatments (Figure 2A; $F = 5.302$, $df = 8, 61$, $P < 0.001$). The *trans*-MeJA treatment increased PI concentration at either 10 μg ($P = 0.030$) or 25 μg ($P = 0.003$) doses. However, the PI concentrations of cis-treated plants were above those of control plants at 2.5 μg ($P = 0.042$), and 25 μg ($P < 0.001$), but only marginally significantly at 10 μg ($P = 0.054$). cis was only more active than trans at the 25 μg level in increasing PI levels ($P = 0.013$).

Nicotine-Inducing Activity. As with PI concentrations, nicotine concentrations varied among the treatments (Figure 2B; $F = 6.462$, $df = 8, 80$, $P < 0.001$). Treatment with cis only marginally increased nicotine concentrations above those in control treatments at 2.5 μg ($P = 0.068$), 10 μg ($P = 0.068$), and 25 μg ($P = 0.074$). In contrast, trans treatments significantly increased nicotine concentration at 10 μg ($P = 0.001$) and 25 μg ($P < 0.001$) and trans was more active than cis ($P = 0.003$) at the highest application amount (25 μg). In summary, while cis was more active at the highest concentration in eliciting PIs, trans was more active in eliciting nicotine production than cis.

Hornworm Performance. Larvae fed leaves treated with the different epimers had equivalent mortality rates (Figure 2C), but their masses differed after they had fed on leaves from the different treatments for 2 and 4 days (Figure 2D; $F = 4.703$ and 2.705, $df = 8, 56$, $P < 0.001$ and $P = 0.014$, respectively). After 2 days, larvae consuming leaves treated with either 2.5 and 25 μg trans or 2.5, 10, and 25 μg cis were smaller than those feeding on control leaves (P's $\leq$ 0.030). The application of 0.5 μg of cis did not affect larval growth ($P = 0.083$). Only at the 10 μg level did larval masses differ significantly between trans and cis treatments ($P = 0.005$). After 2 days of consuming a systemically located leaf, larvae consuming leaves from plants treated with 25 μg trans or cis weighed less than those on leaves from control plants (P's $= 0.014$ and 0.051,

respectively). Larvae fed leaves from the 10 μg cis treatment grew less than those consuming leaves from the 10 μg trans treatment ($P = 0.049$). While cis may be slightly more active than trans, the effects are not consistent at all treatment levels.

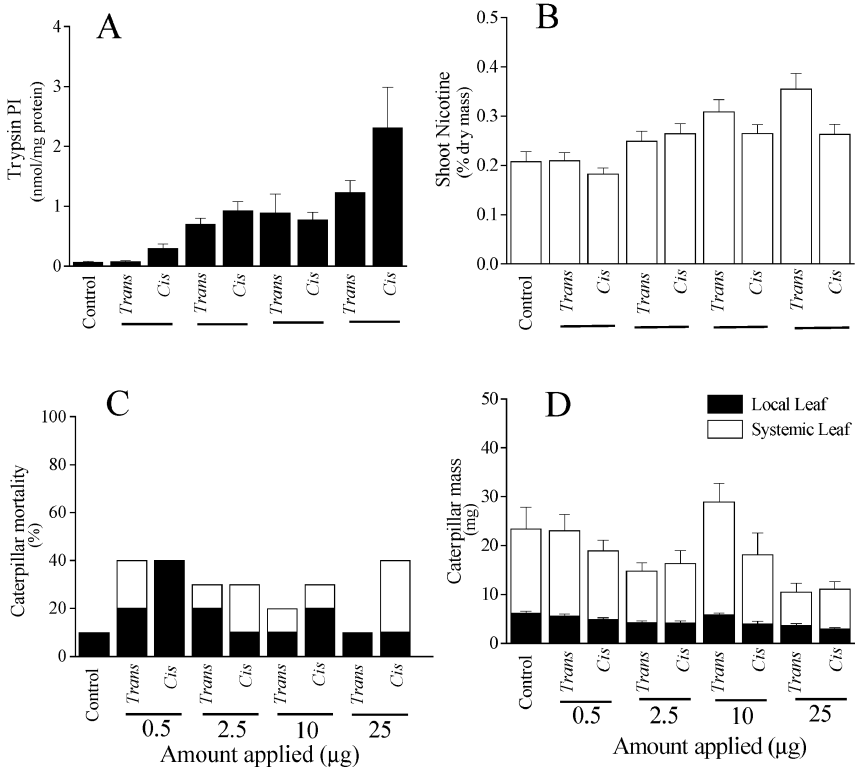

FIG. 2. A: Trypsin PI levels (mean +1 SEM) in leaves of *N. attenuata* plants treated 2 days earlier with lanolin applications of either *trans*- or *cis*-MeJA at 0.5, 2.5, 10, or 25 μg per plant. See Methods section for an estimate of applied values of each MeJA epimer that includes epimerization of *trans*- to *cis*-MeJA during application. Control plants received only treatments of lanolin. B: Shoot nicotine levels of plants 4 days after treatment applications. C: Mortality of *M. sexta* larvae on the treated (black bar) and systemic leaf (white bar), harvested 2 and 4 days, respectively, after treatments were applied. Percentage mortality is expressed as the cumulative number of larvae in each treatment that died of the initial 10 larvae. D: Mass of *M. sexta* larvae 2 days after feeding on the treated leaf (black bar) and 2 days after feeding on the systemic leaf (white bar).

Activity of cis- *and* trans-*MeJA in Aqueous Spray Applications*

PI-Inducing Activity. PI concentrations of the first leaf harvested were different between the treatments (Figure 3A; $F = 4.850$, $df = 6, 37$, $P = 0.001$), with significant differences between the control and the trans high ($P < 0.001$), cis high ($P = 0.001$), and trans–cis low ($P = 0.005$) treatments. No differences between the trans and cis, at either the low dose ($P = 0.87$) or high dose ($P = 0.51$) were found.

In contrast, PI levels in the second leaf harvested were not different among the treatments (Figure 3B; $F = 1.785$, $df = 6, 37$, $P = 0.13$). The results do not support the differences between epimers observed in the lanolin treatment or the hypothesis that the trans to cis switch represents a uniquely active signal.

Nicotine-Inducing Activity. Nicotine concentrations in the first leaf harvested were different between the treatments (Figure 3C; $F = 3.026$, $df = 6, 39$, $P = 0.016$). The trans–cis low treatment increased nicotine concentrations above that found in the control treatment ($P = 0.005$), while those in the trans high and trans–cis high treatments were only marginally greater than that of controls ($P = 0.053$ and 0.062, respectively). At low and high doses, trans and cis treatments were not significantly different. However, nicotine levels in the trans–cis low treated leaves were greater than those in the trans low ($P = 0.02$) and cis low ($P = 0.001$) treatments.

Nicotine concentrations in the second leaf harvested were different among the treatments (Figure 3D; $F = 2.893$, $df = 6, 38$, $P = 0.020$). Only the trans–cis high was higher than controls ($P = 0.003$). The nicotine concentration in the trans–cis high treatment was also greater than that in trans high ($P = 0.030$) and cis high ($P = 0.003$). In summary, the trans to cis switch appears to elicit nicotine production more effectively than either epimer alone.

Hornworm Performance. Overall, larval mortality remained lower and larval weight gain larger than in the lanolin-treatment experiment (Figure 3E). Larval masses among the different treatments were not significantly different after 2 days of consuming the first leaf (Figure 3F; $F = 0.978$, $df = 6, 36$, $P = 0.454$). However, larval masses among all of the treatments were significantly different after 4 days of consuming treated leaf material, 2 days on the first harvested leaf and 2 days on the second harvested leaf (Figure 3F; $F = 2.486$, $df = 6, 36$, $P = 0.04$). Larvae feeding on leaf material from the trans high, cis high, and trans–cis high treatments weighed less than those feeding on leaves from control treatments (P's $= 0.005$, 0.048, and 0.026, respectively). There were no differences between the trans, cis, or trans–cis treatments at either dosage level (P's ≥ 0.34). Differences in larval growth between the different MeJA treatments was not observed after larvae fed on the second leaf for 2 days.

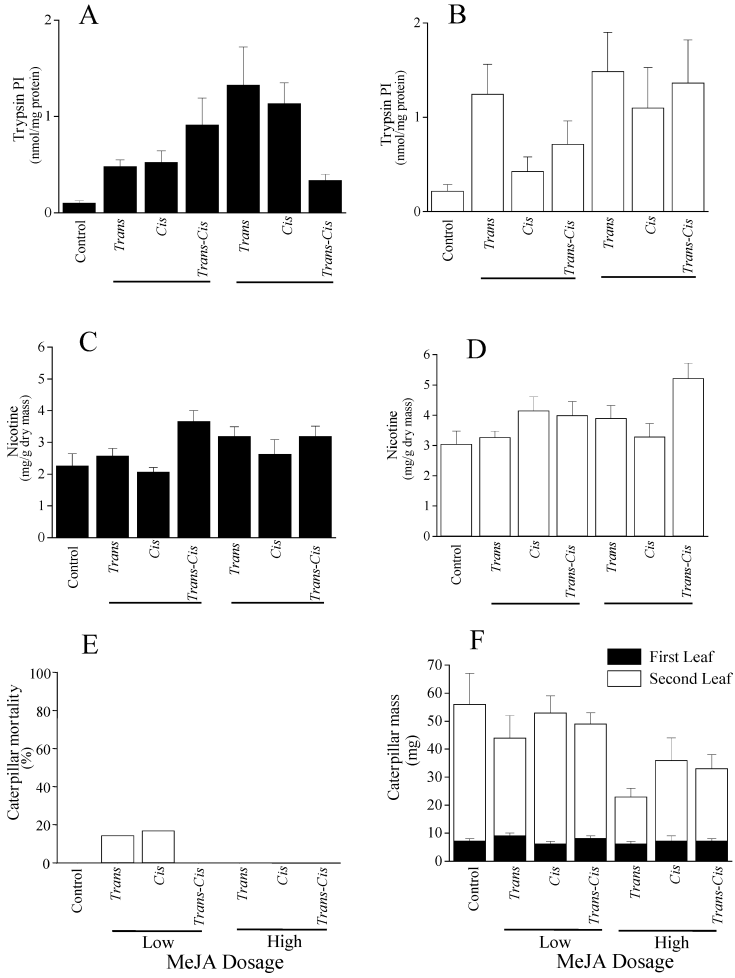

FIG. 3. Trypsin PI (A, B) and nicotine (C, D) levels (mean +1 SEM) in the first fully expanded leaf harvested from each plant 7 days after initiation of treatments (2 days after the first cis application in trans–cis treatment), and in the leaf at the next youngest node 2 days after the first harvest (9 days after initiation of treatments), respectively. Plants were treated daily with either 1 μg (low) or 5 μg (high) of their respective epimers. See Methods section for an estimate of applied values of each MeJA epimer that includes epimerization during application. E: Percentage mortality of *M. sexta* larvae on the first (black bar) and second leaf (white bar), harvested 7 and 9 days after treatments were initiated, respectively. Mortality is expressed as the cumulative number of larvae in each treatment that died of the initial 7. F: Mass of *M. sexta* larvae 2 days after feeding on the first leaf (black bar) and 2 days after feeding on the second leaf (white bar).

In summary, our results are not consistent with the hypothesis that cis is biologically more active than trans in eliciting resistance against this specialist herbivore.

DISCUSSION

Studies of plant-plant communication generally fail to treat plants with relevant quantities of the chemical of interest in a manner that mimics the natural release. Experimental treatments are often several orders of magnitude higher than natural exposures and plant responses are tested in small, enclosed containers. For example, Birkett et al. (2000) placed 2.5 mg *cis*-jasmone (their putative interplant signal) on a filter paper in a closed container with 9 plants for 24 hr, during which time the compound completely volatilized. No values are reported for the natural release rate of *cis*-jasmone from *Ribes nigrum* (the focus of the study), but the experimental release rate (104.17 μg/hr, assuming an equal emission rate over the exposure interval) is 4 orders of magnitude greater than that measured from six varieties of cotton (2.1 $\pm$ 4.0 to 19.3 $\pm$ 13.7 ng/hr; Loughrin et al., 1995). Similarly, Arimura et al. (2000a) exposed excised lima bean leaves to 10 μg of their test compounds for 3 or 24 hr in a 7-l sealed chamber. When infested with spider mites, lima bean plants release approximately 99.2, 122, and 164.4 ng/hr/plant of (*E*)-β-ocimene, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), respectively (Dicke et al., 1999). It is not clear how quickly the volatiles were emitted from the cotton wool. In addition to using high exposures, both studies contained plants (or excised leaves) in small air-tight containers under illuminated conditions in which plants would likely draw down the CO_2 concentration below their CO_2 compensation point, increase stomatal openings, and further exaggerate the exposure of the "receiver" leaves to volatile signals. In summary, the experimental procedures used in laboratory tests of interplant signaling have tended to favor the accumulation of volatile compounds in the headspace of receiver plants, rather than having them disperse, as would occur in natural environments.

Of plants examined for MeJA production, *Artemisia* and *Jasminum* are unique in that they release large quantities of MeJA and, unlike most plants, wounding is not required for production (Hildebrand et al., 2000). The pools of JA are substantial (Table 1), among the highest concentration reported for any plant (Farmer, 1994; Mueller, 1997) and are nearly three orders of magnitude greater than that found in undamaged *N. attenuata*. Additionally, even larger pools of MeJA exist; approximately 96 μg/g with a trans–cis ratio of 20:80 (Table 1). It is unknown how MeJA is formed within the cell, but work in snapdragon has identified a methyl ester-forming enzyme, *S*-adenosyl-L-methionine/benzoic acid carboxyl methyl transferase, responsible for the formation of the volatile methyl

ester, methyl benzoate, from benzoic acid (Dudareva et al., 2000). Presumably, a similar enzyme methylates the large pools of free JA in the plant prior to its release. The clean-up procedure for the JA determinations epimerizes the endogenous JA pool (Baldwin et al., 1997), so it is not possible to determine whether the endogenous JA pool is predominantly cis or trans. However, the majority of the evidence points to *cis*-JA as the first biosynthetic product in plants (Mueller and Brodschelm, 1994). This is further supported by the high proportion of MeJA existing as cis in undamaged sagebrush leaves (Table 1). Large pools of JA are likely to progress toward their thermodynamic equilibrium of 92:8, possibly as a consequence of epimerization promoted by unspecific or specific protein binding in the plant cell (Mueller and Brodschelm, 1994). It is likely that these large pools of JA and MeJA provide an unlimited source for MeJA emission from sagebrush.

To be an effective airborne signal, the active component must contain accurate information and remain at physiologically active levels over a biologically-relevant distance. Much like the release of the "green leaf" volatiles [also proposed as possible airborne signals (Bates and Rothstein, 1998; Engelberth et al., 2004)], the increased emission of MeJA and, in particular, *cis*-MeJA is tightly associated with damage, occurring immediately after damage (Figure 1A) and only from damaged tissues (Figure 1B). Moreover, the amount of MeJA at the site of release (sagebrush) is not significantly reduced for at least 40 cm (Figure 1C), suggesting that the damage-induced enrichment of MeJA in the immediate headspace of an attacked sagebrush is not likely to be diluted at biologically relevant distances. This contrasts with the expected exponential decay in airborne concentrations with distance as volatiles diffuse from a point source (Figure 1C inset). Sagebrush plants are, however, not point sources and their canopy provides a structure that may affect local air currents. In summary, our data support the hypothesis that *A. tridentata* is capable of releasing substantial quantities of volatile *cis*-MeJA and the release of *cis*-MeJA fits some of the expectations for an effective airborne signal. Whether or not receiver plants respond is the second question we addressed.

We developed two procedures that delivered *cis*- and *trans*-MeJA in realistic quantities directly to plants: (1) in a lanolin paste, which delivered a whole-plant dose to a defined leaf area in a single exposure and, (2) in an aqueous spray, which delivered smaller amounts to the entire canopy in a series of applications to simulate an extended exposure. The two treatment methods may also deliver the two isomers with different probabilities of epimerization before they enter the plant. Lanolin applications may provide a lipophilic route into the plant that avoids the enolization required for epimerization (Mueller and Brodschlem, 1994). Enolization occurs during protonation of the C-6 keto group, which might occur with a greater frequency in the aqueous spray application. Headspace trapping (trans–cis ratio of 63:37) of the lanolin containing the cis treatment with an original trans–cis

ratio of 5:95, provided evidence for significant epimerization (61% of cis) either in the lanolin or during volatilization from the lanolin into the air. A similar analysis of the aqueous sprays revealed less epimerization (34% of cis). However, it is not clear which application procedure simulates the epimerization that occurs when plants are exposed to airborne MeJA. Lanolin applications provided some support that *cis*-MeJA is biologically more active than trans because concentrations of PIs and larval mortality were greater and larval mass lower in plants treated with cis (Figure 2A and C). However, aqueous sprays did not support these conclusions, but rather demonstrated that trans was approximately as active as cis in eliciting PI accumulation (Figure 3A and B). Previous studies testing the different epimers in various bioassays suggest that each may activate different jasmonate receptors and elicit different responses (Koda et al., 1992; Weiler et al., 1993). For example, growth inhibition and production of the secondary metabolites, paclitaxel and baccatin III, in *Taxus* cells is differently regulated by the different MeJA epimers (Yukimune et al., 2000). We conclude that the different MeJA epimers may trigger different responses in plants, but their overall activity does not differ dramatically. Moreover, prior exposure to trans, as would occur when plants grow next to undamaged sagebrush, does not dramatically sensitize plants to a short-term exposure to cis, as would occur when sagebrush is damaged (Figure 3A–F). In summary, the amount of MeJA applied to plants, rather than the particular epimer applied, was the most important determinant of plant responses in these laboratory experiments. Whether the quantities used realistically represent natural exposures deserves further discussion.

A damaged sagebrush plant releases approximately 40–80 ng/g/hr MeJA (Karban et al., 2000) from its damaged tissues. It is reasonable to assume that on average, an attacked sagebrush plant would have 10 g of damaged leaves in its canopy releasing at this rate. Such a damaged sagebrush plant would release 400–800 ng/hr MeJA and if a nearby tobacco plant received this entire dose for as long as the herbivores attacked the sagebrush, a plant would receive an amount comparable to that received in the aqueous spray treatments, which delivered 1 or 5 μg MeJA each day. At these doses, both PI and nicotine concentrations were elevated above controls, and growth of *M. sexta* larvae was negatively influenced. While both of these traits are known to be correlated with plant fitness and resistance in natural populations of *N. attenuata* (Baldwin, 1998; Glawe et al., 2003), it is unclear whether the responses are sufficiently strong to account for the decreased herbivory observed in the Karban et al. (2000) study. In a field study (Baldwin, 1998), 500 μg applications of MeJA (90.1: 8.3; trans–cis) to the rhizosphere of plants were required to induce nicotine concentrations comparable to that elicited by foliar wounding. In this study the MeJA was delivered in 10 ml of water to the soil surrounding the plant's roots, and it is not clear how much actually came in contact with plant roots or was volatilized and assimilated by the shoot. It is clear, however, that the entire quantity of MeJA released from a plant will not be directly

deposited onto a single neighboring tobacco plant. Without trapping containers to contain the headspace from a damaged sagebrush, the quantities of MeJA trapped were about 10-fold less than those trapped from undamaged sagebrush and 10- to 50-fold less than those from the damaged sagebrush (Figure 1C). At these concentrations (1–3 ng/hr) we have no evidence that MeJA elicits resistance or resistance-related traits. A field experiment was set up to examine responses of natural populations of tobacco to the two epimers, but unfortunately, an outbreak of *M. quinquemaculata* and *M. sexta* prevented us from detecting differences in MeJA-treated plants compared to controls. All plants in the experiment were completely consumed shortly after treatment applications (Preston et al., 2001).

Additional field studies are needed to determine whether sagebrush's wound-induced MeJA release effects resistance in nearby growing tobacco. Moreover, it will be important to quantify MeJA release in response to herbivore attack and not simply to mechanical wounding, which was used in the Karban et al. (2000) study. Other wound-induced volatile releases are amplified and quantitatively altered when herbivores cause the wounding or herbivore-specific salivary factors are added to wounds (De Moraes et al., 1998; Halitschke et al., 2000, 2001). However, since wounding does not increase endogenous MeJA or JA levels above the already high constitutive titers found in sagebrush (Hildebrand et al., 2000), it would be surprising if herbivory dramatically altered release rates.

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