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Methyl jasmonate is blowing in the wind, but can it act as a plant–plant airborne signal?

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Abstract

Interplant communication in nature is beginning to look like a reality with the field demonstration that tobacco plants downwind of damaged sagebrush suffer less herbivory, a response that appears to be mediated by an airborne signal. Sagebrush constitutively releases methyl jasmonate (MeJA), a compound that is highly active in inducing a number of physiological responses in plants. Damage increases the absolute quantity of the MeJA released as well as the proportion of MeJA in the isomeric *cis* form. Several studies have shown that volatile MeJA, when released in sufficient quantities, can simulate responses elicited by direct MeJA applications. Additionally, the thermodynamically unstable *cis* isomer, which is responsible for the characteristic jasmine odor, is thought to be the biologically active form of MeJA. To examine the hypothesis that the *cis*-MeJA release is responsible for the apparent interplant communication, we developed methods to: (1) entrain sagebrush constituents in water which preserved the isomeric shift in the MeJA released after damage; (2) chemically manipulate the MeJA *trans* : *cis* ratio; and (3) isolate nearly pure *cis*-MeJA by HPLC. These treatments were applied as aqueous sprays to a natural population of tobacco plants, however, an outbreak of specialist herbivores consumed all treated plants and chemical analysis on previously harvested treated leaf material was inconclusive. The hypothesis is currently being carefully investigated with laboratory experiments.

Keywords: *Nicotiana attenuata*; *Artemisia tridentata* ssp. *tridentata*; *cis*-MeJA; *trans*-MeJA; Epimerization; Water entrainment; Inter-plant communication

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1. Introduction

Using a number of experimental manipulations, Karban et al. (2000) demonstrated that airborne communication between plants may be possible in a natural setting, outside laboratory bell jars. *Nicotiana attenuata* Torr. ex Wats. (Solanaceae) plants transplanted in close proximity (within 15 cm) to artificially damaged sagebrush suffered reduced levels of herbivory as compared to tobacco plants transplanted near undamaged sagebrush (Karban et al., 2000). The active signaling component(s) emanating from the damaged sagebrush responsible for the physiological changes in the neighboring tobacco plants remains unknown. Sagebrush constitutively releases a complex mixture of volatiles (at least 23 identified compounds including representatives from several chemical categories including monoterpenes, sesquiterpene lactones, coumarins and flavonoids; Muller et al., 1966; Kelsey et al., 1978; Personius et al., 1987), with a large number of constituents exhibiting a quantitative change after damage and hence are candidates for potential signals (Fig. 1A, B). Determination of the active component is a daunting task considering the chemical and the environmental complexities involved, but recently a number of plant-derived volatiles have been proposed to mediate plant–plant airborne signaling.

2. Possible signals

Included among the compounds that are thought to be involved in interplant communication are two jasmonates [*cis*-jasmone (Birkett et al., 2000) and methyl jasmonate (MeJA; Farmer and Ryan, 1990)], the methyl ester of a phenolic compound often linked to pathogen attack [methyl salicylate (MeSA; Shulaev et al., 1997)], several terpenes [β-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*E*, *E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT; Arimura et al., 2000)], and some C_6-C_{10} alkenals and alkanals (Zeringue, 1992). Interestingly, insects may also utilize many of these substances as airborne signals for intraspecific communication. *Cis*-jasmone, MeSA, as well as two C_8 alkanals are emitted from male *Amauris ochlea* butterfly hairpencils (Petty et al., 1977) and MeJA is emitted from male oriental fruit moth hairpencils [*Grapholitha molesta* (Busck.)] as pheromones which attract receptive females (Nishida et al., 1982; Baker et al., 1991). Of these proposed signals, only MeJA is detectable in volatile collection from sagebrush, as determined by comparison with chemical standards (Figs. 1A, B, 2A, B; Karban et al., 2000).

Previous work has suggested that MeJA could be released in sufficient quantities from excised sagebrush [*Artemisia tridentata* Nutt. ssp. *tridentata* (Asteraceae)] foliage to effect an increase in proteinase inhibitor (PI) production in tomato cuttings [*Lycopersicon esculentum* (Solanaceae)] sharing the same air-space in a bell jar (Farmer and Ryan, 1990). Induced PI production was strongly correlated with herbivore resistance in tomato (Orozco-Cardenas et al., 1993). Several studies have shown that plants exposed to volatile synthetic MeJA initiate JA-induced responses. Exposure to volatile MeJA induces alkaloids in *Catharanthus* and *Cinchona* seedlings

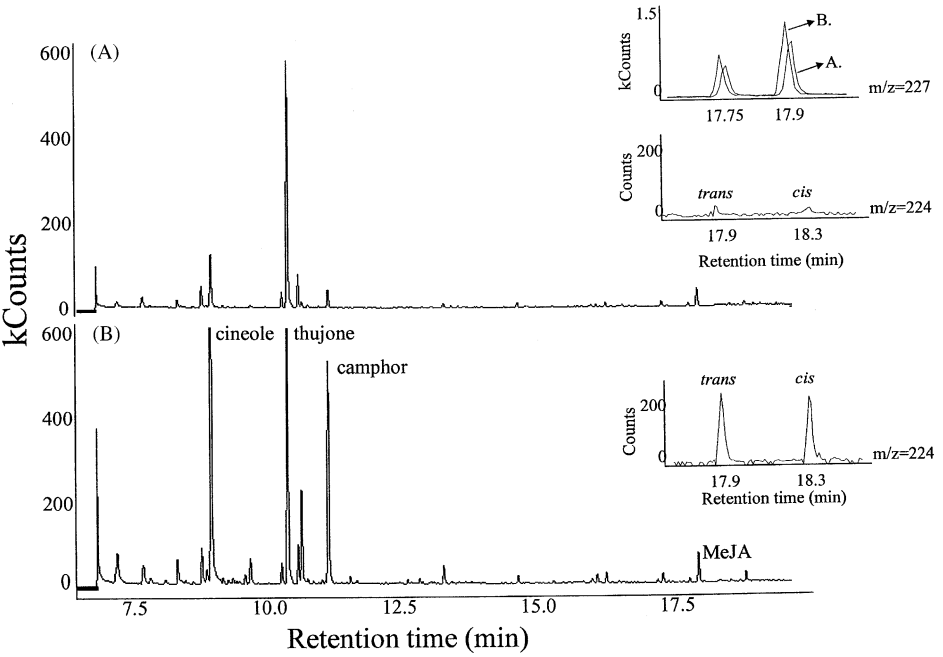


Fig. 1. The GC–MS chromatograms of volatiles collected from the headspace of undamaged sagebrush (A) and artificially damaged sagebrush (B). Branches from sagebrush plants were enclosed in 3.75-l plastic cones, with an open bottom (22 cm i.d.) and top (7 cm i.d.). Volatile collection and analysis of the traps followed methods detailed in Karban et al. (2000), with minor modifications. The sagebrush volatiles were collected for 3 h and the internal standard [710 ng of triple ^{13}C-labeled MeJA (MW = 227)] was added after the air sampling but prior to elution of the traps. Elution was performed using 4 ml of dichloromethane (CH_2Cl_2), gently concentrated to 100 µl before GC–MS analysis. The GC–MS analysis was made using the full-scan mode. We did not attempt to identify all the compounds present in the volatile emission, but in B, we labeled three of the major volatile components, cineole, thujone, and camphor, and one minor component, MeJA. Since the internal standard shares many of the same ions as the natural MeJA, the large *trans* peak (17.9 min) in the main chromatograms represents both natural MeJA plus the internal standard. To more accurately represent the amount of natural *trans* in the samples and its proportion to *cis*, the insets depict the chromatogram of the 224 ion (the molecular ion of MeJA, not contained in the internal standard) and the 227 ion (the molecular ion of the internal standard) in the volatile collections.

(Aerts et al., 1994), insect resistance in cabbage and tobacco (Avdiushko et al., 1997), hydroperoxidase lyase activity and enhanced production of several volatile six-carbon products (Avdiushko et al., 1995), PPOs in tomato (Constabel et al., 1995), indolyl glucosinolates in *Brassica napus* (Doughty et al., 1995), vegetative storage proteins (VSPs) in soybean (Franceschi and Grimes, 1991), and furanocoumarins in *Apium graveolens* (Miksch and Boland, 1996). In *N. attenuata*, exposure to volatile MeJA differentially affected several genes involved in the regulation of both primary and secondary metabolism (Hermsmeier et al., 2001). These changes in transcription levels are likely linked to many of the well-studied JA-induced responses in

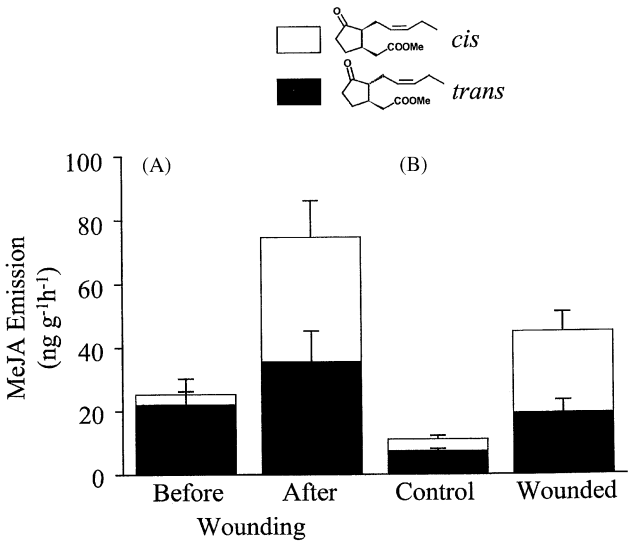


Fig. 2. A. Mean (±SEM) of the two naturally occurring epimers of MeJA [*trans* (black bars); *cis* (white bars)] trapped from the air surrounding replicate *Artemisia tridentata* (Nutt.) ssp. *tridentata* plants growing in a single population before and after a single clipping (A) and from four replicate plants from each of seven populations in which branches from each plant were either clipped or left intact (B). Quantities of volatile MeJA were trapped from air surrounding unenclosed branches for 2 h before and after clipping (A) and simultaneously from clipped and unclipped branches which were enclosed in clear 2-l, open bottom, polystyrene chambers (B). Isomers of MeJA were separated and quantified by GC–MS (methods described in Karban et al., 2000) and expressed as ng trapped h^{-1} g fresh leaf mass of the branch at the time of trapping.

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; Kahl et al., 2000; Halitschke et al., 2000; van Dam et al., 2001; Keinänen et al., in press). Clearly, exposure to volatile MeJA, at sufficiently high concentrations, can elicit the same effects as when MeJA is applied directly to the plant. However, it is unknown if the levels of MeJA that are released by one plant are sufficient to effect changes in a neighboring plant.

3. Criteria for successful signals

For a volatile to function as a plant–plant airborne signal in a natural setting, certain criteria have to be met (Firn and Jones, 1995). First, if the signal is constitutively released, independent of damage, then the signal from damaged plants must be released in significantly greater quantities for the receiver to distinguish it from the signal levels produced by an undamaged plant. Alternatively, qualitative,

rather than quantitative, changes in the signal could provide the information. Second, the signal must be received, and not only emitted, at physiologically active levels. The dilution that occurs as a signal which is released into the environment represents the most onerous challenge for a potential plant–plant signal. Clearly, the greater the distance over which a signal is to function, the greater the released amounts must be. Once these criteria are met, a compound may be considered as a potential airborne signal.

4. MeJA as a possible airborne signal: meeting the criteria

Since MeJA is released by sagebrush irrespective of damage, tobacco has a potential problem in being able to distinguish the signal from background noise. The solution may be in detecting either quantitative or qualitative changes in the MeJA coming from damaged sagebrush. Quantification of MeJA in sagebrush's volatile emission indicates that its release increases 9.2–12.4-fold after damage, with the change being proportionately greater in the putatively more biologically active *cis* isomer (3R, 7S), which increases 6.5–10.8-fold with a corresponding 2.7–1.6-fold increase of the *trans* isomer (Fig. 2A, B; Karban et al., 2000). These changes suggest that these alterations in the amount and isomeric signature of the MeJA emitted could aid tobacco in distinguishing between damaged and undamaged sagebrush and provide the information content of the volatile signal.

Secondly, sagebrush has the potential to release MeJA at a level that may still be physiologically active when it reaches the recipient. Based on the results from sampling that occurred at the time when the MeJA emission is the highest (immediately after damage; C.A. Preston, *unpublished data*), a damaged sagebrush plant releases 40–80 $ng\,g^{-1}\,h^{-1}$ of MeJA (Karban et al., 2000). Sagebrush can grow to be very large, generally 1.5 m in height (Kolb and Sperry, 1999) and may have an approximate fresh leaf mass of 1 kg. This suggests that a damaged sagebrush plant could release MeJA at a rate of 40–80 $\mu g\,h^{-1}$. Responses can be induced in *N. attenuata* with 5 μg of MeJA (unpublished results), an 8–16-fold difference between emission and reception levels. Moreover, the change in the isomeric signature may indicate that the *cis*-isomer in particular, and not the total amount of MeJA, is the active signal. The *trans*:*cis* MeJA ratio changes from approximately 80:20 in undamaged plants to approximately 40:60 in damaged plants (Fig. 2A, B; Karban et al., 2000). *Cis*-MeJA levels increase from 2–6 $ng\,g^{-1}\,h^{-1}$ to 24–48 $ng\,g^{-1}\,h^{-1}$ after damage, indicating that our average-sized sagebrush plant could emit 24–48 $\mu g\,h^{-1}$. If the *cis* isomer is the active isomer, then MeJA may be active at levels as low as 350 ng (assuming that there is 7% *cis* [based on the *trans*:*cis* ratio found in thermodynamically stable MeJA (Beale and Ward, 1998)] in the 5 μg MeJA that it takes to initiate JA-induced responses in *N. attenuata*), a 68–137-fold difference from the amount released by sagebrush and the amount necessary to induce JA-related responses in adjacently-growing tobacco.

Other factors may increase the probability that MeJA functions as an airborne signal. The wax surface on the epidermis of a plant's leaves which protects against

water loss and the entry of pathogens also helps lipophilic substances, such as MeJA, to adhere and accumulate (Bruin et al., 1995). Moreover, the height and density of the sagebrush canopy and the proximity of the *N. attenuata* plants function to reduce the surrounding volume of air and increase the likelihood that MeJA could be received at physiologically active concentrations. To test the hypothesis, we need to take into consideration the specifics of the MeJA isomers that are used for further bioassays.

5. The structure of Ja and MeJA

Jasmonate consists of a cyclopentane ring with a ketone group at the C-6 position and two chiral centers, one located at C-3 and the other at C-7, leading to a pentenyl side chain with a single double bond. There are four possible stereoisomers since the chiral center can have either an *R* or *S* absolute configuration. The mirror image isomers, (3*R*, 7*S*) and (3*S*, 7*R*), have their side chains in a *cis* orientation. These isomers are known as (+)- and (−)-7-iso-JA or (+)- and (−)-epi-JA. The enantiomers, (3*R*, 7*R*)- and (3*S*, 7*S*)-JA or (−)-JA and (+)-JA, have their side chains in the *trans* configuration. For simplicity, we will herein refer to the (3*R*, 7*S*) and (3*S*, 7*R*) isomers as *cis* and the (3*R*, 7*R*) and (3*S*, 7*S*) isomers as *trans*. The C-3 position is nonepimerizable, whereas C-7, being next to the ketone on the cyclopentanone ring, is susceptible to epimerization by enolization of the ketone, which can occur during storage or bioassays (Holbrook et al., 1997). The *cis* orientation is less stable and will epimerize to the more stable *trans* configuration, with the final *trans* : *cis* ratio of approximately 93 : 7 (Beale and Ward, 1998).

6. JA and MeJA, *in planta*

JA and MeJA, are endogenous chemical signals found in many plants. They are credited with signaling a plethora of responses following mechanical, herbivore, or pathogen stress, including the stimulation of senescence and tendril curling, inhibition of growth and germination, and the production of defenses, such as proteinase inhibitors and alkaloids (Gross and Parthier, 1994; Karban and Baldwin, 1997; Creelman and Mullet, 1997). Some details about the biosynthesis of jasmonates are still lacking. Naturally occurring jasmonates have the *R* stereochemistry at C-3 and either *S* or *R* at C-7 (Vick and Zimmerman, 1984). Following the elicitation of *Rauvolfia serpentina* cell cultures, 74% of the biosynthesized JA could be recovered as *cis*-JA when the extraction and sample preparation conditions avoided pH extremes, while JA from unelicited plant cell cultures or undamaged plants consists of an epimeric mixture of the two isomers at thermodynamic equilibrium (Mueller and Brodschelm, 1994). Thus, the immediate product of endogenous biosynthesis of JA appears to be *cis*-JA, which, in turn, is stereochemically determined by the activity of allene oxide cyclase (Ziegler et al.,

2000). Epimerization of JA within the cell may help regulate the lifetime of the active signal, altering its biological activity or affecting its turnover or conjugation (Farmer, 1994), though the rate of epimerization is relatively slow, with the half-life of the *cis* isomer being greater than three days at pH 7 and 25°C (Mueller, 1997). Also, since lipoxygenase is also jasmonate-inducible, jasmonate has the potential to autoregulate its biosynthesis by a feedback mechanism (Beale and Ward, 1998); but recent experiments with barley demonstrated that exogenous applications of MeJA or JA did not lead to increases in endogenous JA biosynthesis (Kramell et al., 2000). While there is still much to learn about the control of *trans*:*cis* ratios, evidence clearly demonstrates that organisms can regulate it.

7. Activity of JA and MeJA in exogenous applications

When applied exogenously, both free acids and methyl esters are active. The biological activities of jasmonate as well as the characteristic odor of MeJA are generally attributed to *cis*-MeJA/JA (Acree et al., 1985; Mueller, 1997; Beale and Ward, 1998). Commercially available synthetic MeJA is composed of both natural and synthetic isomers: 47.5% *trans* (3*R*, 7*R*; natural), 47.5% *trans* (3*S*, 7*S*; synthetic), 2.5% *cis* (3*R*, 7*S*; natural), and 2.5% *cis* (3*S*, 7*R*; synthetic) (Nishida et al., 1985). The *R* configuration at C-3 has higher activity in most bioassays than the *S* (Koda et al., 1992; Ward et al., 1999) but biological responses also differed in their sensitivity to each stereoisomer of JA, suggesting differential sensitivity in individual genes (Koda et al., 1992). A fungus–plant interaction appears to be mediated by *cis* form; *Botryodiplodia theobromae* Pat., a common tropical fungus, synthesizes *cis*-JA (Miersch et al., 1987), possibly mediates allelopathic interactions with its host plant (Miersch et al., 1993). If *cis*-MeJA is the active isomer, application of synthetic MeJA may overestimate the amount of MeJA required for initiating JA-responsive signaling cascades. However, studies examining the different isomers have not given clear support to the hypothesis that the *cis* form is responsible for eliciting JA-related responses. Testing the hypothesis that *cis*-JA or *cis*-MeJA is the active signal is difficult due to its instability.

8. Testing the *cis*-hypothesis

While some attempts have been made to prevent epimerization of the *cis* isomer through methylation or fluorination of the C-7 position, these chemical modifications resulted in alteration of the derivative's biological activity (Taapken et al., 1994; Koda et al., 1995, Holbrook et al., 1997). The R group at the C-3 position was found to be essential for biological activity and the addition of the methyl group at C-7 resulted in reduced activity (Holbrook et al., 1997). Alterations of the chemical structure may be unnecessary for determining MeJA's role in plant–plant communication. Following damage, sagebrush emits a greater proportion of the *cis* isomer, which is likely due to either rapid production or the release of sequestered

cis-MeJA. The *cis*-MeJA released by the sagebrush plant would naturally be epimerized in the atmosphere and it is reasonable to test the activity of experimentally-released *cis*-MeJA under the same conditions.

9. Using water entrainments to capture sagebrush volatiles

To examine tobacco's response to sagebrush's volatiles, in particular the MeJA, we entrained the volatiles in water. Briefly immersing sagebrush foliage in water elicits several volatiles that are typically found in headspace trappings of sagebrush canopies (Fig. 3A). Moreover, the increased quantity and the epimeric change in MeJA that occurs after damage can also be captured in water entrainments produced from undamaged and damaged sagebrush branches (Fig. 3B). The experimental advantage of this technique is that we no longer have to work with tobacco growing adjacent to sagebrush, but rather can deliver the sagebrush volatiles directly to distantly-growing tobacco with a high degree of replication.

To determine the importance of the MeJA epimeric ratio in damaged sagebrush emissions, we devised a method of chemically increasing the epimerization rate by adjusting the solution's pH, which effectively altered the *trans* : *cis* ratio in damaged sagebrush water entrainments to a ratio similar to that found in undamaged sagebrush water entrainments. In acidic aqueous solutions, the epimerization rate of *cis*-JA is rapid, with an approximate half-life of 17.5 h at pH 1, as compared to a half-life greater than 400 d at pH 6 (Mueller, 1997). We epimerized the damaged sagebrush water entrainment by adding hydrochloric acid and subsequently neutralizing it with potassium hydroxide. For the sagebrush water entrainment treatments used in a field experiment (see Fig. 5), the mean ($\pm$SEM) *trans* : *cis* ratios of MeJA ($n = 5$) were: undamaged sagebrush $84.59 \pm 1.62 : 13.81 \pm 1.67$, damaged sagebrush $51.48 \pm 1.30 : 47.52 \pm 1.02$, and epimerized damaged sagebrush $71.36 \pm 7.29 : 28.37 \pm 7.15$. The epimerized damaged sagebrush water entrainment had a *trans* : *cis* ratio similar to that of the undamaged sagebrush water entrainment. By comparing the effects of damaged sagebrush water entrainment and the epimerized entrainment on tobacco plants, we hope to examine the importance of the epimeric form of MeJA in eliciting JA-induced responses. However, this procedure may also alter other components in the sagebrush water entrainments and therefore needs to be tested alongside treatments that examine plant responses to the pure isomeric forms of MeJA.

10. HPLC separation of synthetic MeJA

Pure *cis*-MeJA is not available commercially and the closest available substance is "extra epi"-MeJA from Bedoukian Research (Danbury, CT, USA), which has a *trans* : *cis* ratio of approximately 80 : 20. To produce a solution with a higher proportion of the *cis* isomer, we used HPLC to separate the "extra epi"-MeJA into nearly pure *trans* and *cis* isomers (Fig. 4). Similar methods have been used to isolate

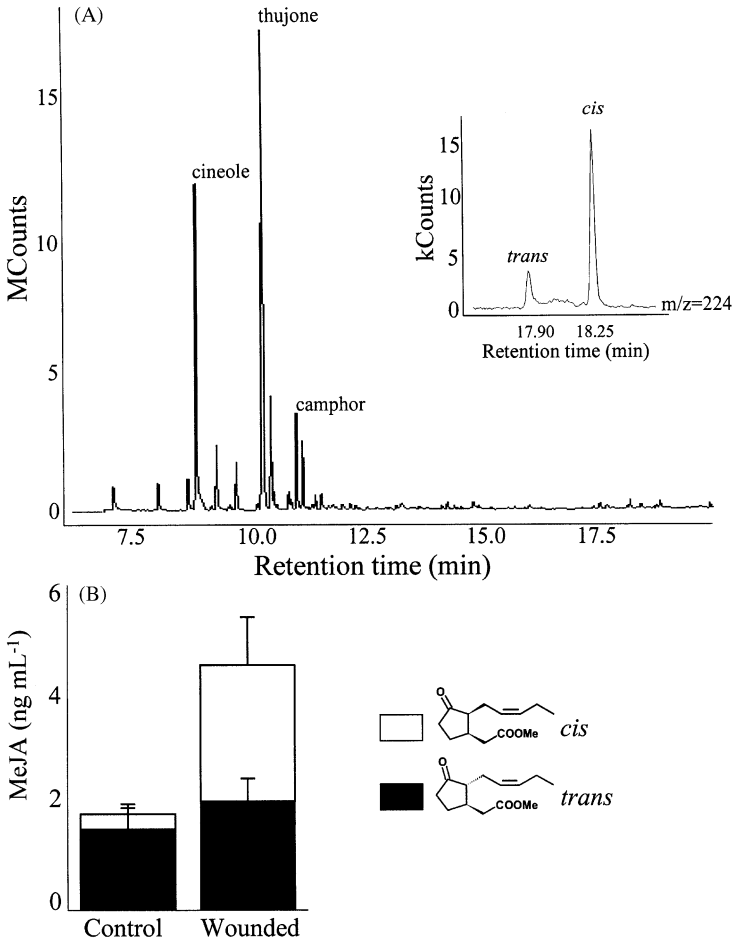


Fig. 3. A. The GC-MS chromatogram of components entrained in water from dipped *Artemisia tridentata* (Nutt.) ssp. *tridentata* foliage. Sagebrush foliage collected from a single sagebrush population in southwestern Utah was transported to the laboratory, where it was stored at −20°C. In the laboratory, 25 g of sagebrush branches were dipped into 100 ml of distilled water. To determine that sagebrush components were entrained in the water, 10 ml of this sagebrush entrainment was twice extracted with 10 ml of CH_2Cl_2, pooling the solvent layers in a separate vial. The solvent was allowed to evaporate and the extracted substances were reconstituted in 100 µl of CH_2Cl_2. The GC–MS analysis followed methods detailed in Karban et al. (2000) except that the full scan mode that was used. Though we did not attempt to identify all the compounds in the chromatogram, three major compounds (cineole, thujone, and camphor) also found in the volatile collections (Fig. 1A, B) are labeled. The peaks reflecting MeJA are too small to be seen in the total ion chromatograph. The inset shows the selected ion chromatograph of the 224 ion, depicting the MeJA isomers. B. To determine whether wounding also effected a differential release of isomers that could be entrained in water, branches from 17 sagebrush plants from a single population in southwestern Utah were either wounded or left undamaged and immediately misted with distilled water. By spraying water onto the leaves of the sagebrush, approximately 10 ml of plant surface run-off was collected from wounded and unwounded branches in glass containers and analyzed by GC–MS. To quantify MeJA, 710 ng of a triple ^{13}C-labeled MeJA internal standard was added prior to the solvent extraction. Mean amounts (±SEM) of MeJA entrained are expressed as ng ml^{-1} of *trans* (black bars) and *cis* (white bars).

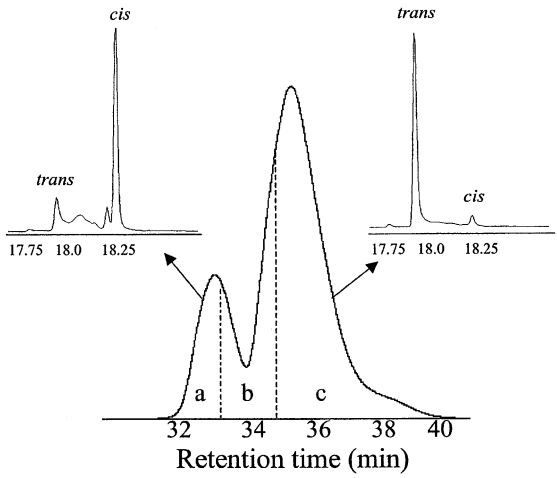


Fig. 4. "Extra epi"-MeJA (80 : 20 *trans* : *cis* epimeric ratio; Bedoukian Research, Danbury, CT, USA) was separated into fractions highly enriched in the *trans* and *cis* isomers by HPLC (center chromatogram). The isomers were separated using a μBondapak column (30 mm long, 3.9 mm i.d.; RP-18 125 Å 10 μm; Waters, Milford, MA, USA), with an isocratic mobile phase [eluent 70 : 30 (v : v) acetonitrile : water] at a flow rate of 1.5 ml min^{-1} (Varian 9012Q pump), and detected by the absorbance at 200 nm (UV detector Varian 9050). Three fractions were collected (a–c). Fractions a and c consisted of nearly pure *cis* (left inset) and *trans* (right inset), respectively, and fraction b contained a mixture of *trans* and *cis*. Each isomer was partitioned into *n*-hexane and quantified for further experiments.

the four stereoisomers of MeJA (Okamoto and Nakazawa, 1992; Nishida and Acree, 1984; Acree et al., 1985), but our method enabled only the separation of the dominant and naturally-occurring *trans* and *cis* forms (3*R*, 7*R* and 3*R*, 7*S*, respectively). By repeated collections, we acquired a *cis*-MeJA solution with an approximate *trans*:*cis* ratio of 5 : 95.

11. Delivering the MeJA to the plant

Because the *cis*-MeJA can be easily epimerized to *trans*-MeJA, we had to consider how to deliver the MeJA to the plant so that it would remain in the *cis* form and also simulate a volatile exposure. Using volatile exposures under field conditions would introduce too many uncontrolled variables to be an effective experimental treatment, particularly the release rate, the amount received by the treatment plant as well as the possible exposure of neighboring plants. Direct application of MeJA to plants using lanolin pastes (Baldwin et al., 1996) or aqueous sprays (Laue et al., 2000) effectively elicits JA-related responses while delivering reproducible quantities to individual plants with a low probability of exposing neighboring plants. Of these two techniques, aqueous sprays are more similar to volatile exposures since they can

deliver MeJA lightly over the whole plant surface, while lanolin can only be applied to a few cm^2 of leaf tissue.

In the laboratory, we determined the amount of epimerization that occurs during this procedure. An aliquot of the fractionated *cis*-MeJA stock solution in *n*-hexane was transferred to a glass bottle and after the solvent evaporated, the *cis*-MeJA was suspended in water by vigorous shaking and transferred to a glass perfume spray bottle with a plastic dispenser. Ten ml of the MeJA solution was sprayed into a glass scintillation vial 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. The *trans* : *cis* ratio after spraying was $36.9 \pm 2.5 : 63.1 \pm 2.5$. Though epimerization did occur, the proportion of *cis*-MeJA was equivalent to that released by damaged sagebrush (Fig. 2A, B). We conclude that aqueous sprays can deliver *cis*-MeJA to plants.

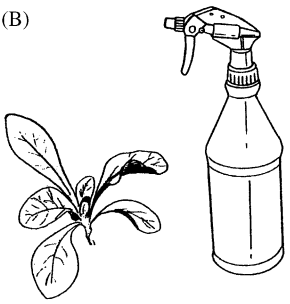
12. Testing *cis*-MeJA in the field

With methods of (1) entraining sagebrush volatiles in water which captured the change in the *trans* : *cis* ratio that occurs with damage, and (2) epimerizing sagebrush entrainments so that *trans* : *cis* ratio in the damaged sagebrush water entrainment resembles that from undamaged plants, as well as (3) having nearly pure solutions of both *trans*- and *cis*-MeJA and (4) a technique for treating plants reproducibly and independently, it was now possible to examine if MeJA, in particular *cis*-MeJA, is the active airborne signal between damaged sagebrush and neighboring tobacco plants. We designed a large field experiment (Fig. 5) using a natural population of *N. attenuata* located in a one-year-old burn in SW Utah. Treatments of undamaged and damaged sagebrush water entrainments were compared with each other and with different quantities of *cis*-MeJA (Fig. 5A). Treatment comparisons were made in triplicates, with one plant in each triplicate assigned as a control and receiving only a water spray without any chemical additions, except in one comparison (triplicate comparison 3, 10, 11), where the control was water that had undergone the same base/acid treatments as the epimerized damaged sagebrush water entrainment (Fig. 5C). Effects from applications of undamaged and damaged sagebrush water entrainments were compared with each other (comparison triplicate 1–3) and effects from the damaged sagebrush water entrainment were compared with those produced by different amounts of *cis*-MeJA (comparison triplicates 1, 3, 4; 1, 3, 5; 1, 3, 6). To directly compare the effects of the two isomers, *trans*- and *cis*-MeJA were applied within three triplicate comparisons (1, 4, 7; 1, 5, 8; 1, 6, 9). Different quantities were applied (1) to ensure an induction of JA-related responses and (2) to test if a differential response (i.e. if one isomer was more active than the other) remained consistent at different levels of induction. Finally, the damaged and the epimerized damaged sagebrush water entrainments were compared to determine if the activity of the entrainment was affected by epimerization, further testing the hypothesis that *cis*-MeJA is the active component in sagebrush emissions (comparison triplicate 3, 10, 11).

(A)

Trt. #	Treatments
1	Water
2	Undamaged Sagebrush Entrainment
3	Damaged Sagebrush Entrainment
4	5 μg *cis*-MeJA
5	10 μg *cis*-MeJA
6	50 μg *cis*-MeJA
7	5 μg *trans*-MeJA
8	10 μg *trans*-MeJA
9	50 μg *trans*-MeJA
10	Epimerised Damaged Sagebrush Entrainment
11	Epimerised Water

(B)



(C)

Comparison	Triplicate (by Trt. #)	*n*
Undamaged and Damaged Sagebrush Entrainments	1, 2, 3	14
Damaged Sagebrush Entrainment and Different Amounts of *cis*-MeJA	1, 3, 4	12
	1, 3, 5	13
	1, 3, 6	13
trans-MeJA and *cis*-MeJA, at Different Amounts	1, 4, 7	11
	1, 5, 8	12
	1, 6, 9	11
Damaged Sagebrush Entrainment, Before and After Epimerisation	1, 10, 11	13
	3, 10, 11	12

Fig. 5. Using a natural population of *N. attenuata* located in a one-year-old burned area in the Great Basin Desert of SW Utah, we compared responses of tobacco plants to applications of 11 different aqueous treatments (A). The treatments were applied as water sprays, with each plant receiving its treatment in 2 sprays, which delivered a total volume of approximately 2 ml (B). Undamaged sagebrush water entrainments (trt. #2) were produced by dipping 100 g of cut sagebrush branches (with minimal damage to the leaves during the harvest) in 300 ml of tap water, without submersing the cut stem. Damaged sagebrush water entrainments (trt. #3) were produced in a similar fashion, but the leaves were clipped prior to dipping in water. Some of the damaged sagebrush water entrainment was epimerized (trt. #10), restoring a *trans*:*cis* isomeric ratio of MeJA to levels similar to those in undamaged sagebrush water entrainments, by adding 1 ml of 1 N hydrochloric acid to 10 ml of the extract, allowing the solution to react for 24 h, and neutralizing with 5 N potassium hydroxide to pH 7. Tap water was treated similarly as a control (trt. #11). Stock dilutions of synthetic MeJA (lot 05310-0168; Aldrich, Steinheim, Germany) and the *cis*-MeJA from the HPLC separation were made using *n*-hexane. Stock solutions were aliquoted into 20-ml glass scintillation vials and the solvent was allowed to evaporate. Distilled water was added to make 5, 10, and 50 μg ml^{-1} solutions of *cis*- (trt. #'s 4, 5, 6, respectively) and *trans*- (trt. #'s 7, 8, 9, respectively) MeJA. To ensure that the MeJA was dispersed in the water, the scintillation vials were vigorously shaken before filling the spray bottles and spray bottles were shaken before each treatment application. Treatments were applied twice, with 5 days between the first and second applications. Relevant treatment comparisons were made by assigning plants to triplicates (C). At the beginning of the experiment, three plants were assigned to each treatment comparison, based on proximity to each other (all plants in a triplicate were less than 50 cm apart), equal rosette size, and absence of previous herbivore damage. One plant in each triplicate was assigned as a control and received only sprays of water without any chemical additions except in one comparison (comparison triplicate 3, 10, 11), where the control was water that underwent the same acid/base treatments as the 'epimerized' sagebrush entrainment. There were 45 replicates of each triplicate comparison. The *n* value (C) represents the number of replicates in each triplicate comparison where all treated plants had a single leaf harvested for nicotine analysis.

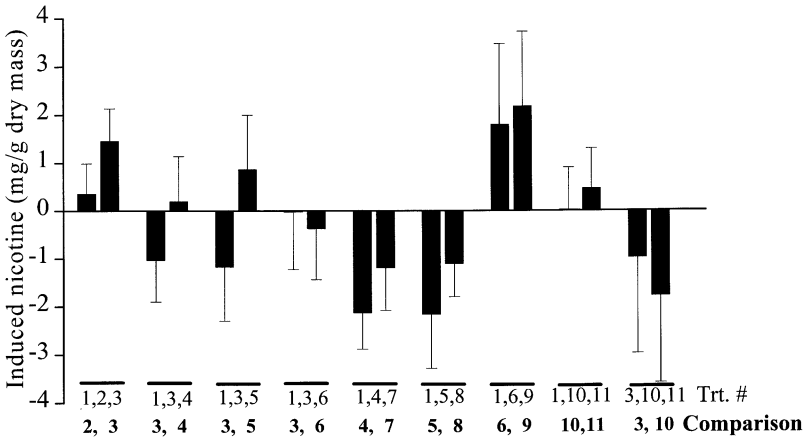


Fig. 6. Mean (±SEM) induced nicotine levels for plants sprayed with different treatments (trt. #'s defined in Fig. 5A) and grouped in triplicate comparisons (described in Fig. 5C). Induced differences were determined for each triplicate comparison by subtracting the nicotine concentration of the control plant (treatment 1 in all comparisons except 3, 10, 11, where 11 is the control) from each of the two nicotine concentrations of the treated plants.

The experiment was intended to measure the consequences of the different treatments on tobacco plants by comparing concentrations of a herbivore defense metabolite (nicotine), herbivory, survival, and lifetime viable seed production. However, an outbreak of two specialist herbivores, *Manduca quinquemaculata*, the tomato hornworm, and *M. sexta*, the tobacco hornworm, occurred. In the experiment, which included 1215 plants (45 replicates of 9 triplicate comparisons), not a single plant survived the resulting herbivore challenge which saw nearly all tobacco plants consumed at this field site consumed (C.A. Preston and A. Keßler, personal observation). Consequently, the leaves that were harvested within all the triplicate comparisons from a subset of the total number of replicates (see Fig. 5C for *n* values) prior to the herbivore outbreak provided the only results from this experiment: nicotine concentrations in all treatment comparisons (Fig. 6). The sink–source transition leaf was harvested from rosette-stage plants four days after the second treatment application, with adequate time for systemic induction of JA-related responses (Baldwin and Schmelz, 1996). The harvested leaves were placed individually into coin envelopes, transported from the field site in a portable cooler and stored at $-15°C$ before being transported to the laboratory, where they were freeze-dried prior to analysis. Nicotine analysis was performed following methods detailed in Keinänen et al. (in press), with a few modifications. From each freeze-dried leaf, 20 μg of tissue from the leaf tip was placed into a 2.0-ml screwtop microcentrifuge tube containing 0.9 g of Lysing Matrix D (Q · Biogene, Heidelberg, Germany). To each tube, 1 ml of the extraction buffer was added and the mix was homogenized for 90 s at $6.5\,m\,s^{-1}$ using the FastPrep® System (FP120; Q · Biogene, Heidelberg, Germany). The homogenate was centrifuged and the supernatant was

transferred to a 1.5-ml vial for HPLC analysis. There were no significant differences between the induced nicotine levels (nicotine concentration of each treatment minus the nicotine concentration of the control in each triplicate comparison) within any of the treatment comparisons (Fig. 6; Ps $\geqslant 0.21$). The treatments were either insufficient to induce nicotine, or alternatively, the treatments possibly induced resistance so that the control plant in each group was preferentially attacked by herbivores, subsequently inducing its nicotine levels before the leaves were harvested. An initial census of damage did not indicate that herbivore damage by orthopteran nymphs and adults or hornworm larvae was greater on the control plants, but damage by some herbivores such as weevil beetles which attack the undersides of the leaves, could remain undetected (A. Keßler, personal observation). The short time interval between the treatment applications and the *Manduca* outbreak reduced our ability to accurately assess the effects of our treatments in this field experiment.

13. Plant–plant communication: are we there yet?

In contrast to communication between animals, which is readily observed in behavioral responses of the receiver, research in inter-plant communication is hampered by the lack of quantifiable and ecologically-relevant response variables in the receivers. In short, the behavior of animals makes the study of inter-species communication a more tractable subject with animals than it is with plants. However, with the ability to measure large-scale transcriptional responses in plants with arrays, it may become easier to study relevant response variables in plants. Karban et al.'s (2000) study has provided convincing, repeatable evidence that plants transplanted within 10–15 cm of a damaged sagebrush plant exhibit increased resistance to generalist herbivores. Whether this is an example of an evolved inter-plant communication, whereby the receiver gains a fitness benefit of responding, remains to be seen. Though benefits can be discerned for the tobacco plant, it may not be always beneficial to respond to this signal. For example, if herbivores do not move from the sagebrush to the tobacco, i.e. if they are sagebrush specialists, then the induction of defenses could have negative fitness consequences for the tobacco plant, reducing its competitive ability and seed production (Baldwin, 1998; Baldwin and Hamilton III, 2000). On the other hand, if the herbivore does moves (or others subsequently arrive), the volatile-elicited defenses may benefit the tobacco by deterring herbivory (Baldwin, 1998). Moreover, it is not clear if an adequate proportion of the *N. attenuata* population grows in sufficient proximity to sagebrush to receive the volatile signal (i.e. within 10–15 cm) to have selected these responses. In the Great Basin Desert of SW Utah, between-plant distances measured at three *N. attenuata* populations located in recently burned areas bordered by sagebrush populations (containing tens of thousands of plants each) a single rosette plant was found growing closer than 30 cm to an unburned sagebrush (C.A. Preston and I.T. Baldwin, personal observation). At this distance, the active airborne signal would likely be diluted to physiologically inactive levels. In California, however, *N. attenuata* is found growing within 15 cm of established sagebrush (R. Karban,

personal communication), but such circumstances still represent the minority of *N. attenuata*'s niches. In the Karban et al. (2000) study, all of the experimental plants were transplanted next to established sagebrush plants to attain sufficient levels of replication. However, the possibility that other species may respond similarly to the emissions of sagebrush makes this an attractive system to continue the exploration of plant–plant communication (Karban, this issue).

Further investigation is needed to ascertain that MeJA, specifically the *cis* isomer, is the active component released by sagebrush. In-depth laboratory experiments, using many of the same treatments from our field experiments, will elucidate the role which *cis*-MeJA plays in mediating airborne interactions between sagebrush and neighboring tobacco.

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