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# Conversion of airborne nerolidol to DMNT emission requires additional signals in *Achyranthes bidentata*

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ABSTRACT

**DMNT biosynthesis was proposed to proceed via (*E*)-nerolidol in plants a decade ago. However, (*E*)-nerolidol function as airborne signal/substrate for in-vivo biosynthesis of DMNT remains to be investigated and the regulation of DMNT production and emission is largely unknown. We address both of these aspects using *Achyranthes bidentata* model plant in conjunction with deuterium-labeled $d_5$-(*E*)-nerolidol, headspace, GC-FID, and GC/MS-based absolute quantification approaches. We demonstrate that airborne (*E*)-nerolidol is specifically metabolized in-vivo into DMNT emission, but requires airborne VOC MeJA or predator herbivore as additional environmental signal. In addition, we provide new insight into the complex regulation underlying DMNT emission, and highlight the importance of studying multiple environmental factors on emission patterns of plant VOCs and their mechanistic regulation.**


## 1. Introduction

Plants emit a blend of volatile organic compounds (VOCs) in response to environmental stimuli, including predators and pathogens, as a part of its elaborated self-defense mechanisms [1–5]. Herbivores are well-studied natural predators with respect to plant defense response and VOC emissions [5–9]. Emitted VOC types and their amounts vary from plant to plant and under environmental conditions [5,6,10–13]. Depending on the plant species, dominant terpene VOCs are (*E*)-β-ocimene, linalool, (*E*)-α-bergamotene, (*E*)-β-farnesene, and β-caryophyllene [3,5,10,14]. In addition to these terpenes, plants emit an irregular $C_{11}$ terpene VOC, known as homoterpene (3*E*)-4,8-dimethy-1,3,7-nonatriene (DMNT) [5,12,15,16]. The biosynthetic pathways are basically defined for most VOCs, but the underlying regulation for their production and emission remains largely unknown, including DMNT.

The DMNT biosynthesis has been proposed to occur via nerolidol as an immediate precursor (Fig. 1A) [17–20]. Nerolidol is a sesquiterpene alcohol and a VOC constituent in the headspace of several flowers [21] and plants. Recently, the enzyme catalyzing nerolidol into DMNT was identified and characterized [19] (Fig. 1A). However, unlike DMNT, which is one of the defensive VOCs involved in plant-insect interaction [15], nerolidol itself is not considered as an active compound in plant defensive systems. Furthermore, two questions remain to be answered: (i) does nerolidol function as an airborne signal? and (ii) is the airborne nerolidol metabolized into DMNT?

Here, we have addressed the above questions using *Achyranthes bidentata* (hereafter called Achyranthes) plant as a model system [13] in combination with deuterium-labeled $d_5$-(*E*)-nerolidol (Fig. 1B), VOC collections in headspace, GC-FID, and GC/MS-based absolute quantification approaches. The $d_5$-(*E*)-nerolidol was used to track its airborne nature and in-vivo metabolic product in Achyranthes. Novel findings presented in this study demonstrate nerolidol as an airborne VOC and its efficient in-vivo metabolic conversion specifically into DMNT emission, providing a better understanding on mechanistic regulation of the DMNT emission.

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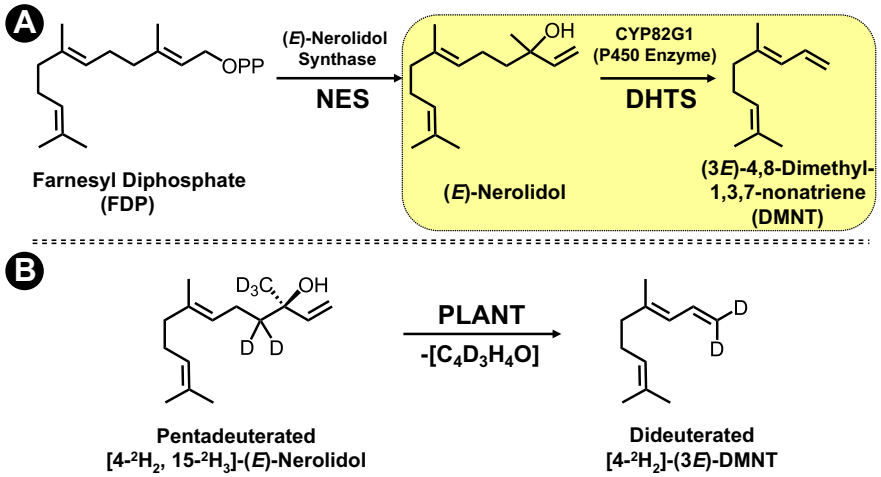


**Fig. 1.** Proposed biosynthesis pathway of DMNT and synthesized $d_5$-(*E*)-nerolidol. (A) The biosynthesis pathway proposed for DMNT in plants via (*E*)-nerolidol. (B) The structure of synthesized $d_5$-(*E*)-nerolidol and it metabolic conversion to $d_2$-DMNT in Achyranthes plant. The CYP82G1 is a unique enzyme in the plant CYP82 family of cytochrome P450 monooxygenase (P450) with a function as DMNT/TMTT (C16-homoterpene (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene) homoterpene synthase. Details are mentioned in the text. Abbreviations: NES, (*E*)-nerolidol synthase; and DHTS, DMNT homoterpene synthase.

## 2. Materials and methods

### 2.1. Chemicals

*Trans*-methyl jasmonate (*trans*-MeJA) and nerolidol (mixture of *E*-/*Z*-isomers) were purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA). The penta-deuterated [4-$^2H_2$, 15-$^2H_3$]-(*E*)-nerolidol was synthesized as reported previously [22]. All other chemicals were of analytical grade.

### 2.2. Plant materials and biological replication

Seeds of Achyranthes plant (*Achyranthes bidentata* var. *tomentosa*) were collected at Tenno Katagami (Akita, Japan), and grown under the conditions described previously [13]. Shoots of Achyranthes with fully opened four leaves (about 12-cm-tall) were also used as experimental materials. A total of five independent biological replications were performed for each experiment.

### 2.3. Exposure of Achyranthes plant or its shoot to airborne VOCs

MeJA and/or $d_5$-(*E*)-nerolidol (2 mg) was applied to a paper disk (5-mm-diameter). The paper disk was then hung in the headspace of a glass container (1 L) having whole Achyranthes plant or its shoot (Supplementary Fig. 1).

### 2.4. Larvae and herbivore treatment

Eggs of *Spodoptera litura*, kindly provided by Biological Research Laboratories (Nissan Chemical Industries Ltd., Shiraoka, Saitama, Japan), were reared on an artificial wheat-germ diet (Insecta LF-S; NOSAN Corporation, Yokohama, Japan) at 24 °C. Three sets of experiments comprising Achyranthes shoots with opened leaves and whole plant were designed for herbivory treatment.

#### 2.4.1. Experiment design 1: application of larval regurgitant to leaves

Larvae were transferred to feed on Achyranthes leaves for two days before regurgitant collection. Larvae were placed in a glass Petri dish and a capillary glass tube (1.0-mm-diameter) was introduced into the mouth of a larva. Regurgitant was aspirated and trapped in a glass bottle (5 mL). Collected regurgitant (10 μL) in the trap was applied on Achyranthes leaf within the assigned mark (8 marks per leaf) for 24 h; marks were made by pressing the leaf with the Pasteur pipette (1.5-mm-diameter). These leaves with larvae regurgitant were also exposed to airborne $d_5$-(*E*)-nerolidol for 24 h to examine their combined effect on DMNT and emission of other VOCs.

#### 2.4.2. Experiment design 2: application of sixth-instar larvae alone or with airborne $d_5$-(*E*)-nerolidol

Five sixth-instar larvae were placed on leaves of Achyranthes shoot and allowed to feed for 30 min. To expose the fed leaves to airborne $d_5$-(*E*)-nerolidol for 24 h, stems of the shoots were transferred inside a glass container containing 10 mL of water (Supplementary Fig. 1).

#### 2.4.3. Experiment design 3: application of sixth-instar larvae on leaves of whole plant alone or with airborne $d_5$-(*E*)-nerolidol

Three-week-old Achyranthes plants (15- to 20-cm-tall) were transplanted together with roots and soil to plastic pots (6-cm-diameter) and grown further for two weeks. Ten sixth-instar larvae were placed on leaves and allowed to feed for 2 h, followed by transfer of the plants to a glass container for exposure with airborne $d_5$-(*E*)-nerolidol for 24 h.

### 2.5. VOC collections and analyses

VOCs in the headspace of glass container were collected using solid-phase micro-extraction (SPME) fibers (65 μm Stable Flex PDMS/DVB, Supelco Co., PA, USA) and analyzed by GC-FID (Shimadzu GC-2010, Kyoto, Japan) or GC-MS (PerkinElmer Turbo Mass, Shelton, CT, USA) as described previously [13].

## 3. Results

### 3.1. DMNT emission in the presence of nerolidol, methyl jasmonate, and herbivore

To investigate whether airborne nerolidol is metabolized into DMNT emission, Achyranthes leaves were exposed to airborne $d_5$-(*E*)-nerolidol by supplying it to the headspace of glass container (Supplementary Fig. 1). Amounts of DMNT and other VOCs emitted in the headspace were quantified by GC-FID. Neither DMNT nor other VOCs were detected from exposed or unexposed leaves (data

not shown). Previously, it was shown that Achyranthes leaves emit DMNT as a minor VOC in response to airborne MeJA [13]. Given this report and MeJA as a naturally occurring and well-studied VOC, Achyranthes leaves were exposed to airborne MeJA alone or in combination with airborne $d_5$-(*E*)-nerolidol. As expected, DMNT emission was detected (marked by broken rectangle) along with the other nine VOCs from the MeJA-exposed leaves (Fig. 2A, marked by broken rectangle and C). Importantly, a 3.4-fold increase was found only in the DMNT emission from leaves exposed to a combination of MeJA and $d_5$-(*E*)-nerolidol airborne signals (Fig. 2A and D) compared to that of airborne MeJA alone (Fig. 2A and C); emission patterns of the other nine VOCs were dramatically suppressed over airborne MeJA (Fig. 2A). Identified 13 VOCs were as indicated in the total ion chromatograms: green leaf volatiles [GLVs: methyl 2-(*E*)-hexenoate (1), 3-(*Z*)-hexenyl acetate (2), and 2-(*E*)-hexenyl acetate (3)]; monoterpenes [(*E*)-β-ocimene (4) and linalool (5)]; homoterpene [DMNT (6)]; and sesquiterpenes [7 through 13: (*E*)-β-caryophyllene, (*E*)-α-bergamotene, sesquisabinene, (*E*)-β-farnesene, α-humulene, (*E*,*E*)-α-farnesene, and β-bisabolene, respectively] (Fig. 2).

Next, to know whether molecular signal(s) other than the VOC MeJA supports metabolization of nerolidol to DMNT emission, a natural predator herbivore and its larval regurgitant were used. To note, a large body of evidence has revealed that herbivore secrete elicitors including fatty acid-amino acid conjugate (FAC), which trigger highly complex defense response and VOC emissions in plants, including DMNT [5–7,12,23]. Leaves were first exposed to larval regurgitant of *S. litura* alone or with airborne $d_5$-(*E*)-nerolidol (Fig. 2B). Larval regurgitant alone elicited the emission of DMNT (6) as a minor VOC versus the dominant (*E*)-β-farnesene (10) and (*E*,*E*)-α-farnesene (12) VOCs (Fig. 2B). However, larval regurgitant together with airborne $d_5$-(*E*)-nerolidol caused a 6.4-fold increase specifically in the DMNT emission compared to larva regurgitant. Again, no increase in emission of the other nine VOCs was found, but rather their emissions were suppressed.

Similarly, *S. litura* larvae feeding on leaves for 30 min alone elicited DMNT emission as a minor VOC versus the major (*E*)-β-ocimene (4), (*E*)-β-farnesene (10), and (*E*,*E*)-α-farnesene (12) VOCs (Fig. 3A; marked by broken rectangle). The leaf area chewed up by larvae of *S. litura* was recorded to be about 13 mg per leaf of its 3 g fresh weight, suggesting that even a minor damage by *S. litura* larvae is sufficient to trigger the DMNT emission. However, when these chewed leaves were exposed to airborne $d_5$-(*E*)-nerolidol, the magnitude of DMNT emission was dramatic with up to

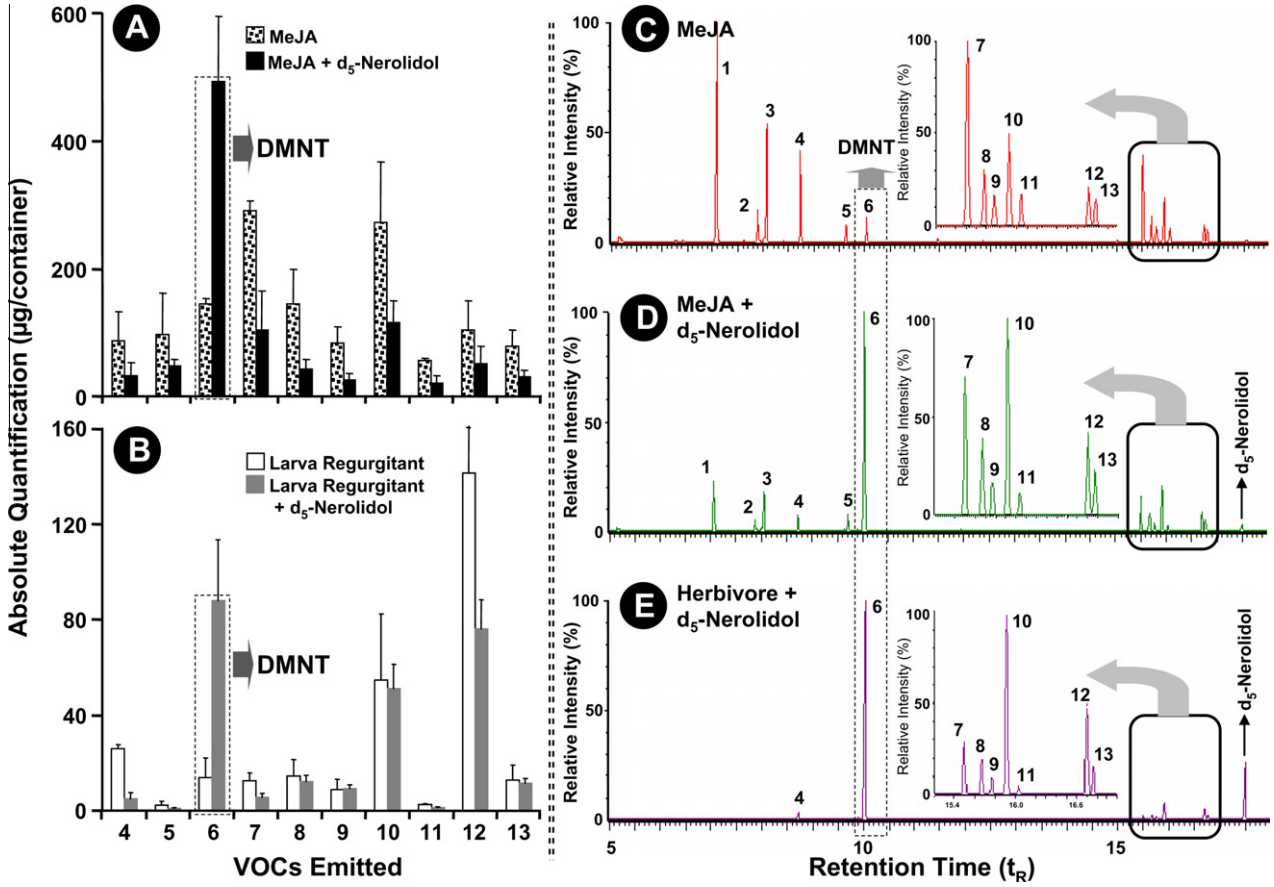


**Fig. 2.** Absolute quantification of VOCs emitted by Achyranthes leaves upon exposure to airborne MeJA, airborne $d_5$-(*E*)-nerolidol, larval regurgitant of herbivore *S. litura* or their simultaneous combinations at 24 h. (A) Exposure to MeJA alone or with $d_5$-(*E*)-nerolidol. (B) Exposure to larval regurgitant alone or with $d_5$-(*E*)-nerolidol. (C), (D), and (E) are total ion chromatograms ($t_R$ 5.0–18 min) of GC/MS-analyzed VOCs at 24 h emitted by Achyranthes leaves upon exposure to MeJA, MeJA plus $d_5$-(*E*)-nerolidol, or herbivore plus $d_5$-(*E*)-nerolidol, respectively. A segment of chromatogram ($t_R$ 15.5–17.0 min; rectangular with solid line) was enlarged (inset) to clearly present peaks 7 through 13 and to detect basal peaks, if any. Assigned numbers in (A) through (E) correspond to: 4, (*E*)-β-ocimene ($t_R$ = 8.69); 5, linalool ($t_R$ = 9.68); 6, DMNT ($t_R$ = 10.00); 7, (*E*)-β-caryophyllene ($t_R$ = 15.51); 8, (*E*)-α-bergamotene ($t_R$ = 15.68); 9, sesquisabinene ($t_R$ = 15.78); 10, (*E*)-β-farnesene ($t_R$ = 15.93); 11, α-humulene ($t_R$ = 16.05); 12, (*E*,*E*)-α-farnesene ($t_R$ = 16.71); and 13, β-bisabolene ($t_R$ = 16.78). Other prominent peaks 1, 2, and 3 were the VOCs identified as methyl 2-(*E*)-hexenoate ($t_R$ = 7.02), 3-(*Z*)-hexenyl acetate ($t_R$ = 7.84), and 2-(*E*)-hexenyl acetate ($t_R$ = 8.01), respectively. In chromatograms (D) and (E), peak for $d_5$-(*E*)-nerolidol ($t_R$ = 17.48) was also detected and marked. Concerted action of airborne $d_5$-(*E*)-nerolidol on dramatically enhanced emission of DMNT is highlighted with rectangles (broken line). None of these VOCs was detected in the headspace samples of control Achyranthes leaves or exposed to only airborne $d_5$-(*E*)-nerolidol. In order to quantify the VOCs amounts, GC-FID analysis was carried out. The absolute amount of each VOC was calculated using *n*-octane as an internal standard. Data shown are derived from five independent biological replicates and were used to calculate means and SE. Error bars represent standard errors.

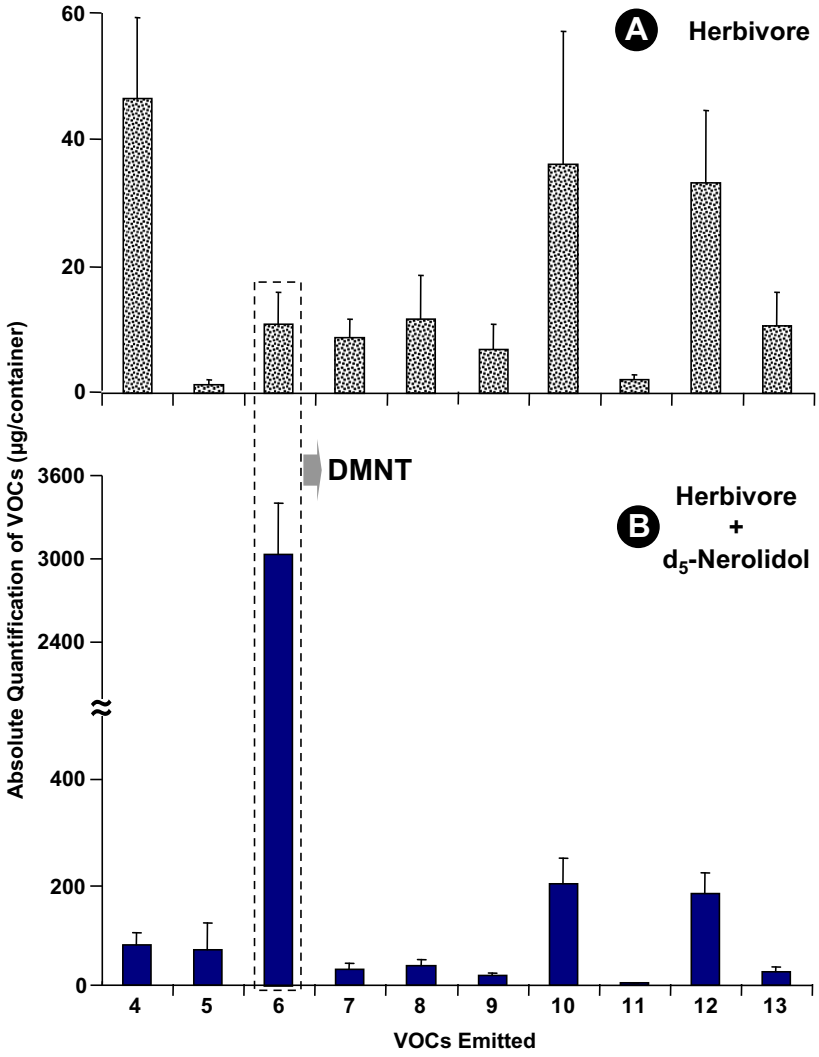


**Fig. 3.** Absolute quantification of VOCs emitted by Achyranthes leaves upon herbivore *S. litura* larvae feeding followed by exposure to airborne $d_5$-(*E*)-nerolidol. (A) Effect of individual *S. litura* larvae feeding on leaves. (B) Effects of sequential combination of *S. litura* larvae feeding of leaves for 30 min and airborne $d_5$-(*E*)-nerolidol exposure for 24 h. Rest is the same as described in Fig. 2, including the assigned numbers (4 through 13) for emitted and identified VOCs.

290-fold increase as the major VOC in comparison with herbivore *S. litura*-elicitation alone (Fig. 3B and Fig. 2E). Moreover, this sequential combination showed additive effect on emission patterns of the other nine VOCs. It is likely that signals were triggered by larvae chewing the leaves. These signals may facilitate efficient metabolism of airborne nerolidol into DMNT biosynthesis and emission.

Taken together, the findings demonstrate that airborne nerolidol is metabolized to biosynthesize primarily DMNT and its emission in Achyranthes, but requires yet unknown factor(s) or molecular signal(s) derived from airborne VOC MeJA, larval regurgitant of *S. litura*, or *S. litura* larvae. Moreover, increase in DMNT emission affects considerably the emission patterns of at least the nine VOCs (4–5 and 7–13 in Figs. 2 and 3).

### 3.2. Plant conversion of airborne $d_5$-(*E*)-nerolidol into DMNT emission

To extend the above finding at the whole plant level, experiments of *S. litura* larvae feeding and airborne MeJA were separately conducted again with airborne $d_5$-(*E*)-nerolidol (Supplementary Fig. 2). Leaves chewed by the larvae *S. litura* for 30 min are shown in Supplementary Fig. 2B. The larvae chewed up to 4% of the total leaf area. The measured weight of leaf was on average 2.985 ± 0.645 g, and larvae fed 12 ± 7 mg of the leaf during the feeding period. As shown in Fig. 4, the DMNT dominant chromatograms were again obtained in either experimental condition (A and B). Hence, airborne nerolidol is also metabolized in-vivo specifically into DMNT and emitted at the whole plant level.

### 3.3. De-novo synthesized $d_2$-DMNT derives from airborne $d_5$-(*E*)-nerolidol

The GC/MS chromatograms in Figs. 2 and 4 showed the DMNT peaks at 10 min. A typical MS spectrum is shown for DMNT emitted by leaves in response to sequential combination of *S. litura* larvae feeding and airborne $d_5$-(*E*)-nerolidol (Fig. 5). In the MS spectrum, characteristic fragment ions were found at *m*/*z* 152, 137, 109, 83, and 69. These fragmentation patterns match well with a previous report on the $d_2$-DMNT fragmentation [22]. A simultaneous combination of airborne MeJA and airborne $d_5$-(*E*)-nerolidol also showed exactly the same mass spectrum and fragmentation patterns (data not shown). The expanded chromatograms within an inset in Fig. 5 show the relative intensity of deuterated versus undeuterated DMNT by monitoring at *m*/*z* 152 and *m*/*z* 150, respectively. Their calculated peak areas revealed that $d_2$-DMNT amount was about 20 times higher than DMNT in case of MeJA plus $d_5$-(*E*)-nerolidol airborne signals (Fig. 5B) or *S. litura* larvae plus airborne $d_5$-(*E*)-nerolidol (Fig. 5C). No $d_2$-DMNT was de-

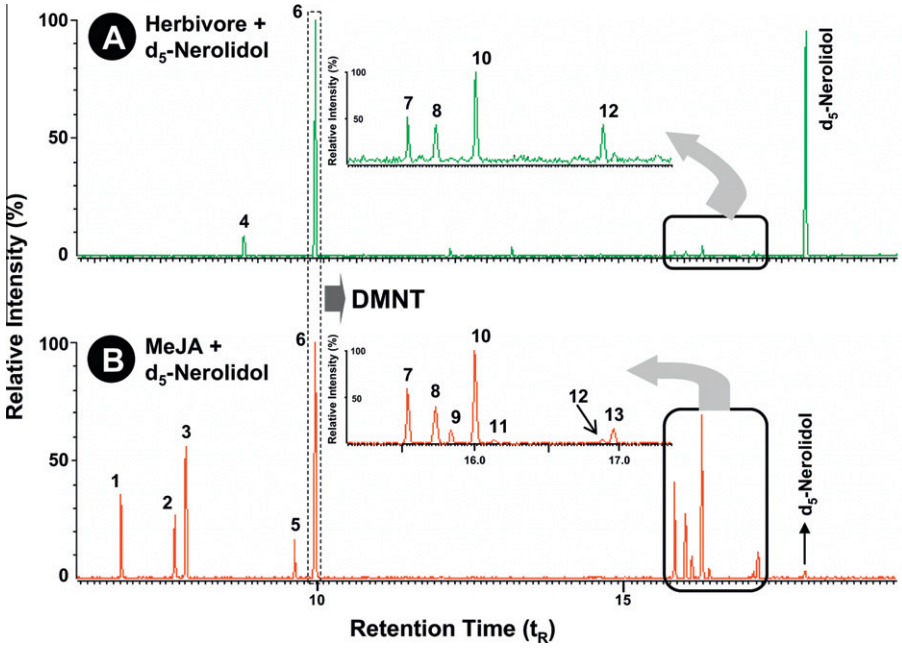


**Fig. 4.** GC/MS chromatograms of the VOCs emitted by Achyranthes (whole plant). (A) sequential exposure to *S. litura* larvae feeding and airborne $d_5$-(*E*)-nerolidol and (B) simultaneous exposure to MeJA and $d_5$-(*E*)-nerolidol airborne signals at 24 h. Peaks numbering in the chromatograms correspond to those assigned in Fig. 2 for total ion chromatograms and quantified VOCs. Rest is the same as described in Fig. 2.

tected with airborne MeJA alone (Fig. 5A). Together, our results demonstrate that the DMNT peak at 10 min in the chromatograms mainly consists of $d_2$-DMNT, which is de-novo biosynthesized from airborne $d_5$-(*E*)-nerolidol.

## 4. Discussion

### 4.1. (E)-Nerolidol is an airborne signal for DMNT biosynthesis

Plant-emitted VOCs are known to mediate intra- and inter-plant communications [1,24–27]. However, a few of them have been experimentally shown to function as airborne signal for such communications. MeJA is one such VOC that elicits other VOC emissions and defense-related genes/proteins [1,13,25,27]. MeJA was recently demonstrated as an airborne VOC [13]. It was shown that airborne MeJA is converted essentially to JA and JA-Ile in the receiver Achyranthes plant, leading to VOC emissions and induction of de-novo jasmonate production [13,24]. Evidence provided in this study adds up (*E*)-nerolidol as another novel airborne signal for biosynthesis and emission of DMNT in Achyranthes. But unlike airborne MeJA, (*E*)-nerolidol requires additional environmental signal(s) to metabolize itself into DMNT and to elicit altered pattern of VOC emissions.

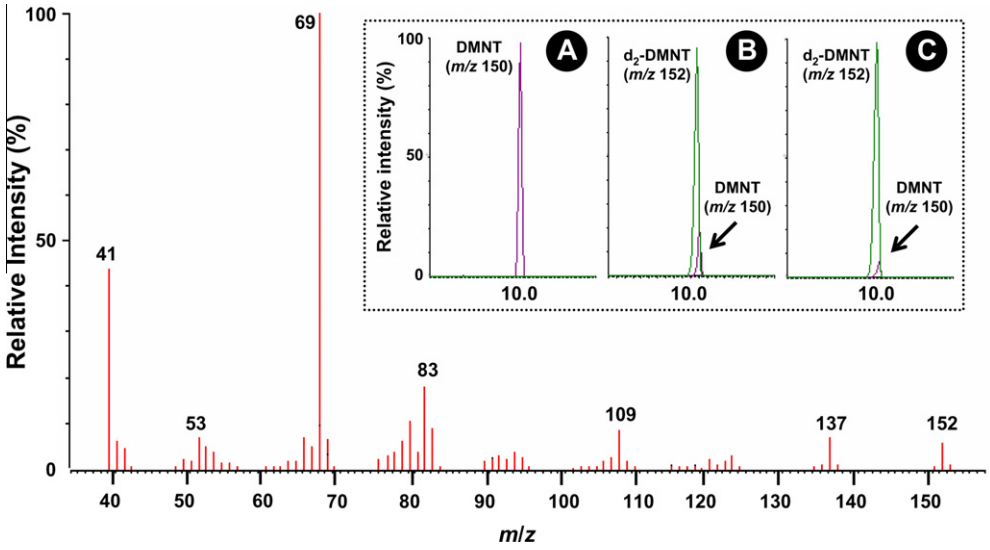


**Fig. 5.** MS spectrum of $d_2$-DMNT emitted by Achyranthes leaves exposed to *S. litura* larvae and then to airborne $d_5$-(*E*)-nerolidol. In an inset, expanded mass chromatograms show selected ion chromatograms at *m*/*z* 150 and *m*/*z* 152 analyzing molecular ions of DMNT and $d_2$-DMNT, respectively. Treatments are: (A) airborne MeJA; (B) airborne MeJA plus airborne $d_5$-(*E*)-nerolidol; and (C) *S. litura* larvae plus airborne $d_5$-(*E*)-nerolidol.

### 4.2. Underlying complex regulation of DMNT: an emerging view

Available reports suggest that DMNT production/emission is differentially regulated in plants in response to various environmental factors [3,5,12]. For example, it is a dominant VOC of spider mites-infested lima bean [15], but a minor VOC of maize [16], *Arabidopsis* [19], and Achyranthes-exposed to airborne MeJA [13]. These studies led to propose the importance of (*E*)-nerolidol synthase (NES) over DMNT homoterpene synthase (DHTS) [15] or both [16] in regulation of DMNT biosynthesis (Fig. 1A).

Our new findings integrated to a proposed model imply that induction or activation of DHTS enzyme by MeJA or herbivore as a second environmental factor appears to be critical to de-novo metabolize the airborne (*E*)-nerolidol into DMNT and to influence the patterns of VOC emissions (Supplementary Fig. 3B and C). This statement is based on the fact that airborne (*E*)-nerolidol, as a single factor, fails to metabolize into DMNT (Supplementary Fig. 3A). Indeed, DHTS enzyme was recently shown to be induced by herbivory [19]. Our view is in agreement with the assumption that only NES induction is not sufficient for significant increase in the DMNT emission [16] and hence, plants must receive the exogenous (*E*)-nerolidol to perform this task. Given this scenario, it is highly likely that plants receive airborne (*E*)-nerolidol to bypass its de-novo biosynthesis.

### 4.3. Multiple environmental factors influence volatile emission patterns

In nature, plants rarely experience a single environmental stimulus, but rather are exposed to multiple stimuli simultaneously or sequentially. Although individual (single) stress has been well studied for VOC emissions, very few studies have been performed to quantitatively profile VOC emissions under multiple factors in the laboratory or the field [3,5]. Two or more stress factors could have additive, opposing, or priority effects on the pattern of VOC emissions. As discussed in the above sections, airborne (*E*)-nerolidol, as a single factor, was not sufficient to be metabolized into DMNT. But when airborne (*E*)-nerolidol is combined with the second stress factor [MeJA, larval regurgitant (containing elicitors including FAC), or herbivore larvae], the airborne nerolidol was efficiently metabolized. Moreover, combination of airborne nerolidol with MeJA or larvae regurgitant as abiotic factors had an attenuation effect on VOC emissions except for DMNT (Fig. 2), whereas with biotic factor as herbivore larvae showed an additive effect on VOC emissions (Fig. 3). In line with this finding, the additive effects of spider mites (biotic) and ozone (abiotic) or herbivore damage (biotic) and pathogen infection (biotic) has been reported on VOC emissions, including DMNT [28,29]. These findings demonstrate the importance of studying multiple factors to discover new patterns and functions of plant VOCs.

## 5. Concluding remarks

In conclusion, our experimental approach demonstrates nerolidol as a novel airborne signal for de-novo biosynthesis of DMNT in Achyranthes, suggesting the importance of multiple environmental stimuli in better understanding their functions and patterns of VOC emissions. A similar approach could be applicable to large number of uninvestigated VOCs (such as GLVs, terpenes, and MeSA) to know their airborne functions and finding new pathways of metabolic conversion/biosynthesis and crosstalk.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.04.026.

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