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Exogenous ACC enhances volatiles production mediated by jasmonic acid in lima bean leaves

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Abstract We report the synergistic effects of exogenous 1-aminocyclopropane-1-carboxylic acid (ACC) and jasmonic acid (JA) on production of induced volatiles by excised lima bean leaves. Application of ACC alone to leaves induced trace amounts of volatiles. ACC positively affected three JA-induced volatiles, (*E*)- and (*Z*)-β-ocimene, and (*Z*)-3-hexenyl acetate. The ethylene inhibitor, silver thiosulfate, inhibited the production of these compounds. The results suggest synergistic effects of JA and ACC on inducible volatile production by lima bean leaves. Furthermore, lima bean leaves treated with JA plus ACC became more attractive to predatory mites, *Phytoseiulus persimilis*, than those treated with JA alone.

Key words: 1-Aminocyclopropane-1-carboxylic acid; Ethylene; Jasmonic acid; Herbivore-induced plant volatile; *Phytoseiulus persimilis*; *Phaseolus lunatus*

1. Introduction

Plants have developed various systems for direct and indirect defenses against attacks by different herbivore species. Direct defenses include production of toxins, repellents, and/or digestibility reducers constitutively or in response to herbivore attacks [1–3]. In contrast, indirect defense enhances the effectiveness of carnivorous natural enemies of herbivores. Plants emit so-called herbivore-induced plant volatiles (HIPVs), which attract carnivorous natural enemies of herbivores [4–6]. Such volatiles serve as a cue to direct predators into the vicinity of their prey.

Despite the accumulation of ecological studies on plant–carnivore interactions through HIPVs, few studies have been devoted to the molecular mechanisms of HIPVs production. Accumulation of jasmonic acid (JA) is elicited in higher plants in response to herbivore attack [3,7,8]. In response to exogenous JA, lima bean leaves release volatiles (JA-induced volatiles). The JA-induced volatiles are similar in their chemical composition to volatiles from lima bean leaves infested with *Tetranychus urticae* [9], and both types of volatiles are attractive to predatory mites, *Phytoseiulus persimilis* [10]. These findings suggest that the JA-dependent signaling pathway might be involved in the production of HIPVs [10–12]. Recently, it has been suggested that not only JA but also salicylic acid (SA) is involved in the emission of HIPVs from lima bean leaves infested with *T. urticae* [13]. In general, SA is considered an important mediator of plant resistance to pathogenesis, but not herbivore infestation [14]. The role of such signaling molecules for volatile production in plants thus seems to be more multifunctional than initially expected.

We recently demonstrated that gene transcripts are comprehensively induced in lima bean leaves in response to *T. urticae* infestation [15]. Three of the fragments detected were assumed to be from gene transcripts for *S*-adenosylmethionine synthetase, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, and ACC oxidase. These genes encode enzymes that catalyze the biosynthesis of ethylene [16], one of the signals that mediates direct defence responses against mechanical injury [17], pathogens [18], and herbivory [3]. In our previous study, we observed active ethylene production by lima bean plants infested with *T. urticae* [19]. Here, we demonstrated that ACC, a precursor of ethylene, enhances the release of JA-induced volatiles from lima bean leaves. Moreover, exogenous application of both JA and ACC to lima bean leaves attracted more predatory mites of *T. urticae* than those treated with JA alone. We infer that both ethylene and JA are synergistically involved in the signal cascade(s) of *T. urticae*-induced volatile production.

2. Materials and methods

2.1. Plants and mites

Lima bean plants (*Phaseolus lunatus* cv. Sieva) were reared in plastic pots (diameter = 12 cm, depth = 10 cm) in a climate-controlled room (25 ± 2°C, 16L-8D). Two to 3-week-old seedlings with fully developed primary leaves were utilized for the experiments. Herbivorous mites (*T. urticae*) and carnivorous mites (*P. persimilis*) were obtained from a laboratory-maintained culture. *T. urticae* were reared on kidney bean plants (*Phaseolus vulgaris* cv. Nagauzuramame) and *P. persimilis* were reared on *T. urticae* living on kidney bean plants in the room described above.

2.2. Applications of chemicals to lima bean leaves

For chemical treatments, a detached primary lima bean leaf, includ-

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Abbreviations: JA, jasmonic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; STS, silver thiosulfate; DMNT, (*E*)-4,8-dimethyl-1,3,7-nonatriene; SA, salicylic acid; HIPV, herbivore-induced plant volatile

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ing its petiole, was placed in a glass vial (6 ml) filled with aqueous solution containing JA (0.3 mM; Wako, Kyoto, Japan) and ACC (0.1–10 mM; Wako). We used silver thiosulfate (STS, 0.5 mM; Palace Chemical Co., Ltd., Yokohama, Japan) as an inhibitor. Experiments were conducted in the climate room (25 ± 2°C, 50–70% R.H, 16L-8D; 2150 lux, fluorescent lights) and the leaves were treated for 24 h.

2.3. Chemical analysis of volatile compounds

Two leaves for each treatment were placed in a lidded glass bottle (2 l) for 30 min. We collected the volatiles in the bottle using 100 mg Tenax-TA resin (20/35 mesh; GL Science, Japan) packed in a glass tube (3.0 mm i.d. × 160 mm length) for 1 h (100 ml/min). The volatiles were eluted with 2 ml diethyl ether. *n*-Eicosane (0.5 μg) was added to the eluate as an internal standard. The eluate was concentrated with a stream of gaseous N_2 and injected into the injection port (250°C) of a gas chromatography–mass spectrometer (GC: Hewlett Packard 6890 with an HP-5MS capillary column: 0.25 mm i.d., 30 m length, 0.25 μm film thickness, MS: Hewlett Packard 5973 mass selective detector, 70 eV). The GC oven temperature was programmed to rise from 40°C (5 min hold) to 280°C at 15°C/min. JA-induced volatiles were defined as the volatiles that were found in the headspace of treated leaves but were not found (or found in trace amounts) in that of untreated leaves. The chemical structure of each compound was elucidated by comparison of the mass spectra and the retention time with those of authentic chemical samples. (*E*)-β-Ocimene, (*Z*)-β-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) were generously provided by W. Boland (Max Planck Institute of Chemical Ecology). (*Z*)-3-Hexenyl acetate, linalool, and limonene were purchased from Wako Chemical Company. The chemical structures of two compounds (α-copaene and β-caryophyllene) were estimated by comparison with data in the mass spectra database (Wiley 275 version C. 00. 00, Wiley, New York, USA) and that of the Laboratory of Ecological Information, Kyoto University.

2.4. Bioassay using Y-tube olfactometer

A Y-tube olfactometer was used to test the responses of predatory mites (*P. persimilis*) toward volatiles from five leaves treated with compounds for 24 h (for details of the olfactometer, see [20]). Air containing volatiles from treated or control leaves was sent at 2.5 l/min into the olfactometer. Adult female mites were starved for 24 h before the bioassays were initiated and were then individually positioned at the starting point of an iron Y-shaped wire that was fixed at the center of a Y-tube. When a mite reached the end of either arm in the olfactometer, we judged that the mite made a final choice. In contrast, when a mite did not make a final choice within 5 min, we recorded it as no choice. We switched the arm after every five assays. Olfactometer tests were conducted four times with new odor sources. The data obtained were subjected to a χ^2 test. The null hypothesis was that predators had a 50:50 distribution across the two odor sources.

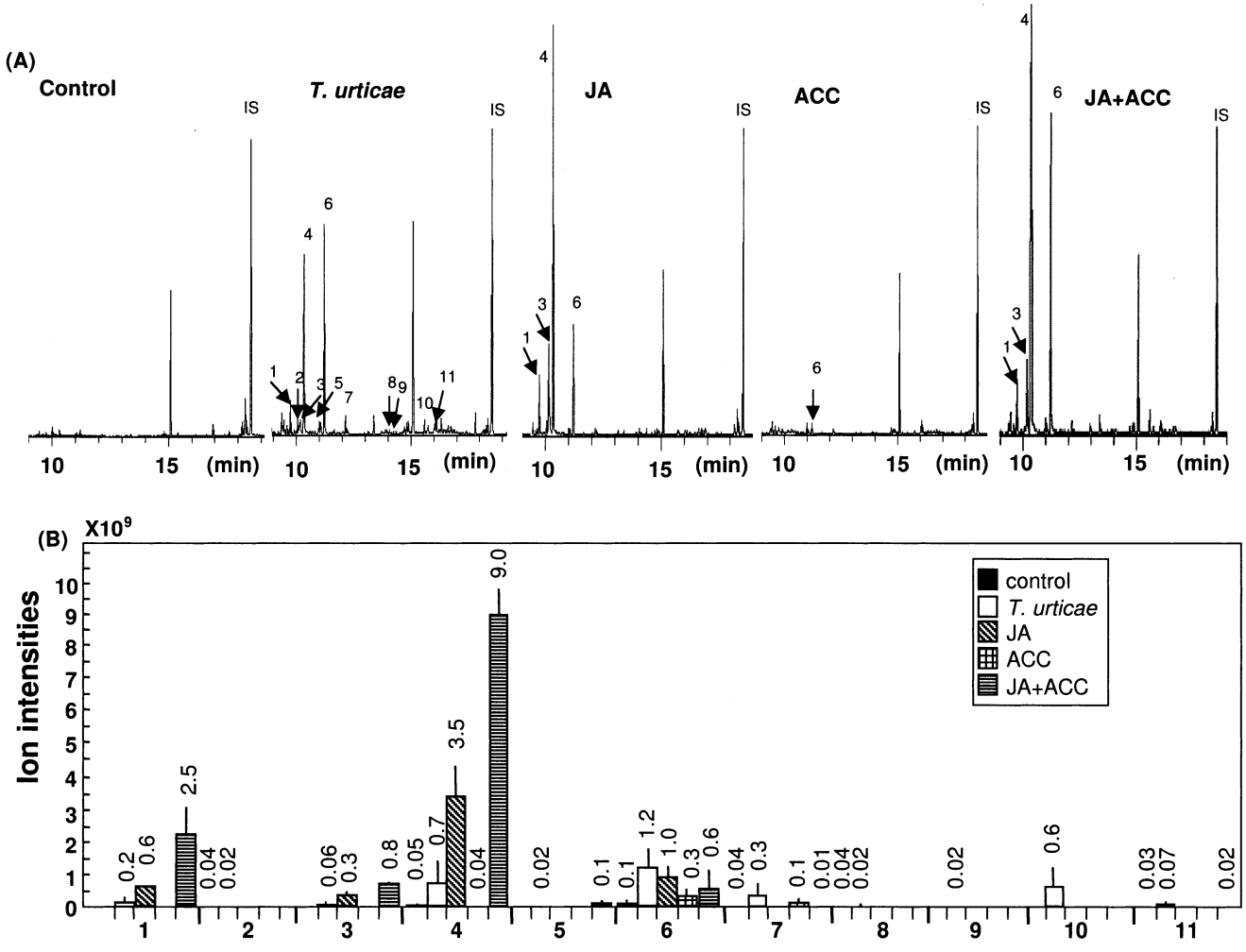

Fig. 1. A: GC–MS profiles and (B) relative amounts of induced compounds in the headspace of lima bean leaves treated with water (control), infested by 100 adult female *T. urticae*, or treated with JA (0.3 mM), ACC (1 mM) or JA (0.3 mM)+ACC (1 mM), for 24 h. Bars represent standard error ($n = 4$). Compound names: 1. (*Z*)-3-hexenyl acetate, 2. limonene, 3. (*Z*)-β-ocimene, 4. (*E*)-β-ocimene, 5. linalool, 6. DMNT, 7. MeSA, 8. α-copaene, 9. β-caryophyllene, 10. TMTT, 11. unidentified compound.

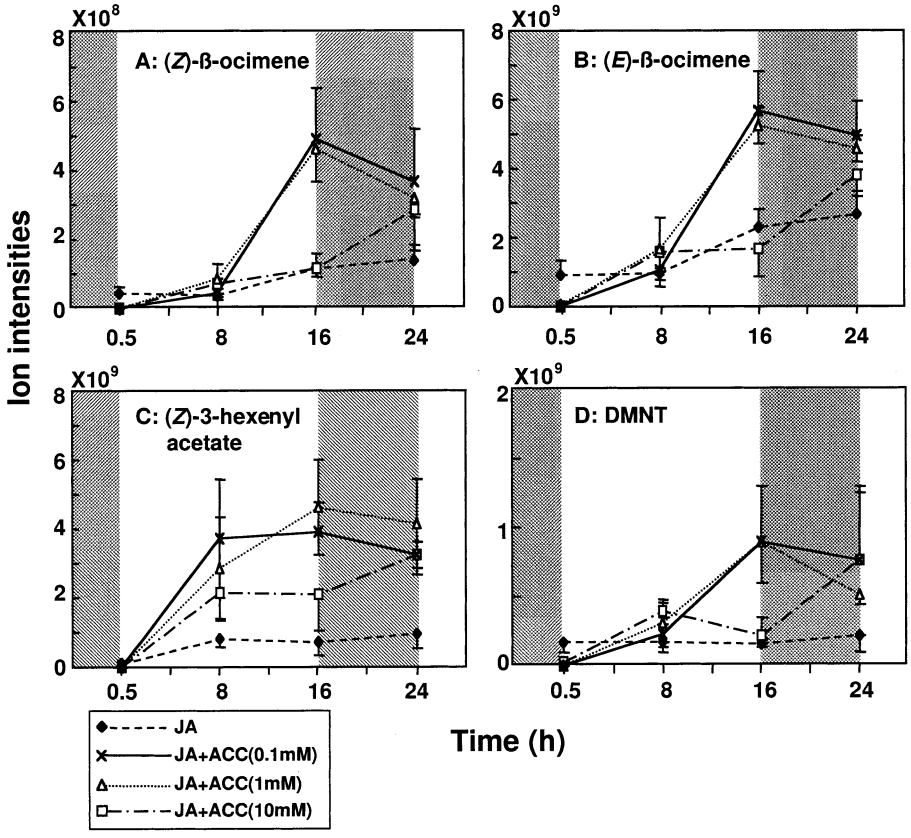

Fig. 2. Transition of volatile emissions from leaves treated with JA and various concentrations of ACC. Relative amounts of induced (A) (*Z*)-β-ocimene, (B) (*E*)-β-ocimene, (C) (*Z*)-3-hexenyl acetate and (D) DMNT found in the headspace of lima bean leaves treated with 0.3 mM JA plus 0–10 mM ACC. The volatiles were collected 0.5, 8, 16, and 24 h after each application. White areas and dotted areas represent light and dark conditions, respectively. Vertical bars represent standard error ($n = 3$).

3. Results and discussion

3.1. Effects of ACC on JA-induced volatile emissions

Application of JA (0.3 mM) to two lima bean leaves for 24 h elicited the emission of (*E*)-β-ocimene, (*Z*)-β-ocimene, DMNT and (*Z*)-3-hexenyl acetate (Fig. 1), which were also found in the headspace of two lima bean leaves infested with *T. urticae* (100 females per leaf) for 24 h. The leaves infested by *T. urticae* also released limonene, linalool, TMTT, methyl salicylate (MeSA) and an unidentified minor compound, which were not found in the headspace of JA-treated leaves. The biosynthesis of TMTT is induced by 12-oxo-phytodienoic

Table 1
Analysis of variance of the effects of JA and ACC treatment (Fig. 2) and time to the measurement on the production of induced volatiles by lima bean leaves

	Source	d.f.	*F*	*P*
(*Z*)-β-Ocimene				
	1 Treatment	3	3.959	0.0165
	2 Time to measurement	3	17.277	<0.0001
	1×2	9	2.276	0.0421
	Error	32		
(*E*)-β-Ocimene				
	1 Treatment	3	4.831	0.0069
	2 Time to measurement	3	36.107	<0.0001
	1×2	9	3.275	0.0062
	Error	32		
DMNT				
	1 Treatment	3	1.246	0.3095
	2 Time to measurement	3	4.457	0.0100
	1×2	9	1.091	0.3963
	Error	32		
(*Z*)-3-Hexenyl acetate				
	1 Treatment	3	5.705	0.0030
	2 Time to measurement	3	9.879	<0.0001
	1×2	9	1.013	0.4503
	Error	32		

acid (an early intermediate of the octadecanoid pathway), but not JA [11]. MeSA is synthesized via the phenolic pathway [21,22]. In contrast, linalool is reported to be induced by JA [10]. Furthermore, Dicke et al. reported that 52 volatiles were emitted from *T. urticae*-infested plants, and 59 volatiles from JA-treated plants [10]. The varying composition of induced volatiles of this study and that of Dicke et al. may be due to different experimental conditions such as JA concentration and duration of JA treatment, and different methods for desorption of the volatiles from the absorbent (solvent desorption in this study and thermodesorption in [10]).

When ACC (1 mM) was applied in the absence of JA, lima bean leaves released few or no plant volatiles (Fig. 1). However, when ACC (1 mM) was applied in addition to JA (0.3 mM), lima bean leaves emitted the same four volatile compounds as emitted from JA-treated leaves, but in increased amounts (Fig. 1). To test the effects of applied ACC concentration, we then treated leaves with JA (0.3 mM) and various concentrations of ACC (0, 0.1, 1, and 10 mM; Fig. 2, Table 1). The headspace of the treated leaves was analyzed every 8 h up to 24 h. We found that emission of (*E*)-β-ocimene, (*Z*)-β-ocimene, DMNT, and (*Z*)-3-hexenyl acetate was induced, but the emission of other volatiles was not recorded in the headspace of JA+ACC-treated leaves of this experiment. The treatment and time to measurement significantly affected emission of (*Z*)-β-ocimene, (*E*)-β-ocimene, and (*Z*)-3-hexenyl acetate (Fig. 2, Table 1). By contrast, only the time to measurement significantly affected emission of DMNT (Fig. 2, Table 1), suggesting that ACC might not be involved in the production of DMNT although it was involved in the production of (*Z*)-β-ocimene, (*E*)-β-ocimene, and (*Z*)-3-hexenyl acetate. Interestingly, the amounts of (*E*)-β-ocimene and DMNT were higher in JA-treated leaves than in JA+ACC-treated leaves 0.5 h after application. Thus, ACC may exert negative effects on (*E*)-β-ocimene and DMNT production in very early stages of induction.

After the addition of 0.5 mM STS to a solution of JA and ACC, the emission of JA+ACC-induced (*E*)-β-ocimene, (*Z*)-β-ocimene, and (*Z*)-3-hexenyl acetate was significantly reduced (Fig. 3). We therefore inferred that at a minimum, ACC-derived ethylene positively influences de novo synthesis of JA-inducible β-ocimene and (*Z*)-3-hexenyl acetate. O'Donnell et al. proposed that ethylene functions downstream of, or in parallel with JA in transcriptional regulation of defense genes [17].

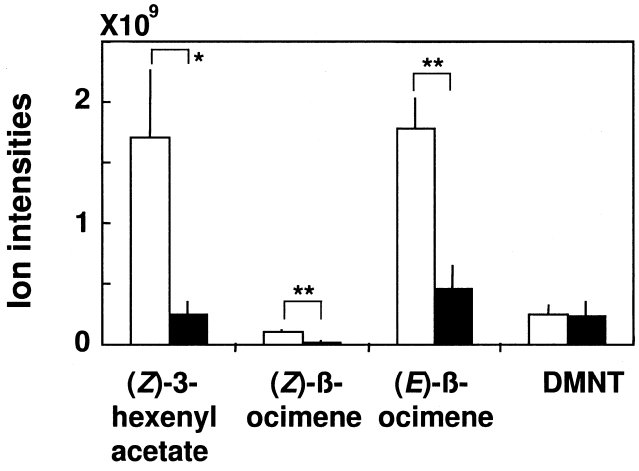

Fig. 3. Relative values of induced volatiles detected in the headspace of lima bean leaves treated for 12 h with JA+ACC or JA+ACC+STS. Bars represent standard error ($n=8$). *$0.01<P<0.05$, **$0.001<P<0.01$ (Mann–Whitney U test).

3.2. Attractiveness to a natural enemy of T. urticae

When we applied 0.3 mM JA solution to lima bean leaves, female *P. persimilis* showed no preference for the odor of JA-treated leaves over the untreated control leaves (χ^2 test, $P>0.05$; Table 2). Previous studies have shown that *P. persimilis* is attracted to the odor of lima beans coated with 1 mM JA, whereas mites show no preference for lima beans treated with 0.1 mM JA [10]. In contrast, when we applied 0.3 mM JA and 1 mM ACC to leaves, *P. persimilis* showed significant preference for the odor from JA+ACC-treated leaves over that from the control leaves ($\chi^2=19.24$, $P<0.001$; Table 2). When we applied only ACC to leaves, *P. persimilis* showed no odor preference ($\chi^2=0.346$, $P>0.05$; Table 2). Thus, the odor from JA+ACC-treated leaves was more attractive than the odor from JA-treated leaves (2×2 contingency test, $\chi^2=4.53$, $P<0.05$) or ACC-treated leaves (2×2 contingency test, $\chi^2=6.64$, $P<0.01$). The increased amounts of (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene and (*Z*)-β-ocimene after ACC treatment would partly explain the increased attractiveness of the JA-induced blend of volatiles. However, when offered singly, (*Z*)-3-hexenyl acetate has no potential to attract *P. persimilis* in the Y-tube olfactometer [23]. Whether (*Z*)-3-hexenyl acetate exerts a positive synergistic effect on the attractiveness of JA+ACC-treated leaves remains unclear. The

Table 2
Results of individual olfactometer tests of *P. persimilis* to volatiles emitted from treated leaves vs. those from uninfested control leaves

Experiment	Odor source	Experiments				
		first	second	third	fourth	Total
JA-treated leaves vs. control leaves						
	JA-treated leaves	16	18	16	10	60
	Control leaves	14	10	14	9	47
	No choice	0	2	0	1	3
ACC-treated leaves vs. control leaves						
	ACC-treated leaves	16	17	15	7	55
	Control leaves	12	13	15	9	49
	No choice	2	0	0	4	6
JA+ACC-treated leaves vs. control leaves						
	JA+ACC-treated leaves	21	21	22	14	78
	Control leaves	9	9	8	6	32
	No choice	0	0	0	0	0

Values represent numbers of *P. persimilis* that made a final choice.

ratio of (*E*) and (*Z*) isomers of β-ocimene is reported to be important in determining the attractiveness of β-ocimene [23]. In this study, the ratio of the (*E*) isomers was greater than 90%, and mixtures of this ratio are reported to be attractive when 0.2, 2 or 20 μg of synthetic β-ocimene isomers is offered to *P. persimilis* in the Y-tube olfactometer [23].

4. Conclusions

In the present paper, we showed that ACC and JA mediate the signal cascade(s) of several induced volatiles produced by lima bean leaves and affect the attractiveness of leaves to carnivores. ACC is a metabolic precursor of ethylene [16] and is generally used instead of ethylene for treatment of plants. In addition, we observed active ethylene production by lima bean plants infested with *T. urticae* (600 *T. urticae*/five plants) (plantlets infested for 1 day, 1.53 nl/l/plant; uninfested plantlets, 0.34 nl/l/plant: [19]). We therefore inferred that ethylene could act as a signaling mediator of volatile biosynthesis in lima beans in response to herbivore attack. Moreover, ERELEE4 (ethylene responsive enhancer element [24]) was identified at the 1-kb upstream genomic region of a myrcene/(*E*)-β-ocimene synthase gene in *Arabidopsis* [25]. This finding suggested that ethylene may regulate genes encoding terpene synthases at the transcriptional level in plants. However, it has also been reported that ethylene does not affect the release of (−)-*cis*-α-bergamotene induced by methyl JA in tobacco plants [3]. In the present paper, we found that the production of DMNT was not affected by ACC. Thus, clarification of the mechanisms for the production of HIPVs will require further study.

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