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Field Evaluation of the Bacterial Volatile Derivative 3-Pentanol in Priming for Induced Resistance in Pepper

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Abstract Plants are defended from attack by emission of volatile organic compounds (VOCs) that can act directly against pathogens and herbivores or indirectly by recruiting natural enemies of herbivores. However, microbial VOC have been less investigated as potential triggers of plant systemic defense responses against pathogens in the field. *Bacillus amyloliquefaciens* strain IN937a, a plant growth-promoting rhizobacterium that colonizes plant tissues, stimulates induced systemic resistance (ISR) via its emission of VOCs. We investigated the ISR capacity of VOCs and derivatives collected from strain IN937a against bacterial spot disease caused by *Xanthomonas axonopodis* pv. vesicatoria in pepper. Of 15 bacterial VOCs and their derivatives, 3-pentanol, which is a C8 amyl alcohol reported to be a component of sex pheromones in insects, was selected for further investigation. Pathogens were infiltrated into pepper leaves 10, 20, 30, and 40 days after treatment and transplantation to the field. Disease severity was assessed 7 days after transplantation. Treatment with 3-pentanol significantly reduced disease severity caused by *X. axonopodis* and naturally occurring *Cucumber mosaic virus* in field trials over 2 years. We used quantitative real-time polymerase chain analysis to examine *Pathogenesis-Related* genes associated with salicylic acid (SA), jasmonic acid (JA), and ethylene defense signaling. The expression of *Capsicum annuum Pathogenesis-Related protein* 1 (*CaPR1*), *CaPR2*, and *Ca protease inhibitor2* (*CaPIN2*) increased in field-grown pepper plants treated with 3-pentanol. Taken together, our results show that 3-pentanol triggers induced resistance by priming SA and JA signaling in pepper under field conditions.

Keywords 3-Pentanol · Pepper · Defense priming · ISR · PGPR · Systemic acquired resistance · Volatile organic compound

Abbreviations

PGPR	Plant growth-promoting rhizobacteria
ISR	Induced systemic resistance
VOC	Volatile organic compound

Introduction

Plants are defended from attack by emission of volatile organic compounds (VOCs). Volatile organic compounds can act directly against pathogens and herbivores, or can indirectly through the recruitment of natural enemies of herbivores (Heil and Silva Bueno 2007; Kost and Heil 2006; Rayko et al. 2008; Stout et al. 2006). Plants not directly under attack can respond to VOCs released by a neighboring plant and can stimulate their own induced resistance mechanisms against possible insect attack (Kessler and Baldwin 2001). For example, when wounded by herbivores, certain plants produce methyl jasmonate (MeJA), which acts both as an alarm system for neighboring plants and as a kairomone to attract herbivore enemies (Creelman and Mullet 1997). However, not all plants release MeJA, suggesting that it is not a general plant volatile defense signal. Alternative compounds include green leafy volatiles (GLV), which are derived from C18 fatty acids (linolenic and linoleic acid). After conversion to a

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hydroperoxide by a lipoxygenase, these are cleaved into C12 and C6 components by hydroperoxide lyase. Depending on the C18-substrate, hydroperoxide lyase produces either (*Z*)-*3*-hexenal or hexanal, both of which function as airborne infochemicals in specific plant–insect and plant–plant communications (Hatanaka 1993; Kessler and Baldwin 2001). In addition, plant infection by microbial pathogens stimulates plant biosynthesis of the phenolic defense hormone salicylic acid (SA) and its volatile form, methyl salicylate (MeSA), which acts as a potential signal to induce expression of defense-related genes in neighboring plants (Shulaev et al. 1997; Thaler et al. 2010).

Insect and plant species both produce 3-pentanol, suggesting that this volatile may play a role in intra- (insect sex pheromones) and inter-specific interactions such as the induction of resistance (Bukovinszky et al. 2005; Gatti Liguori et al. 2008; Vitta et al. 2009). For example, 3-pentanol is released from wild *Brassica oleracea* plants and their sprouts in response to *Pieris rapae* caterpillar and *Plutella xylostella* feeding, respectively (Bukovinszky et al. 2005; Gols et al. 2011). Chemical ecological examination has shown that 3-pentanol acts as an insect pheromonal component in *Megaplatypus mutatus*, *Triatoma infestans*, and *T. brasiliensis* (Funes et al. 2009; Gatti Liguori et al. 2008; Manrique et al. 2006; Vitta et al. 2009). Behavioral studies have shown that 3-pentanol is a sex pheromone component in the ambrosia beetle *M. mutates* (Coleoptera: Curculionidae: Platypodinae) (Gatti Liguori et al. 2008). Male *M. mutates* release a sex pheromone that is composed mainly of (+)-sulcatol, sulcatone, and 3-pentanol. These VOC are, however, rarely detected in microbial cultures. *Megaplatypus mutatus* is native to South America but recently was introduced into Italy, where it has caused serious economic problems in agricultural systems such as fruit and poplar tree fields (Funes et al. 2011). In addition, 3-pentanol has been found to be part of a sex pheromone emitted by female *T. infestans* metasternal glands that promote the aggregation of males around the mating couple (Crespo and Manrique 2007). A comprehensive compositional profile of VOCs from soil bacteria (*Bacillus* spp.) has revealed the production of 1-pentanol but not 3-pentanol (Farag et al. 2006).

Several synthetic and microbially derived chemical compounds have been shown to protect crop and model plants against diverse plant pathogens and herbivores (Lyon 2007). This capacity is referred to as induced resistance (Kloepper et al. 2004), and the active compounds that trigger this phenomenon are reported to be released by rhizosphere bacteria (rhizobacteria) (Lee et al. 2012; Lyon 2007; Van Loon et al. 1998; Van Loon 2007). A new type of induced resistance referred to as ISR was described by three independent groups. Induced systemic resistance is elicited by plant growth-promoting rhizobacteria (PGPR) that increase plant defense mechanisms (Alstrom 1991; Van Peer et al. 1991; Wei et al. 1991). Exploitation of ISR in the agricultural field is hampered by microbial stability under natural conditions. To overcome these limitations, the identification of bacterial determinants with similar ISR effects has been attempted through *in vitro* and small-scale greenhouse experiments. A series of experiments with rhizobacteria, mostly *Pseudomonas* and *Serratia* spp., identified diverse bacterial determinants that contribute to ISR, including siderophores, lipopolysaccharides (LPS), 2,4-diacetylphloroglucinol (DAPG), and other bacterial metabolites and cell wall components (De Meyer et al. 1999; Duijff et al. 1997; Iavicoli et al. 2003; Leeman et al. 1995; Ongena et al. 2005; Ryu 2013; Rvan Peer et al. 1991). A new class of agro-chemical, namely, benzothiadiazol (BTH), that induces plant defense reactions against a broad spectrum of pathogens was developed subsequently and released commercially (Tally et al. 1999). Another characteristic phenomenon of induced resistance is "defense priming", which is "the physiological condition in which plants are able to better or more rapidly mount defense responses, or both" (Conrath et al. 2006). *Bacillus amyloliquefaciens* strain IN937a, which is an endophyte that thrives inside plant tissues, stimulates induced systemic resistance (ISR) via the emission of VOCs (Kloepper et al. 2004; Ryu et al. 2003a). Bacterial volatiles from *B. amyloliquefaciens* strain IN937a have been used previously to prime plant resistance, but volatile compounds such as 2,3-butanediol and acetoin were eliminated as the possible active protectants. To elucidate the signaling networks involved in ISR stimulated by VOCs from strain IN937a, a series of mutant and transgenic *Arabidopsis* plant lines were tested by Ryu et al. (2003a, 2004). All the mutants showed ISR when pre-treated with strain IN937a VOCs, which indicated that IN937a-mediated ISR was not dependent on the major plant defense hormones SA, jasmonic acid (JA), or ethylene. The use of bacterial VOCs in the agricultural field has been limited to date (Farag et al. 2013). More recently, it has been demonstrated that a bacterial volatile from *B. amyloliquefaciens* strain IN937a and its derivatives 2,3-butanedione and 3-pentanol increase plant defense against a bacterial pathogen (*Pseudomonas syringae* pv. lachrymans) and a sucking insect aphid on open-field cucumber seedlings (Song and Ryu 2013).

The aim of this study was to assess the utility of VOCs for disease control in a field pepper crop. While synthetic resistance triggers like BTH (known as Actigard® in USA and BION® in Europe) were commercialized previously, the products often fail to gain market share due to their negative effects on plant growth and yield, referred to as "allocation fitness costs" or "trade-offs" (Heil et al. 2000). Some microbial products have been tested for use as ISR triggers. Of these, 2,3-butanediol, which is a major bacterial VOC that elicits strong ISR in *Arabidopsis thaliana*, was found to be ineffective in crops such as pepper plants. A demand remains for the commercial development of effective bioactive compounds

for use on crop plants. Our previous VOC profiling study led us to analyze VOCs from *B. amyloliquefaciens* strain IN937a because it produces a larger number (>35) of bacterial VOCs than other PGPR strains (Farag et al. 2006; Ryu et al. 2004). In the current study, we screened commercially available bacterial volatiles and their derivatives for the capacity to elicit induced resistance against bacterial spot caused by *Xanthomonas axonopodis* pv. vesicatoria in pepper seedlings. Priming of plant defense responses induced increased expression of pathogenesis-related genes including *CaPR1*, *CaPR4*, and *CaPIN*. Severe disease symptoms distinct from those caused by the bacterial pathogen were observed at the end of the growing season. Investigation of the plants revealed mosaic and shoe-string symptoms. These symptoms were characteristic of infection by *Cucumber mosaic virus* (CMV), a common and economically important viral disease of pepper in South Korea. The severity of diseases caused by *X. axonopodis* pv. vesicatoria and by naturally occurring CMV were significantly reduced at the end of 2 year field experiments. Our results suggest that a bacterial volatile derivative can be used to elicit induced resistance by defense priming of SA and JA signaling in a pepper crop plant under large field conditions.

Methods and Materials

Pepper Growth Chamber Experiments Pepper plants (*Capsicum annuum* L. cv. Bukwang) were cultivated in a growth chamber at 25 °C under a 16/8 h L/D photoperiod. Application of chemical treatments to elicit induced resistance in pepper were performed as previously described (Kang et al. 2007; Song et al. 2013a, b). Individual pepper seedlings were treated with a 50 ml root drench consisting of 10 μM or 100 nM concentrations of 1-pentanol, ethylbutyric acid, 2-methyl-1-propanol, 2-pentanone, 2-pentyl furan, 3-acetyl-propanol, 3-methyl-2-butanone, 3-methyl-1-butanonol, 3-pentanol, glyoxylic acid monohydrate, butyl acetate, indole, isoamyl acetate, isoamyl alcohol, or isovaleric acid. Benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester was provided by Syngenta Korea. Other chemicals were purchased from Sigma Aldrich Co. BTH, which is used as a commercial resistance trigger (Actigard® in the USA and BION® in Europe) was used as a control at 0.5 mM. Two VOCs (3-pentanol and 2,3-butanediol) were evaluated further for induction of ISR at three concentrations: 1 mM, 10 μM, and 100 nM. For pathogen challenge, a culture of the virulent *Xanthomonas axonopodis* pv. vesicatoria obtained from Dr. Doil Choi, Seoul National University, S. Korea, (OD_{600}=0.04 in 10 mM $MgCl_2$) was pressure-infiltrated into pepper leaves using a needleless syringe 1 week after root drenches were applied, as described previously (Kang et al. 2007; Oh S-K et al. 2006). Disease severity in leaves was assessed 7–10 day after pathogen challenge using the following scale: 0, no symptoms; 1, yellowish color; 2, chlorosis only; 3, necrosis and chlorosis; 4, partial necrosis of the inoculated area; and 5, complete necrosis of the inoculated area (Yi et al. 2013). Numbers of bacteria were measured at 0, 3, and 7 days after pathogen infiltration, as described previously (Song et al. 2013a). Each treatment was applied to eight plants and the experiment was repeated three times.

Pepper Field Trials Field trials were conducted in Cheongwon-gun, Chungcheongbuk-do, South Korea (36° 35′ 32.27″ North, 127° 30′ 34.75″ East) in April 2010 and April 2011. All necessary permits were obtained from the private landowner. Pepper seeds (*Capsicum annuum* L. cv. Bukwang) were surface-sterilized with 5 % sodium hypochlorite (NaOCl) for 10 min and then rinsed five times with sterile distilled water. Seeds were then placed on MS medium (0.22 % Murashige & Skoog salts including vitamins, 1.5 % sucrose, 0.8 % plant agar, pH 5.8) in a transparent sterile container. Pepper seedlings were cultivated in a dark growth chamber at 25±3 °C. Germinated pepper seeds were transferred to low nutrient soil and cultivated in a greenhouse for 3 weeks. To test induced resistance under field conditions, 3-weeks-old seedlings were soaked in water (control), 1 mM 3-pentanol, or 0.5 mM BTH solution for 1 h, and then transplanted to the field at a spacing of 40 cm. Before transplanting, rows were covered with black and white polyethylene plastic film to control weeds.

Ten days after application of water, BTH, or 3-pentanol, bacterial suspensions of 10^6 colony forming units (CFUs)/mL of *X. axonopodis* pv. vesicatoria were infiltrated into the underside of pepper leaves using the needleless syringe method as described above. Seven days after pathogen challenge, bacterial disease severity was assessed as described previously (Yang et al. 2009).

Severity of naturally occurring CMV was assessed on a 0–5 scale as follows: 0 = no symptoms; 1 = mild deformation and mosaic of the youngest two leaves; 2 = pronounced leaf deformation and mosaic of the youngest two leaves with progression of symptoms into sequentially older leaves; 3 = pronounced leaf deformation and mosaic progression beyond the two youngest leaves with all leaves expressing some form of CMV-induced symptoms; 4 = similar symptoms as described for a rating of 3, with plants also being stunted in growth (where stunting includes both reduced internode extension and smaller leaves); and 5 = severe stunting with the majority of leaves being small, severely deformed, and tightly bunched together (Song et al. 2013a).

To test whether 3-pentanol and BTH influenced the growth of plants under field conditions, shoot length was measured 134 day post-transplanting (dpt). Pepper fruit yield per plant was harvested and measured 98 dpt.

Pepper plants were cultivated on 20 cm high × 30 cm wide × 880 cm long rows. Single-row treatment plots consisted of 23 plants, and were replicated four times in a completely randomized design. Ten plants per replicate were randomly selected for assessment of disease and plant growth.

Virus Detection by RT-PCR For diagnosis of viral disease, test samples were selected from areas of the plant that exhibited symptoms of disease. Tissue samples were ground in 50 mM $NaHPO_4$ (pH=7.0) buffer. An ImmunoStrip (Agdia, USA) was inserted into the tissue lysates and incubated in the sample extract for 30 min. RT-PCR was used to confirm CMV infection using the following primers: 5′-CGGCGGAAGACCATGATTT-3′ and 5′-CCTTCCGCCCATTCGTTAC-3′. *CaActin* was used as a normalizing control. Primers for *CaActin* were designed against GenBank database sequence AY572427.1 and were as follows: 5′-CACTGAAGCACCCTTGAACCC-3′ and 5′-GAGACAACACCGCCTGAATAGC-3′.

RT-PCR and Quantitative RT-PCR Following inoculation with *X. axonopodis* pv. vesicatoria, plants were returned to the growth chamber, and leaf tissue was harvested 0, 3, and 6 h post-inoculation (hpi). To minimize stress-induced gene expression, intact leaves were selected and immediately frozen in liquid nitrogen. Frozen tissues were used for total RNA extraction with Tri reagent (Molecular Research Center) according to the manufacturer's instructions. DNase-treated total RNA (2 μg) was used for first-strand cDNA synthesis with oligo-dT primers and Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Enzynomics, Korea). PCR reactions were performed according to the manufacturer's instructions. The expression of candidate priming genes was analyzed using the following primers: 5′-ACTTGCAATTATGATCCACC-3′ (*CaPR1*-F), 5′-ACTCCAGTTACTGCACCATT-3′ (*CaPR1*-R); 5′- TTTTAGCTATGCTGGTAATCCGCG-3′ (*CaPR2*-F), 5′- AAACCATGAGGACCAACAAAAGCG-3′ (*CaPR2*-R); 5′-AACTGGGATTGAGAACTGCCAGC-3′ (*CaPR4*-F), 5′-ATCCAAGGTACATATAGAGCTTCC-3′ (*CaPR4*-R); 5′-TGGGACTTTCATTTGTGAAGGAGAG-3′ (*CaPIN2*-F), 5′-GACACAGTGAATAGGCAATATTTGG-3′ (*CaPIN2*-R); 5′-GGGATCCAATGTCTGCGTTTGGAAAATTGG-3′ (*CaGLP1*-F), 5′-TGGATCCCATTTCTTAGGTGCCAACCTTGA-3′ (*CaGLP1*-R); and 5′-TGGGACTTTCATTTGTGAAGGAGAG-3′ (*CaPIN2*-F), 5′-GACACAGTGAATAGGCAATATTTGG-3′ (*CaPIN2*-R) (Song et al. 2013a; Yang et al. 2009, 2011). *CaActin* was used as a control, and was assessed using the following primers: 5′-CACTGAAGCACCCTTGAACCC-3′ and 5′- GAGACAACACCGCCTGAATAGC-3′. A Chromo4 real-time PCR system (BIO-RAD) was used for quantitative RT-PCR. Reaction mixtures consisted of cDNA, iQTM SYBR® Green Supermix (BIO-RAD) and 10 pmoles of each primer. The thermocycler parameters were as follows: initial polymerase activation of 10 min at 95 °C, and then 40 cycles of 30 s at 95 °C, 60 s at 55 °C and 30 s at 72 °C. Conditions were determined by comparing threshold values in a series of dilutions of the RT product, followed by a non-RT template control and a non-template control for each primer pair. Relative RNA levels were calibrated and normalized to the level of *CaActin* mRNA (GenBank accession no. AY572427).

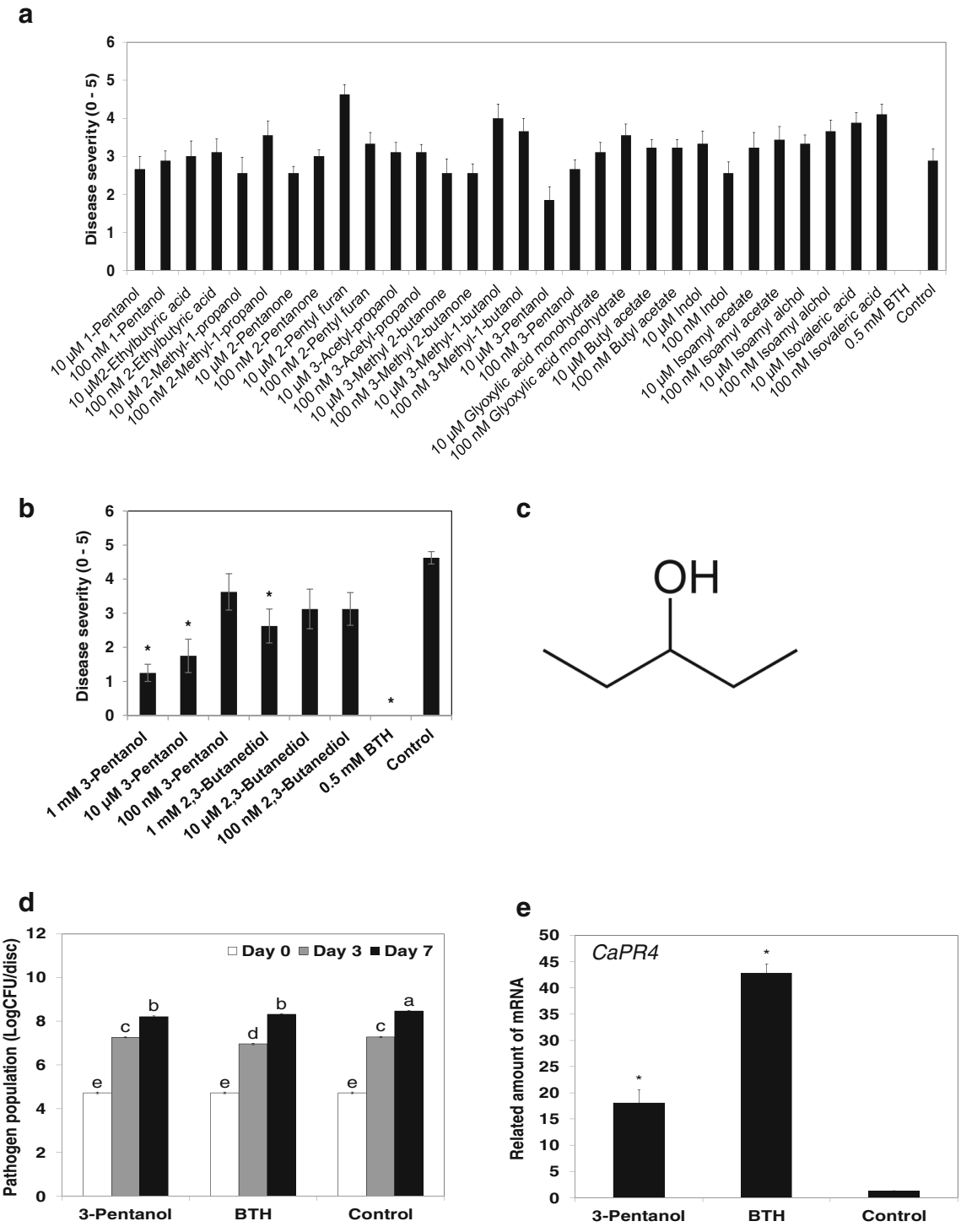
Statistical Analysis Data were assessed using analysis of variance (ANOVA) tests with JMP software version 4.0 (SAS Institute Inc., Cary, NC, USA; URL: www.sas.com). The significance of chemical treatment effects was determined by the magnitude of the F value at $P \leq 0.05$. When a significant F value was obtained for treatments, separation of means was accomplished using Fisher's protected least-significant difference test (LSD) at $P \leq 0.05$. Replicated trials had similar outcomes, and one representative trial for each experiment is reported here.

Results

Screening of Bacterial VOCs and their Derivatives for Elicitation of Induced Resistance and Defense Priming under Greenhouse Conditions In our previous study (Farag et al. 2006), we identified more than 30 bacterial volatiles and their derivatives from a number of PGPRs, including

Fig. 1 Induced resistance by bacterial volatiles and their derivatives in the greenhouse. (**a**) Pepper seedlings were treated by direct drench application with 50 ml of 10 μM and 100 nM 1-pentanol, ethylbutyric acid, 2-methyl-1-propanol, 2-pentanone, 2-pentyl furan, 3-acetyl-propanol, 3-methyl-2-butanone, 3-methyl-1-butanonol, 3-pentanol, glyoxylic acid monohydrate, butyl acetate, indol, isoamyl acetate, isoamyl alcohol, or isovaleric acid. Similar treatments with 0.5 mM benzothiadiazol (BTH) and sterile distilled water were used as positive and negative controls, respectively. Panel (**a**) depicts disease severity (0–5) caused by virulent *Xanthomonas axonopodis* pv. vesicatoria at 7 days post-inoculation (dpi). (**b**) Pepper seedlings were treated by direct drench application with 50 ml of 1 mM, 10 μM, and 100 nM 3-pentanol and 2,3-butanediol. Treatment with 0.5 mM BTH and water were used as positive and negative controls, respectively. Panel (**b**) depicts disease severity (0–5) caused by virulent *X. vesicatoria* in chemically treated plants vs. controls at 7 dpi. Panel (**c**) shows the chemical structure of 3-pentanol. Panel (**d**) indicates bacterial numbers at 0, 3, and 7 dpi. The results of qRT-PCR (panel **e**) show induction of *CaPR4* gene expression by 3-pentanol and BTH treatment at 6 h post *X. vesicatoria* inoculation (hpi) in plants treated with 1 mM 3-pentanol and 0.5 mM BTH. The housekeeping gene *CaActin* was used for normalization. Asterisks in panels a, b, and e indicate significant differences between chemical and control treatments according to LSD at a significance level of $P=0.05$. The different letters in panel (**d**) indicate significant differences between treatments ($P<0.05$, LSD). Error bars represent means±SEM. Sample size: $N=8$ plants per treatment. The data in panels (**b**), (**d**), and (**e**) are taken from one of three replicated trials that produced similar results. Numbers indicate the mean of five replicates, and the mean of three leaves/plant per replicate

B. amyloliquefaciens strain IN937a. In the current study, we screened 15 commercially available volatiles for their capacity to reduce severity of bacterial leaf spot on greenhouse pepper plants. Disease severity was assessed 7 days after infiltration of leaves with *X. axonopodis* pv. vesicatoria. In this initial screen, the only volatile to effectively reduce disease was 3-pentanol at a concentration of 10 μM (ANOVA: numerator *d.f.* = 31, denominator *d.f.* = 569, F=7.8074, P<0.001, N=8) (Fig. 1a). Further tests showed that the minimum effective 3-pentanol concentration was 10 μM (ANOVA: numerator *d.f.* = 7, denominator *d.f.* = 63, F=11.9051, P<0.001, N=8) (Fig. 1b). The chemical formula for 3-pentanol is shown in Fig. 1c. The most effective 3-pentanol concentration (1 mM) was used in subsequent experiments. A derivative of 3-pentanol elicited induced resistance but did not have the same effect in pepper (data not shown). The other derivative, 1-



pentanol, did not reduce symptom development compared to the water control (Fig. 1a). An additional volatile, 2,3-butanediol, which elicited induced resistance in *Arabidopsis* and tobacco, also stimulated induced resistance in pepper plants (Fig. 1b).

Proliferation of the introduced *X. axonopodis* pv. vesicatoria was examined 0, 3, and 7 days after leaf bacterial infiltration. On day 3, bacterial numbers in plants treated with 3-pentanol did not differ from those of the control plants, whereas BTH treatment reduced the bacterial population compared to the control (Fig. 1d). On day 7, bacterial growth was significantly lower in plants treated with 3-pentanol or BTH than in those drenched with water. The bacterial populations on day 7 in plants treated with 3-pentanol and BTH were 1.7 and 2.1×10^8 CFU/leaf disk, respectively, while those of the control were 2.9×10^8 CFU/leaf disk (Fig. 1d). The differences in the bacterial populations between 3-pentanol and water-treated plants could be associated with differences in symptom development (day 3 and day 7) (Fig. 1d). To determine whether 3-pentanol and BTH induced expression of plant defense-related genes, transcription of the pepper induced resistance-related marker gene *CaPR4* was assessed after pathogen challenge (Song et al. 2013a; Yang et al. 2009). Three hpi with the pathogen, *CaPR4* expression was 14.7-fold higher in 3-pentanol-treated plants than in water control plants and 35-fold higher in BTH-treated plants than in controls (Fig. 1e). These results indicate that under greenhouse cultivation, root inoculation with 3-pentanol protects pepper seedlings via induction of plant systemic defenses, as indicated by the expression of a defense-related gene.

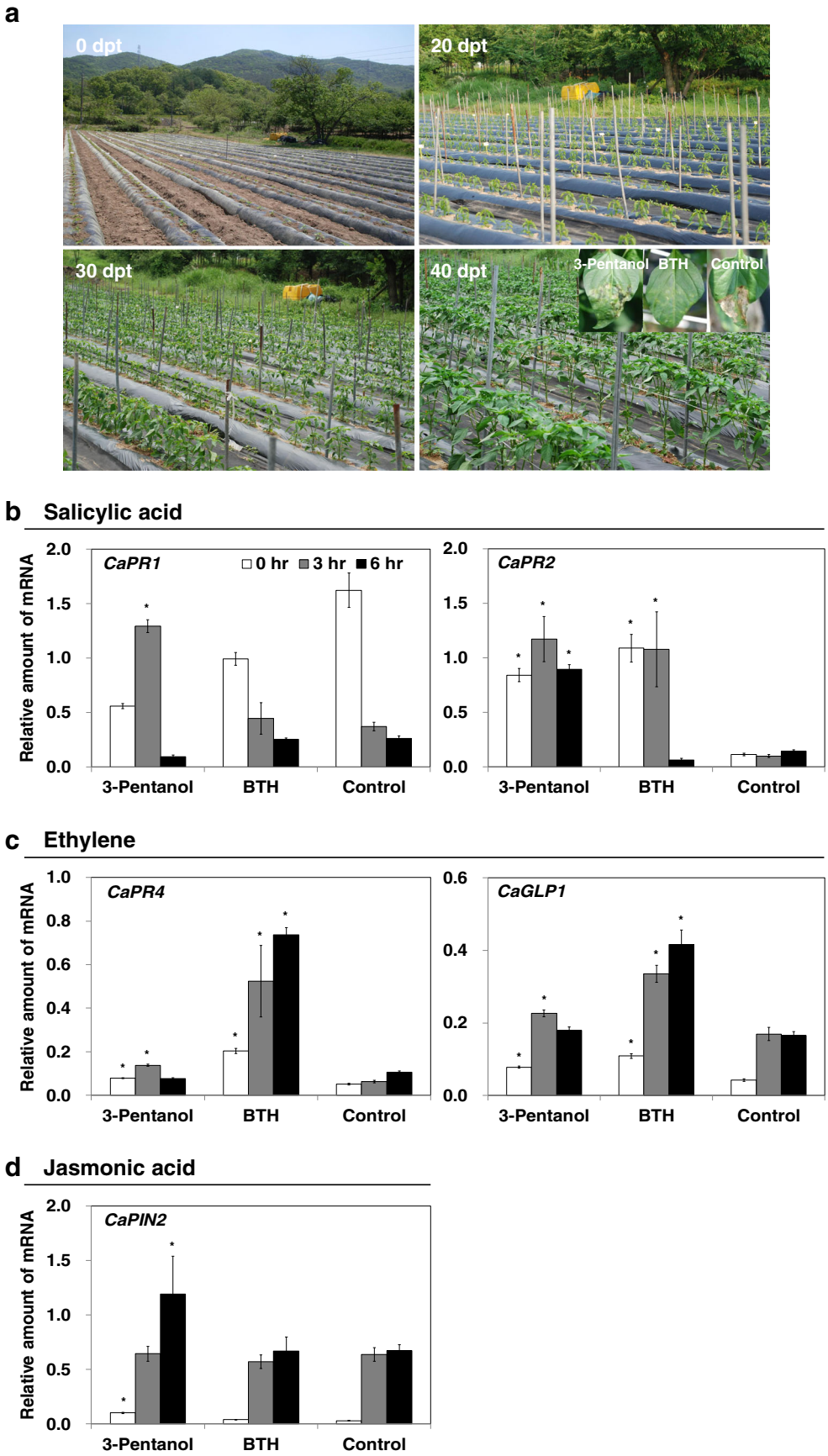
Assessment of 3-pentanol-elicited Induced Resistance under Field Conditions Next, we wished to determine whether 1 mM 3-pentanol could be used for disease management under field conditions. Although we previously confirmed that 3-pentanol successfully suppressed *Pseudomonas syringae* and aphids in a small pot experiment (Song and Ryu 2013), open-field conditions are challenging for the application of VOCs due to possible evaporation and loss of the product. We designed a field trial in a commercial pepper field that has been in use for more than 10 years. Plants were assessed for disease 10, 20, 30, and 40 days after transplantation to the field. All the disease indexes of plants treated with 3-pentanol were significantly lower than those of the control plants except on day 10 (Table 1). In 2010, the disease indexes 10, 20, 30, and 40 dpt were 4.4, 3.1, 1.7, and 0.7, respectively, in 3-pentanol-treated plants and 4.7, 4.5, 4.9, and 4.0, respectively, in the water controls. Treatment with 0.5 mM BTH was used as a positive control. In 2011, the disease indexes on days 20, 30, and 40 were 0.7, 0.7, and 1.7, respectively, in 3-pentanol-treated plants and 2.5, 2.7, and 3.8, respectively, in the water-treated controls (Table 1). Minimal disease symptoms were detected in BTH-treated plants at the indicated time points (Table 1, Fig. 2a). Assessment of *CaPR1* and *CaPR4* expression showed no differences between treatments at 3 and 6 hpt, *CaPR1* expression was significantly ($P=0.05$) higher in 3-pentanol-treated plants than in control plants. No differences in the expression of these genes were detected at 40 dpi (Fig. 2b). To further investigate defense signaling in treated pepper plants, we measured the expression of the following marker genes: *CaPR1* and *CaPR2* for SA signaling, *CaPR4* and *CaGLP1* for ethylene signaling, and *CaPIN2* for JA signaling. *CaPR1* expression was higher in 3-pentanol-treated plants than in control plants 3 hpi (Fig. 2b), and *CaPR2* expression was significantly ($P=0.05$) up-regulated in 3-pentanol-treated plants compared to control plants at all-time points (Fig. 2b). Expression of *CaPR4* and *CaGLP1* marginally increased in response to 3-pentanol treatment, but RNA levels were not statistically different from water-treated

Table 1 Induced resistance against *Xanthomonas axonopodis* pv. vesicatoria by 3-pentanol at 10, 20, 30, and 40 day post-treatment in pepper plants

Years	Days post-transplanting	Disease severity (0–5)		
		3-Pentanol	BTH	Control
2010				
	10	4.4^{a}	0.0^{b}	4.7^{a}
	20	3.1^{b}	0.0^{c}	4.5^{a}
	30	1.7^{b}	0.4^{c}	4.9^{a}
	40	0.7^{b}	0.0^{c}	4.0^{a}
2011				
	10	ND	ND	ND
	20	0.7^{b}	0.1^{c}	2.5^{a}
	30	0.7^{b}	0.1^{bc}	2.7^{a}
	40	1.7^{b}	1.0^{b}	3.8^{a}

Disease severity was measured 7 days after pathogen challenge. Bacterial suspensions of 10^6 colony forming units (CFUs)/mL of *X. axonopodis* pv. vesicatoria were infiltrated into the underside of pepper leaves at 10, 20, 30, and 40 day after treatment with 1 mM 3-pentanol, 0.5 mM benzothiadiazol (BTH), or water (control) treatment in the field when the pepper seeldings were transplanted in 2010 and 2011. Differing letters on the three treatments for the same assessment day indicate significant differences between treatments ($P<0.05$; LSD). *ND* not determined

Fig. 2 Elicitation of pepper defense-related genes by 3-pentanol under field conditions. (**a**) Images show representative pepper growth 0, 20, 30, and 40 days post transplantation (dpt). Disease symptoms are shown (40 dpt inset). (**b**) qRT-PCR of salicylic acid signaling marker genes *CaPR1* and *CaPR2*, ethylene markers *CaPR4* and *CaGLP1*, and jasmonic acid marker *CaPIN2* in plants treated with water, 1 mM 3-pentanol, or 0.5 mM benzothiadiazol (BTH). Samples were taken 0, 3, and 6 h after inoculation with *Xanthomonas. vesicatoria*. The housekeeping gene *CaActin* was used for normalization. Asterisks in panels b, c, and d indicate significant differences between chemical and control treatments according to LSD at a significance level of $P=0.05$. Error bars represent means±SEM. Sample size: $N=10$ plants per treatment in 2010


a
0 dpt
20 dpt
30 dpt
40 dpt
3-Pentanol
BTH
Control
b Salicylic acid
CaPR1
0 hr
3 hr
6 hr
CaPR2
Relative amount of mRNA
2.0
1.5
1.0
0.5
0.0
3-Pentanol
BTH
Control
c Ethylene
CaPR4
CaGLP1
1.0
0.8
0.6
0.4
0.2
0.0
0.6
0.4
0.2
0.0
d Jasmonic acid
CaPIN2

controls (Fig. 2c). However, with 3-pentanol treatment, *CaPIN2* transcription doubled in the 6 h after pathogen challenge (Fig. 2d). Expression of *CaPR1*, *CaPR2*, *CaPR4*, and *CaGLP1*, but not *CaPIN2*, was significantly higher in the BTH-treated plants than in the water-treated controls for at least one time point. Taken together, these data indicate that the two defense hormones SA and JA can be related to 3-pentanol-mediated resistance in pepper (Figs. 2b, c, d).

Effect of 3-pentanol on Induced Resistance against Naturally Occurring Diseases and on Plant Yield In 2010, severe disease symptoms were detected in the field trial plants at the end of the season in mid-September. These symptoms were worse in 2011 presumably due to unusually high temperatures and a long rainy season. Detailed analysis of these symptoms showed the presence of spots and specks suggestive of bacterial spot caused by *X. axonopodis* pv. vesicatoria, and mosaic and shoe-string symptoms typical of *Tobacco mosaic virus* or CMV infection. The pathogenic agents were identified using biochemical and PCR techniques. *Xanthoranas axonopodis* pv. vesicatoria was identified through analysis of 16 s rRNA, colony color and morphology on agar media, a pathogenicity test on pepper, and a hypersensitive response test on *Nicotiana benthamiana* (data not shown). The viral agent was identified as CMV using enzymatic and virus-specific primer-based PCR techniques (data not shown). The disease symptoms caused by CMV and *X. axonopodis* pv. vesicatoria on pepper plants treated with 3-pentanol were significantly reduced compared to symptoms on control plants (Fig. 3a, b). Quantification of CMV by qRT-PCR showed a 4-fold lower amount of CMV RNA on 3-pentanol-treated plants than on control plants (data not shown). The positive control BTH treatment elicited SAR against *X. axonopodis* pv. vesicatoria but not against CMV (Fig. 3a, b). In our previous research, we frequently observed that the strong induction of induced resistance caused a yield penalty referred to as the "allocation fitness cost" (Song et al. 2013a; Yang et al. 2011). We, therefore, examined plant growth parameters including foliar height and fruit yield. The shoot length (height) did not differ between 3-pentanol and control plants, while shoot length was reduced with BTH treatment only in 2011 (Fig. 3c). No reduction in fruit yield was observed with 3-pentanol treatment compared to water-treated control plants, but BTH treatment consistently and substantially reduced pepper yield (Fig. 3d).

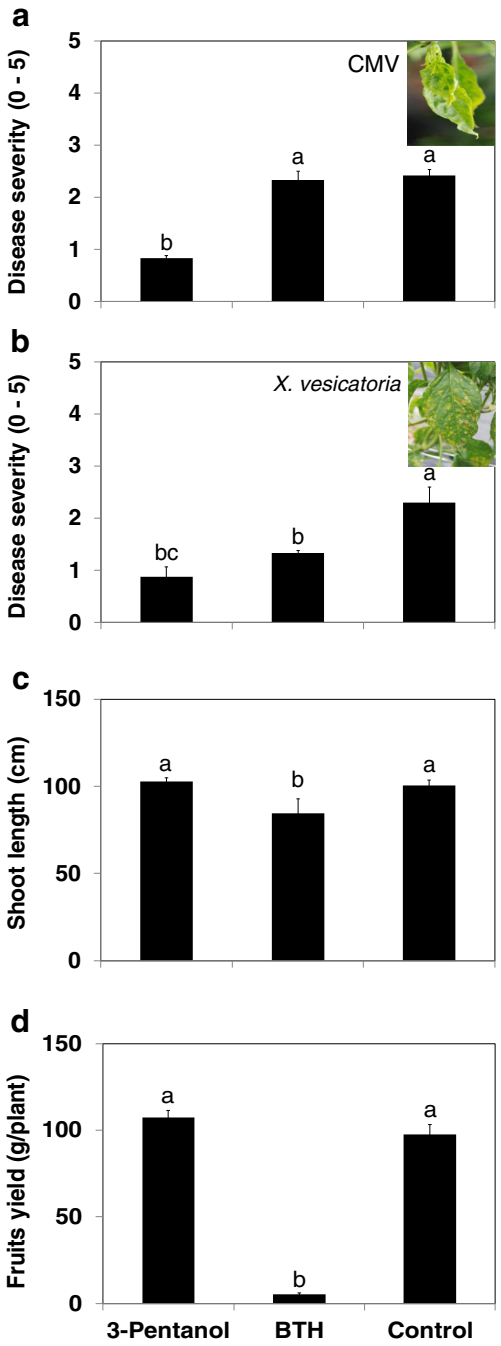


Fig. 3 Induced resistance and growth response to 3-pentanol under field conditions. (**a**) Induced resistance against *Cucumber mosaic virus* (CMV). Disease symptoms caused by naturally occurring CMV were evaluated 134 days post transplantation (dpt). (**b**) Induced resistance against *X. vesicatoria*. Typical disease symptoms were evaluated 134 dpt. (**c**) Shoot length was measured 134 dpt. (**d**) Peppers were harvested 98 dpt and fruit yield per plant was determined. Different letters indicate significant differences between treatments ($P<0.05$, LSD). Error bars represent means±SEM. Sample size: $N=10$ plants per treatment

Discussion

Several compounds and cell wall derivatives previously were shown to induce pathogen resistance in plants (Lyon 2007). In the present study, chemical triggers for elicitation of induced resistance in plants via the defense priming machinery were investigated. The amyl alcohol isomer 3-pentanol, which is a component of insect sex hormones, activated plant defense responses against bacterial and viral pathogens under field

conditions. In our previous report, drench application of 3-pentanol successfully protected cucumber plants against *P. syringae* and aphids in large pots in open-field conditions (Song and Ryu 2013). As cucumbers are cultivated only in greenhouses in South Korea, we were unable to assess the protective potential of 3-pentanol in a large-scale field trial using cucumber. Pepper is a common, commercially important field crop in South Korea, and we, therefore, chose pepper to assess the efficacy of 3-pentanol in a large-scale field trial. Our results showed that 3-pentanol modulated defense priming by induction of SA- and JA-related resistance marker genes in pepper. The results were validated by experiments using *Arabidopsis* as a model system. Further studies revealed that defense priming of the SA and JA defense signaling genes could be elicited without physical contact between the plant tissue and 3-pentanol, suggesting that the volatile form of 3-pentanol was sufficient to elicit induced resistance. Our results indicate that 3-pentanol may potentially be used as a chemical elicitor of induced resistance at the large open-field scale.

Many Gram-negative bacteria produce metabolites that can induce resistance in plants. These are known as ISR-inducing bacteria, and include some *Pseudomonas* and *Serratia* spp. (Van Loon et al. 1998). An *N*-alkylated benzylamine derivative from *Pseudomonas putida* BTP1 (Ongena et al. 2005), 2*R*- and 3*R*-butanediol and phenazine derivatives from *Pseudomonas chlororaphis* O6 (Han et al. 2006; Ryu et al. 2003a; Spencer et al. 2003), and 3-acetobyl-3-hydroxyoxindole from *Strobilanthes cusia* (Li et al. 2008) have been reported to elicit ISR. In addition, bacterial volatiles from specific strains of PGPR promote growth and ISR in *Arabidopsis* seedlings without physical contact with plant roots (Ryu et al. 2003a, 2004). Specifically, volatile emissions from two of several PGPR strains that were tested, namely, *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a, promoted plant growth and elicited induced resistance.

The long shelf life of endospore preparations of bacilli has stimulated the development of commercial products that consist of single organisms or a mixture of *Bacillus* spp. such as Yield Shield and BioYield® (Kloepper et al. 2004). Another mixture of bacilli provides significant protection against *Colletotrichum gloeosporioides* in long cayenne pepper (Jetiyanon et al. 2003; Jetiyanon and Kloepper 2002). Similarly, *Bacillus* sp. BS107 exhibited ISR activity against plant pathogens in pepper (Yang et al. 2009). Although the genus *Bacillus*, which is a representative Gram-positive bacterial genus, has rarely been investigated, *B. subtilis* metabolites have been shown to protect plants against pathogens (Ongena et al. 2007). In our previous study, we reported for the first time that 2-aminobenzoic acid, an ISR-related compound produced by strain BS107, was active against soft-rot disease in tobacco caused by *Pectobacterium carotovorum* subsp. *carotovorum* SCC1. Of the aminobenzoic acids tested, 2- and 4-aminobenzoic acids showed ISR activity against the soft-rot pathogen, but 3-aminobenzoic acid was ineffective. By contrast, further evaluation showed that only 3-aminobenzoic acid (also called para-aminobenzoic acid, PABA) was able to mitigate infection by *X. axonopodis* pv. vesicatoria and CMV, which indicated that host specificity of induced resistance was likely (Song et al. 2013a).

Microbial biocontrol agents often produce VOCs such as acetoin, ethanol, isobutanol, isoamyl alcohol, and isobutyric acid (Fernando et al. 2005; Insam and Seewald 2010). To date, only a few VOCs have been examined for their biocontrol and plant growth-promoting effects (Arrebola et al. 2010; Kai et al. 2009; Mercier and Manker 2005; Ryu et al. 2003a). Ryu et al. (2003a) reported that citrus exposure to 3-hydroxy-2-butanone and 2,3-butanediol produced by *B. subtilis* and *B. amyloliquefaciens* resulted in a significant reduction of soft rot caused by *Erwinia carotovora*. Similarly, Arrebola et al. (2010) reported that VOCs, mainly 3-hydroxy-2-butanone (acetoin) produced by *B. subtilis* or *B. amyloliquefaciens*, were effective against the citrus postharvest pathogens *Penicillium digitatum*, *P. italicum*, and *P. crustorum*, and resulted in reductions in mycelial growth, spore production, germination, and germ tube elongation.

We attempted to identify bacterial volatiles and derivatives with the capacity to increase plant resistance. Previous studies on bacterial volatiles as chemical triggers of systemic resistance were mostly carried out in model plants such as *Arabidopsis* and tobacco. In general, bacteria release 10–40 VOCs into the headspace above bacterial colonies grown on complex media. In 2004, 2,3-butanediol and acetoin were identified as the VOCs responsible for decreasing soft-rot symptoms caused by *E. carotovora* subsp. *carotovora*, suggesting the possible use of bacterial volatiles as environmentally sound biochemical agents for agricultural applications. The disadvantages of using bacterial VOCs in agricultural fields are as follows: 1) rapid evaporation rate, 2) differences in effects between *in vitro* and open-field conditions, and 3) lack of stability of target VOCs. With the aim of overcoming these practical limitations, we examined the effect of 3-pentanol under large field conditions in this study. In 2 years of field trials, 3-pentanol at a concentration of 1 mM consistently protected pepper plants, not only against artificially infiltrated bacteria but also against a naturally occurring viral infection (Figs. 1 and 3). We were unable to determine how much 3-pentanol could be evaporated under specific field conditions of temperature and wind flow; however, because the boiling temperature of 3-pentanol is 121 °C, we do not expect its volatility to be enhanced under natural conditions. However, we cannot rule out the possibility that the small amount of evaporated 3-pentanol is the active agent inducing plant defense. Previous research has determined a role for 3-pentanol in insect sex pheromone signaling between physically separated males and females (Gatti Liguori et al. 2008). To our knowledge, no reports have been published describing

microbially produced 3-pentanol, and it is thus unclear why plants recognize an insect pheromone, 3-pentanol, yet elicit systemic defenses against microbial pathogens. We speculate that GC-MS technology may not yet be sensitive enough to detect microbial 3-pentanol. In our previous study, we successfully detected 1-pentanol by employing Solid-phase microextraction-Gas Chromatography–Mass Spectrometry (SPME-GC-MS) to analyze VOCs from diverse species of soil bacteria and *Escherichia coli* (Farag et al. 2013). Chemical conformational changes of carboxyl acid on a core molecule are common biosynthetic reactions in bacterial metabolism. Recent metagenome and microbiome studies have confirmed that less than 1 % of environmental bacteria are culturable. In addition, several studies have shown that gut bacteria produce diverse insect semiochemicals, including pheromones (Davis et al. 2013). We hypothesize that the insect microbiome contains unculturable bacterial groups that have the capacity to produce 3-pentanol. If this hypothesis is true, plants may have developed defenses in response to bacterial 3-pentanol as well as to the insect pheromones.

In addition to the beneficial effects of 3-pentanol against plant disease, no reduction in fruit yield was detected in pepper plants treated with 3-pentanol. Furthermore, vegetative growth as indicated by shoot height did not differ between 3-pentanol-treated and water-treated plants (Fig. 3c, d). Conversely, plants treated with 0.5 mM BTH suffered a small reduction in shoot length and a large reduction in fruit yield. Overall, this is the first report to demonstrate that a VOC component of insect sex pheromones protects pepper plants against bacterial and viral diseases in a large open-field trial. Further experiments are needed to evaluate the efficacy of chemical derivatives related to 3-pentanol or other insect pheromones in the management of plant pathogens under field conditions.

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