

Volatile Dose and Exposure Time Impact Perception in Neighboring Plants

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Abstract Volatiles emitted from stressed plants can induce resistance in healthy neighbors. It remains unknown, however, how plants perceive volatiles and convert them into internal signals. We exposed lima bean (*Phaseolus lunatus* L.) to different concentrations of either of two volatiles, nonanal and methyl salicylate (MeSA), over 6 or 24 h. Plant resistance to the bacterial pathogen, *Pseudomonas syringae*, was increased significantly after exposure to a headspace with two concentrations of nonanal for 6 h, and the same pattern emerged after an exposure over 24 h. By contrast, exposure to a low concentration of MeSA over 6 h did not significantly reduce bacterial infections, whereas exposure to the same concentration over 24 h significantly enhanced resistance. The dose–response relation that was apparent after 6 h of MeSA exposure disappeared in the 24 h treatment, in which the three tested concentrations caused indistinguishable, high levels of resistance to *P. syringae*. A low concentration of a potentially resistance-enhancing volatile sufficed to cause resistance to pathogens in the receiver plant only after long exposure time. Plant-plant signaling appears to involve the accumulation of volatiles in the receiver.

Keywords Induced resistance · Volatile organic compounds · VOCs · Plant pathogenic bacterium · Plant-plant signaling

Introduction

Healthy plants can enhance their resistance to herbivores, pathogens, or abiotic stress when being exposed to volatile organic compounds (VOCs) that are emitted from stressed plant neighbors. This plant-plant signaling has been described for multiple species comprising monocots and dicots (Heil and Karban, 2010). Field studies conducted with alder (*Alnus glutinosa*), wild tobacco (*Nicotiana attenuata*) exposed to clipped sagebrush (*Artemisia tridentata*), or lima bean (*Phaseolus lunatus* L.), demonstrated that plant-plant signaling functions over short distances of 30 cm up to 1 m (Dolch and Tschardt, 2000; Karban et al., 2003; Heil and Adame-Álvarez, 2010). This distance can decrease in the presence of ozone (Blande et al., 2010). All these observations indicate that the underlying signals are rapidly diluted or degraded to inactive concentrations. It remains unknown, however, how long a plant needs to be exposed to which concentration of a volatile, in order to exhibit a detectable response. Here, we aimed at determining the concentration and minimum time of exposure of lima bean to either of two volatiles, nonanal and methyl salicylate (MeSA). Both compounds enhance resistance in lima bean to the bacterial pathogen, *Pseudomonas syringae* pv. *syringae* strain 61, at the phenotypic and gene expression level (Yi et al., 2009).

Methods and Materials

Seeds of lima bean (*Phaseolus lunatus* L.) were collected from a natural population in the coastal area of the state of Oaxaca, Mexico (15°55' N, 097°09' W). *Pseudomonas syringae* pv. *syringae* strain 61 was kindly provided by Dr. Choong-Min Ryu, KRIBB, Daejeon, S.-Korea. The experiments were conducted in a greenhouse mimicking natural

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light conditions and temperature (12/12 h, D/N at 30°C/26°C). Each three *Phaseolus lunatus* plants were placed in closed transparent boxes of 40 l volume and exposed over 6 or 24 h to one of the two volatiles, nonanal or MeSA (purchased from Sigma-Aldrich, México DF, Mexico). Stable concentrations of the volatile in the headspace were achieved by collocating into the chamber a Petri dish with 2 ml of a lanolin paste in which the compound had been dissolved. We used pastes at concentrations of 800 $\mu\text{g l}^{-1}$ or 8 mg l^{-1} of nonanal and 4 mg l^{-1} , 8 mg l^{-1} or 80 mg l^{-1} MeSA. These concentrations were chosen based on earlier observations (Kost and Heil, 2006; Yi et al., 2009) to produce natural concentrations of the respective VOCs in the atmosphere. For comparison: 190 mg l^{-1} of MeSA in lanolin produced the concentration found in the headspace of a lima bean treated with jasmonic acid (Kost and Heil, 2006), whereas plants treated with benzothiadiazole to induce pathogen resistance emitted about three times more MeSA (Yi et al., 2009). After the exposure time, the Lanolin paste was removed and the boxes were ventilated. Plants were challenged (spray-inoculated) with the pathogen 5 d after VOC exposition to quantify resistance by extracting bacteria 4 d post challenge. Two leaves were collected from each plant, weighed, ground with mortar and pestle in 1 ml of sterilized distilled water, and the supernatant was diluted 1:10, 1:100, and 1:1000. The resulting suspensions were plated on Petri dishes containing KB agar medium with 100 $\mu\text{g ml}^{-1}$ of rifampicin, to which the *P. syringae* strain 61 is resistant. The Petri dishes were incubated at 28°C for 48 h, to count colony forming units (CFUs). All experiments were conducted three times independently with three individual plants each.

Results and Discussion

Exposure to the headspace over a lanolin paste with 800 $\mu\text{g l}^{-1}$ or 8 mg l^{-1} of nonanal for 6 h decreased infection rates significantly, and the same pattern emerged after an exposure over 24 h (Fig. 1a). By contrast, exposure to MeSA at a low concentration (4 mg l^{-1}) over 6 h did not significantly reduce bacterial infections, whereas the same concentration caused a significant effect after an exposure over 24 h (Fig. 1b). The dose–response relation that emerged after 6 h of exposure to MeSA (left part of Fig. 1b) disappeared in the 24 h treatment, in which the three tested concentrations of MeSA caused indistinguishable high levels of resistance to *P. syringae* (right part of Fig. 1b). A defined concentration of a potentially resistance-enhancing volatile caused a significant resistance effect only when the time of exposure was long enough. Three concentrations of MeSA, which had quantitatively different effects after 6 h, induced the same (high) level of resistance after

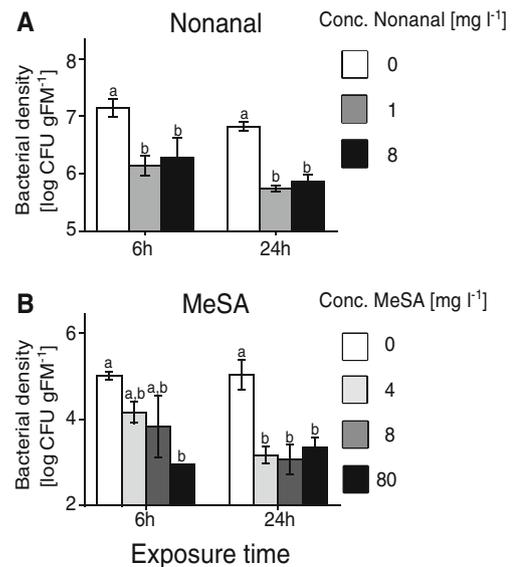


Fig. 1 Bacterial infection in lima bean plants exposed to resistance-inducing volatiles. Bacterial titres [in log (colony forming units) per gram leaf fresh mass] are displayed for *Phaseolus lunatus* plants that had been exposed over 6 or 24 h to different concentrations of nonanal (panel a) and methyl salicylate (MeSA: panel b). Sample size $N=3$ plants per concentration and exposure time; the entire experiment was repeated three times, with similar results. Bars display means \pm standard errors, different letters above bars mark significant differences among treatments ($P < 0.05$ according to least significant difference *post hoc* tests)

24 h of exposure. By contrast, the two concentrations of nonanal tested caused similar levels of resistance, independently of exposure time. In short, it depended on both the concentration and the chemical nature of the compound tested, whether the time of exposure affected the level of the resulting resistance.

Inhibitory effects of exogenous VOCs on the subsequent infection of plants are consistent with two different mechanisms: direct anti-microbial effects and the elicitation of plant-internal pathways for signal perception and transduction. Volatiles can be adsorbed on or in plant leaves (Himanen et al., 2010). Nonanal at a concentration of ca. 8 $\mu\text{g L}^{-1}$ in the growing medium had inhibitory effects on the bacteria, *Bacillus cereus* and *Listeria monocytogenes* (Bisignano et al., 2001), and nonanal at 0.7 $\mu\text{g l}^{-1}$ or MeSA at 0.6 $\mu\text{g l}^{-1}$ in the gas phase inhibited the germination of conidia of the plant pathogenic fungus, *Colletotrichum lindemuthianum*, both *in vitro* and in bean plants (E. Quintana-Rodríguez and M. Heil, unpubl. data). However, Himanen et al. (2010) detected adsorbed hetero-specific VOCs within the first hours after exposing birch (*Betula pendula*) to other plants, whereas our plants were challenged 5 d after VOC exposition. It appears to be unlikely that exogenous VOCs remained in or on leaves over 5 days without being metabolized. By contrast, both VOCs primed resistance-related marker genes in *P. lunatus* under comparable experimental conditions (Yi et al., 2009)

and can thus be involved in a resistance induction. For MeSA, a conversion via salicylic acid-binding protein 2 (SABP2) into salicylic acid (SA) has been brought forward for tobacco (Park et al., 2007) and can be suspected also for lima bean. In this scenario, MeSA or the active hormone, SA, might accumulate in the leaf until a critical threshold level has been reached. For nonanal, the mechanism of its conversion into an active plant-internal signal remains a matter of speculation. We hypothesize that plant-plant signaling can depend on mechanisms that involve the accumulation of volatiles, or of a down-stream signal, in the receiving plant

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