

# One shot-two pathogens blocked

Exposure of *Arabidopsis* to hexadecane, a long chain volatile organic compound, confers induced resistance against both *Pectobacterium carotovorum* and *Pseudomonas syringae*

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**Abbreviations:** VOC, volatile organic compounds; PGPR, plant growth-promoting rhizobacteria; ISR, induction of systemic resistance

Bacteria and plant derived volatile organic compounds have been reported as the chemical triggers that elicit induced resistance in plants. Previously, volatile organic compounds (VOCs), including acetoin and 2,3-butanediol, were found to be emitted from plant growth-promoting rhizobacteria (PGPR) *Bacillus subtilis* GB03, which had been shown to elicit ISR and plant growth promotion. More recently, we reported data that stronger induced resistance could be elicited against *Pseudomonas syringae* pv *maculicola* ES4326 in plants exposed to C13 VOC from another PGPR *Paenibacillus polymyxa* E681 compared with that of strain GB03. Here, we assessed whether another long hydrocarbon C16 hexadecane (HD) conferred protection to *Arabidopsis* from infection of a biotrophic pathogen, *P. syringae* pv *maculicola* and a necrotrophic pathogen, *Pectobacterium carotovorum* subsp *carotovorum*. Collectively, long-chain VOCs can be linked to a plant resistance activator for protecting plants against both biotrophic and necrotrophic pathogens at the same time.

The group of root-colonizing bacteria that is called as plant growth-promoting rhizobacteria (PGPR) confers beneficial effects to plants, including induction of systemic resistance (ISR) against phytopathogens and herbivores.<sup>1-3</sup> Many bacterial determinants for such mechanism from PGPR have been discovered.<sup>4,5</sup> Previous research suggested that components of the bacterial cell membrane and some secondary metabolites contributed to ISR.<sup>1-3,6</sup> In 2003, bacterial volatiles produced from *Bacillus subtilis* strain GB03 were found to act as a determinant for ISR.<sup>7-9</sup> Out of > 30 volatile organic compounds (VOCs) from strain GB03, a C4 hydrocarbon 2,3-butanediol played a critical role in ISR against *Pectobacterium carotovorum* subsp *carotovorum*. A later study revealed that ethylene signaling was essential for elicitation of ISR using *PDF1.2* and *Jin14* indicator genes for ethylene/jasmonate and jasmonate signaling, respectively.<sup>7</sup> Application of volatiles from strain GB03 upto *Arabidopsis* seedlings also caused decrease of disease severity caused by *P. carotovorum*

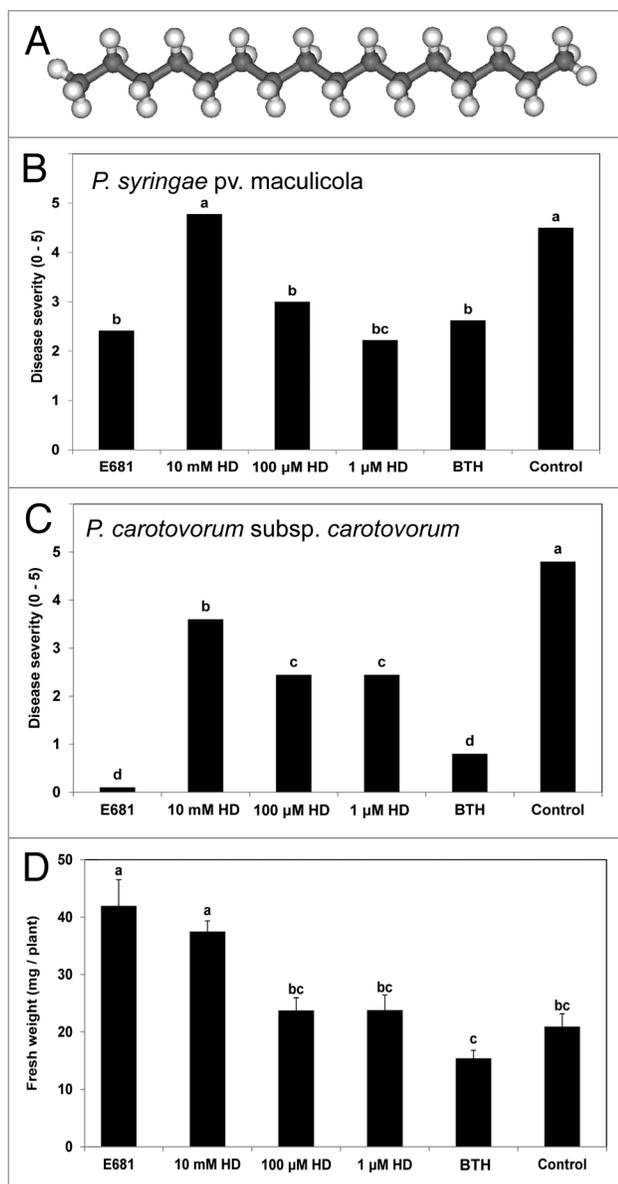
subsp *carotovorum*. In addition to promoting ISR, strain GB03 volatiles elicited an environmental stress resistance against salt by modulating sodium homeostasis in the *Arabidopsis* root.<sup>10</sup> Similarly, 2,3-butanediol, emitted from the Gram-negative bacterium *Pseudomonas chlororaphis* O6, caused stomatal closure resulting in drought resistance.<sup>11</sup> Moreover, the same authors indicated 2,3-butanediol did not effectively induce ISR against *Pseudomonas syringae* pv *tabaci* but found it did successfully suppress soft-rot development caused by *P. carotovorum* in tobacco.<sup>12</sup> Taken together, 2,3-butanediol did not triggers strong ISR against biotrophic bacteria but did against necrotrophic bacteria.

In a search for novel PGPR strains, *Paenibacillus polymyxa* E681 was selected for a promising PGPR that can be utilized as a biocontrol agent and a yield increasing bacterium in different crop species such as cucumber, sesame and pepper plants.<sup>2,13,14</sup> One of the possible explanations for strain E68-mediated enhancement of plant growth and inhibition of plant diseases is

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**Figure 1.** Induced systemic resistance against *Pseudomonas syringae* pv *maculicola* ES4326 and *Penicillium carotovorum* subsp *carotovorum* and plant growth promotion by *Paenibacillus polymyxa* E681 and bacterial volatile hexadecane. (A) Chemical structure of hexadecane. (B) Induced systemic resistance against *P. syringae* pv *maculicola* ES4326 elicited by *P. polymyxa* E681, 10 mM, 100  $\mu$ M and 1  $\mu$ M hexadecane and 0.33 mM BTH using the I-plate system. Disease severity (0 = no symptom, 5 = severe chlorosis) was recorded seven days after pathogen challenge at  $10^8$  cfu/ml on the leaves at 7 d after application of strain E681 and chemicals in roots. (C) Induced systemic resistance against *P. carotovorum* subsp *carotovorum* SCC1 elicited by *P. polymyxa* E681, 10 mM, 100  $\mu$ M and 1  $\mu$ M hexadecane and 0.33 mM BTH using the I-plate system. Disease severity (0 = no symptom, 5 = complete soft-rot) was recorded 24 h after drop-inoculation of pathogen at  $10^7$  cfu/ml. (D) Plant growth responses by *P. polymyxa* E681, 10 mM, 100  $\mu$ M and 1  $\mu$ M hexadecane and 0.33 mM BTH using the I-plate system. Sterile distilled water used as "control." Different letters indicate significant differences between treatments, according to least significant difference at  $p = 0.05$ .

the production of the plant hormones auxin and cytokinin and secondary metabolites polymyxin and 2,3-butanediol.<sup>15-17</sup>

Here, our objective was to examine if volatiles from *P. polymyxa* E681 could elicit ISR against both necrotrophic and biotrophic pathogens. A C16 VOC, hexadecane, was produced only by strain E681 through the careful assessment of the volatiles emitted from two different *Bacillus* spp and strain E681 (Fig. 1A). Our results identified a novel hydrocarbon-based signal molecule from strain E681 as the bacterial determinant(s) involved in ISR in plants.

The pharmaceutical application of hexadecane in the I-plate system confer ISR in *Arabidopsis* against *P. syringae* pv *maculicola* ES4326 as a biotrophic bacterium at seven day-post inoculation and *P. carotovorum* subsp *carotovora* SCC1 as a necrotrophic bacterium at 24 h after drop-inoculation.<sup>7</sup> Previously, indirect application (physical separation between plant and treatment) of any bacterial volatiles did not induce systemic resistance against any biotrophic pathogenic bacteria. In this study, we obtained the first evidence that release of hexadecane can significantly ISR against *P. syringae* pv *maculicola* ES4326 (Fig. 1B). The different concentrations of hexadecane showed different effects against *P. syringae* in comparison with *P. carotovorum*. The highest concentration of hexadecane (10 mM) did not induce ISR against *P. syringae*, although it did against *P. carotovorum* (Fig. 1B). However, disease severity in *Arabidopsis* seedlings exposed to 100  $\mu$ M and 1  $\mu$ M hexadecane was significantly different from 10 mM hexadecane treatment. The indirect treatment of 0.33 mM benzothiadiazole (BTH) reduced symptom development when compared with control (Fig. 1B and C). It is noteworthy that no concentration of hexadecane affected plant growth (data not shown), indicating that hexadecane from VOCs produced by strain E681 plays a role only in ISR. In addition, foliar fresh weights of *Arabidopsis* seedlings were significantly increased by treatment with strain E681 and 10 mM HD compared with water control (Fig. 1D). The treatments of 0.33 mM BTH and 100  $\mu$ M and 1  $\mu$ M HD did not result in significantly higher foliar fresh weights compared with the control (Fig. 1D).

To assess induction of defense gene expression of three marker genes, *Pathogenesis-Related gene 1 (PR1)* for salicylic acid signaling, *PDF1.2* for jasmonic acid signaling and *ChiB* for ethylene signaling.<sup>18</sup> Quantitative RT-PCR technique was applied 0 and 6 h post inoculation of hexadecane into roots and at 0 and 3 h post pathogen challenge on the leaves. Direct application of 0.35 mM hexadecane on the plant roots significantly increased *PR1* gene expression but did not increase *PDF1.2* and *ChiB* gene expressions (data not shown). A hexadecane dose of 0.35 mM, which had the most consistent results across different concentrations of hexadecane, did not show defense priming on the three defense genes (data not shown). The hexadecane treatment upregulated transcription of *PR1* gene in the leaves at a level 4,000-fold higher at 6 h after direct treatment into roots and maintained transcription at lower levels up to 100-fold when pathogen challenged (data not shown).

Using by plant hormonal mutants, we determined that ethylene or cytokinin signaling was necessary for increased plant growth in response to the VOCs emitted by strain E681.<sup>8</sup> We

established that GB03 volatiles triggered ethylene-signaling as revealed from overexpression of *PDF1.2*.<sup>7</sup> In contrast, volatile emission from strain E681 caused to upregulate GUS fused in *PRI* promoter. This result suggests that certain VOCs emitted by strain E681 can be deemed comparable to those released from strain GB03. Surprisingly, strain E681-mediated ISR capacity was greater than in plants exposed to those emitted from strain GB03.<sup>19</sup>

Previously, an analysis of the VOCs released from three bacteria, including *Paenibacillus polymyxa* from potato tubers, showed that dimethylformamide, pentadecene and hexadecane are unique volatiles generated by *P. polymyxa*.<sup>20</sup> This result is in agreement with our previous data.<sup>21</sup> A comparison with our previous data<sup>21</sup> revealed that hexadecane was emitted exclusively from *P. polymyxa*, but not from *B. subtilis* and *B. amyloliquifaciens*. The exclusive bacteria species that reported to emit hexadecane is the cyanobacterium *Oscillatoria perornata*.<sup>22</sup> Although volatiles from fungi and bacteria have widely obtained attention for protecting plants from pathogens, the plant's response to hexadecane has not been thoroughly assessed. Hexadecane is a novel candidate signal molecule that can induce *PRI* expression. How hexadecane was recognized and reacted by plants has not been intensively considered through the use of large scale gene expression techniques.

Previous study revealed a plant growth regulator cytokinin secreted by *P. polymyxa* played an critical role on plant growth promotion in *Arabidopsis*.<sup>17</sup> In addition to cytokinin, the current

results present bacterial volatiles as additional bacterial determinants, corresponding to promotion of seedling growth by *P. polymyxa* E681 (Fig. 1D). Moreover, the strain E681 produces hexadecane out of a blend of volatiles that trigger ISR against biotrophic pathogenic bacterium, *P. syringae*. Hexadecane released by strain E681 induced expression of the *PRI* gene as a greater extent than those released by strain GB03.<sup>19</sup> Our results newly indicate that strain E681 produces hexadecane, which triggers an ISR response that is stronger than previous bacterial volatiles such as acetoin and 2,3-butanediol. The further greenhouse and field experiments can broaden our knowledge to apply bacterial volatiles to protect crop plant against plant pathogens.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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