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Production of plant growth modulating volatiles is widespread among rhizosphere bacteria and strongly depends on culture conditions

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Summary

Recent studies have suggested that bacterial volatiles play an important role in bacterial–plant interactions. However, few reports of bacterial species that produce plant growth modulating volatiles have been published, raising the question whether this is just an anecdotal phenomenon. To address this question, we performed a large screen of strains originating from the soil for volatile-mediated effects on *Arabidopsis thaliana*. All of the 42 strains tested showed significant volatile-mediated plant growth modulation, with effects ranging from plant death to a sixfold increase in plant biomass. The effects of bacterial volatiles were highly dependent on the cultivation medium and the inoculum quantity. GC-MS analysis of the tested strains revealed over 130 bacterial volatile compounds. Indole, 1-hexanol and pentadecane were selected for further studies because they appeared to promote plant growth. None of these compounds triggered a typical defence response, using production of ethylene and of reactive oxygen species (ROS) as read-outs. However, when plants were challenged with the flg-22 epitope of bacterial flagellin, a prototypical elicitor of defence responses, additional exposure to the volatiles reduced the flg-22-induced production of ethylene and ROS in a dose-dependent manner, suggesting that bacterial volatiles may act as effectors to inhibit the plant's defence response.

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Introduction

Plant growth is largely influenced by the presence of bacteria, both underground in the rhizosphere (Bonfante and Anca, 2009) and aboveground in the phyllosphere (Lindow and Brandl, 2003). In the rhizosphere, some bacteria may be neutral or deleterious, but the plant growth promoting rhizobacteria (PGPR) are of particular interest (Lugtenberg and Kamilova, 2009). Many bacteria–plant interactions are based on the direct contact of bacteria with the plant, but interactions at a distance through volatile substances, have emerged as a novel way of signalling between bacteria and plants (Ryu *et al.*, 2003). Bacteria are well-known producers of a diverse blend of volatile compounds (Schulz and Dickschat, 2007), both organic (volatile organic compounds, VOCs) and inorganic (e.g. hydrogen cyanide or carbon dioxide). Some of the bacterial volatiles may simply be toxic to the plant, as exemplified by HCN (Rudrappa *et al.*, 2008; Blom *et al.*, 2011), or other bacterial volatiles such as dimethyl disulfide, ammonia and 3-phenylpropionic acid (Vespermann *et al.*, 2007; Chung *et al.*, 2010; Kai *et al.*, 2010; Kai and Piechulla, 2010). More interestingly, positive effects of bacterial volatiles were described as well: Ryu and colleagues (2003) reported a fivefold growth enhancement of *A. thaliana* when exposed to the volatiles of *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a and they ascribed the effect to *2R-3R*-butanediol and its precursor acetoin, two products of the butanediol fermentation pathway (Syu, 2001). Besides growth promotion, Ryu and co-workers also reported the induction of systemic resistance in *A. thaliana* upon exposure to the complex volatile blend of *B. subtilis* GB03 (Ryu *et al.*, 2004). Similar effects have been described in *Nicotiana benthamiana* (Ryu *et al.*, 2005a; Han *et al.*, 2006; Cortes-Barco *et al.*, 2010a) and *Agrostis stolonifera* (Cortes-Barco *et al.*, 2010b).

With the discovery of the plant growth promoting properties of bacterial volatiles, a search for possible mechanisms has been initiated. Analysis of *Arabidopsis* mutants affected in hormone signalling has indicated possible involvement of cytokinin (Ryu *et al.*, 2003), ethylene (Ryu *et al.*, 2004; Cho *et al.*, 2008), auxin, salicylic acid, brassinosteroids, gibberellins (Ryu *et al.*, 2005b), abscisic acid and jasmonic acid (Cho *et al.*, 2008) in the reaction of

plants to bacterial volatiles, suggesting that volatile-mediated signalling in plants is a complex process. Changes in auxin homeostasis and transport were investigated more thoroughly using microarray analysis and auxin reporter lines (*DR5::GUS*). The results of these investigations suggested an upregulation of auxin production and of expression of transporter genes as well as an increased auxin transport to the roots in response to the volatiles of *B. subtilis* GB03 (Zhang *et al*., 2007).

Until now, studies on bacterial volatiles have mainly focussed on two *Bacillus* species and occasionally on *Pseudomonas* and *Serratia* species, typically grown on the rich culture media routinely used in microbiology laboratories. Moreover, while the effect of some single compounds was tested on plants, the perception of volatiles by the plant and the early signalling events upon exposure to single volatiles has not yet been elucidated. In this study, we explored the volatile-mediated impact of bacteria on plants using a wide array of soil and rhizosphere isolated strains of relevance for plant promotion or biocontrol activities, including members of the genera *Burkholderia*, *Pseudomonas* and *Serratia*. We particularly focused our attention on *Burkholderia* species, because they are commonly present in the soil and have been shown to interact with plants in many different ways, including nitrogen fixation and plant growth promotion as well as plant pathogenicity (reviewed in Coenye and Vandamme, 2003 and Stoyanova *et al*., 2007). We formulated the following hypotheses: (i) the volatile-mediated impact of bacteria on plants is a general phenomenon and is not restricted to the few *Bacillus*, *Pseudomonas* and *Serratia* species investigated before now, (ii) production and activity of volatiles varies depending on the culture medium, (iii) volatiles affect plant growth in a dose-dependent manner, and (iv) the perception of bacterial volatiles by plants is specific for each compound.

To test these hypotheses, we carried out a large assessment study of the growth response of the model plant *A. thaliana* to the exposure of 42 bacterial strains grown on four different culture media. In parallel, we analysed the volatiles produced by each strain on each medium using GC-MS analysis. This led to the identification of approximately 40 volatile organic compounds, which were subsequently tested on *A. thaliana* in different concentrations. We chose three bioactive volatile compounds, indole, 1-hexanol and pentadecane for further study, and we examined whether they elicited some of the responses known to be induced by microbe-associated molecular patterns (MAMPs), which are perceived as 'danger signals' (Boller and Felix, 2009). This was not the case. However, the volatiles modulated the response of *A. thaliana* cells to the prototypical MAMP flg-22, the elicitor-active epitope of bacterial flagellin (Felix *et al*., 1999), indicating that bacterial volatiles may act as micromolecular 'effectors' affecting the basal defence of the plant.

Results

The production of plant growth affecting volatiles is a general feature of rhizosphere bacteria and strongly depends on the culture medium

Forty-two soil-borne bacterial strains were screened on four different media for their volatile-mediated effect on 6-day-old seedlings of *A. thaliana* Col-0. In addition to Luria–Bertani (LB) medium, we used MR-VP medium because it favours butanediol production, MS medium because it has also been used in previous studies (Ryu *et al*., 2003) and Angle medium because it is designed to mimic the soil environment (Angle *et al*., 1991). After 3 weeks plants were harvested and the shoot fresh weight was determined as an estimate of the biomass. The results of this experiment are shown in Table 1. Remarkably, every strain tested led to significantly higher or lower plant biomass in at least one of the media tested. While almost all strains promoted plant growth when grown on MR-VP, even though very few produced butanediol (see Table 2), the strongest effects were observed on LB plates (from 6% of control plant biomass for *Pseudomonas putida*, to 620% for *Pandoraea norimbergensis*). The impact of bacterial volatiles was much less pronounced on the less nutrient-rich media MS and Angle, even though 18 and 12 bacterial strains still significantly promoted plant growth on these two media respectively. Negative effects were only observed on the more nutrient-rich media LB and MR-VP, and plant death (reflected by drastic weight reduction and chlorosis) was only observed when bacteria were grown on LB. Only one strain, *B. pyrrocinia* Bcc171, caused a volatile-mediated plant growth promotion on all four media tested (see Fig. 1). For most of the other strains (e.g. *B. phytofirmans* LMG 22487, *B. phenoliruptrix* LMG 22037, *P. aeruginosa PUPa3* or *P. fluorescens* WCS 417r), highly contrasting effects were observed between volatiles produced on LB and on MR-VP, as is shown for *C. violaceum* in Fig. 1.

The effect of volatiles on plant growth depends on the bacterial dose

In the above-mentioned experiment, we observed variations that seemed to correlate with different colony sizes or variable distance of the plants to the bacterial colony (See Fig. 1B, *B. pyrrocinia* grown on LB, causing chlorosis in leaves most closely exposed to the volatiles). To test the effect of varying amounts of bacteria on the plant's response to volatiles, we performed a dose-response

Table 1. The volatile-mediated effect of 42 soil-borne bacterial strains on the growth of *Arabidopsis thaliana* Col-0.

No.	species	strain	LB	MR-VP	MS	Angle
1	*Bacillus cereus*	B-569	ns	**245**	ns	ns
2	*Burkholderia andropogonis*	LMG 2129	**206**	**190**	**187**	ns
3	*Burkholderia anthina*	LMG 20980	**284**	**180**	ns	ns
4	*Burkholderia caledonica*	LMG 19076	**251**	**253**	ns	ns
5	*Burkholderia caribensis*	LMG 18531	**331**	**427**	ns	ns
6	*Burkholderia caryophylli*	LMG 2155	**251**	**327**	ns	ns
7	*Burkholderia cepacia*	LMG 1222	**358**	**216**	ns	**263**
8	*Burkholderia fungorum*	LMG 16225	ns	**254**	**161**	**155**
9	*Burkholderia gladioli*	LMG 2216	ns	**193**	**127**	ns
10	*Burkholderia glathei*	LMG 14190	ns	**268**	ns	**191**
11	*Burkholderia glumae*	LMG 2196	**347**	**290**	**188**	ns
12	*Burkholderia graminis*	LMG 18924	ns	**375**	**126**	ns
13	*Burkholderia hospita*	LMG 20598	**226**	**236**	ns	ns
14	*Burkholderia kururiensis*	LMG 19447	**335**	**374**	ns	**154**
15	*Burkholderia lata*	LMG 6993	**410**	ns	ns	ns
16	*Burkholderia lata*	LMG 22485	ns	**301**	ns	**160**
17	*Burkholderia phenazinium*	LMG 2247	ns	**311**	**154**	ns
18	*Burkholderia phenoliruptrix*	LMG 22037	*34*	**262**	**152**	**219**
19	*Burkholderia phytofirmans*	LMG 22487	*18*	**356**	**221**	ns
20	*Burkholderia pyrrocinia*	LMG 21822	**315**	**380**	**308**	**281**
21	*Burkholderia sacchari*	LMG 19450	ns	**411**	**225**	ns
22	*Burkholderia sordidicola*	LMG 22029	**311**	**365**	ns	ns
23	*Burkholderia terricola*	LMG 20594	ns	**355**	ns	ns
24	*Burkholderia thailandensis*	LMG 20219	**578**	**303**	ns	ns
25	*Burkholderia tropica*	LMG 22274	**255**	**201**	ns	**203**
26	*Burkholderia xenovorans*	LMG 21463	**167**	**234**	**166**	ns
27	*Cellulomonas uda*		ns	**187**	**132**	ns
28	*Chromobacterium violaceum*	CV0	*14*	**292**	ns	ns
29	*Cupriavidus necator*	LMG 1199	ns	**303**	ns	ns
30	*Escherichia coli*	OP50	ns	**168**	ns	ns
31	*Limnobacter thiooxidans*	LMG 19593	*32*	*74*	ns	ns
32	*Pandoraea norimbergensis*	LMG 18379	**620**	**224**	**163**	ns
33	*Pseudomonas aeruginosa*	PUPa3	*18*	**395**	ns	**143**
34	*Pseudomonas chlororaphis*		*36*	**192**	ns	**143**
35	*Pseudomonas fluorescens*	WCS 417r	*38*	**291**	**168**	**320**
36	*Pseudomonas putida*	ISOf	*6*	**210**	ns	**217**
37	*Serratia entomophilia*	A1MO2	ns	**319**	**134**	ns
38	*Serratia marcescens*	MG1	ns	**282**	**144**	ns
39	*Serratia plymuthica*	IC14	*21*	*86*	**125**	ns
40	*Serratia plymuthica*	HRO-C48	ns	**357**	**324**	ns
41	*Serratia proteamaculans*	B5a	**331**	**373**	ns	ns
42	*Stenotrophomonas rhizophilla*	ep10-p69	ns	**369**	ns	ns

The values represent the percentage of the plant shoot's fresh weight compared with the non-inoculated control. Values < 100 indicate growth inhibition and are printed in *italic*; values > 100 indicate growth promotion and are printed in **bold**; 'ns' means no significant effect. Significance was tested with a Student's *t*-test ($P < 0.05$, $n = 3$–4).

experiment. We chose *C. violaceum* and *B. pyrrocinia* as model strains and varied the amounts of inoculum between 1, 3 and 9 drops of 10 µl of overnight cultures. In addition, 20 µl and 100 µl of the same overnight culture were spread over half of the plate. Plants were grown on MS on the other Petri dish half. The same experiment was performed in the absence of plants to collect volatiles for GC-MS analysis (see below). In the richer media LB and MR-VP, a clear dose-dependency was observed (Fig. 2) but increasing the dose of the inoculum did not influence the results when the nutrient-poor media MS and Angle were used (data not shown). For *C. violaceum*, the killing effect on LB increased with higher bacterial dose and was strongest when bacteria were spread over half a plate. The promoting effect on MR-VP only occurred when a one-drop-inoculum was used, while higher drop numbers or spreading did not cause significant plant growth promotion. Similarly, when *B. pyrrocinia* was grown on LB, growth promotion only occurred with a low bacterial dose (1 drop); intermediate doses (3 drops) did not lead to significant changes, while a higher dose, or spreading the inoculum, caused plant death. On MR-VP, plant growth promotion was highest with three drops of 10 µl, while higher doses or spreading led to the loss of growth promotion, but did not induce deleterious effects as seen on LB. Additionally, the influence of quorum sensing (QS) on the production of bioactive volatiles was tested for both strains, using the QS mutant *C. violaceum* CV026 and a

Table 2. The production of volatile organic compounds by bacteria as measured by GC-MS.

	Production on			
Compound	LB	MR-VP	MS	Angle
Undecane	nd	nd	36	nd
Pentadecane	21	nd	37	nd
1-hexanol	nd	2–7, 10–12, 16–19, 21, 23, 27, 28, 30, 34, 38, 41	1, 2, 6, 7, 14, 18, 21, 23–25, 27, 37	1–3, 5–8, 13, 14, 16–18, 20–24, 26, 30, 38
1-heptanol	1, 5, 9, 12, 17–21, 23, 34, 41	1, 2, 4, 6–14, 16–26, 28–31, 34, 37, 38	6, 16, 18–21, 24, 25, 31, 34, 36, 38	1, 5, 7, 12, 18, 22, 24, 30, 31, 34, 35, 38, 39, 42
2-tetradecanol	1, 25	36	33, 34	nd
2,3-butanediol	nd	28, 41	42	24
3-methyl-1-butanol	1–9, 11–15, 17–19, 20*, 21, 23–27, 29–31, 34–42	1–19, 20*, 21–27, 28*, 30–32, 34–42	20*, 31	39–41
Phenol	4, 20, 28, 41	9, 17, 20*, 25, 32	nd	nd
Phenylmethanol (benzyl alcohol)	1, 2, 5, 6, 8, 9, 11–13, 17, 19–21, 23, 26, 27, 30, 34–36, 41	2, 11–14, 17, 21, 30, 34, 41	2, 6, 21, 23, 24, 27, 30, 34, 36–38, 40, 41	2
2-phenylethanol	1, 2, 4–6, 11, 12, 14, 17–19, 21, 23, 26, 27, 30, 37–39, 41, 42	1, 2, 4–9, 11–15, 17, 18, 20*, 21, 23, 24, 26, 27, 30, 35, 37–42	26, 38, 40, 41	22, 26, 37, 42
4-Methylanisole	37, 38	11, 12, 36	1, 4–6, 8–11, 16, 18, 20, 23, 24, 27, 28, 30, 34, 37	37
1-Butoxy-2-propanol (propylene glycol butyl ether)	3, 9, 15, 20, 28, 38, 41	11, 17, 19, 20*, 23, 25, 28*, 32, 36, 40, 41	nd	20*
2-furaldehyde (furfural)	27, 38	1, 4, 28, 29, 32, 33, 37, 40	34	27, 34
2-furanmethanol (furfuryl alcohol)	nd	nd	1, 2, 6, 20, 21, 23, 24, 30, 36, 37, 40–42	1, 2, 22, 27, 30, 37
Tetrahydro-3-furanmethanol	2, 5, 6, 8–12, 16, 20, 27, 34, 39	3–6, 8–11, 16, 20, 27, 30, 39	2–6, 8–11, 13, 16, 20, 27, 29, 30, 34, 39	2, 4, 5, 9–13, 16, 20, 27, 30, 39
Decanal	nd	nd	13	nd
Undecanal	9, 20	7, 9, 15, 16, 27, 39, 41	nd	16, 18, 20, 39
Tridecanal	nd	nd	nd	20*, 28*
trans-2-dodecenal	20, 30	9, 27, 30	20, 27, 34	16
Phenylacetaldehyde	nd	nd	12	nd
2-nonanone	2, 3, 5, 8–12, 15, 18, 19, 20*, 23, 28*, 32, 34–42	2, 3, 5–9, 11, 12, 20*, 23, 28*, 30, 34–41	6, 7, 9, 11, 13, 20*, 34, 37–39	9, 38
2-decanone	1, 2, 5, 6, 8–10, 14, 16, 17, 20, 24, 27, 37–40	3–6, 8–13, 16, 17, 19, 20, 24, 27, 34, 38, 39	24, 37, 38, 39	6, 24, 27
2-undecanone	2, 5, 8–10, 12, 17–19, 20*, 23, 25, 26, 28*, 32, 35, 36, 38–42	5–9, 11–13, 15, 17, 19, 20*, 23, 28*, 30, 32, 35–41	9, 34, 37–41	2, 16, 38, 39
2-tridecanone	10, 12, 38, 39	12, 30	39	1–4, 8, 16, 18, 30, 34, 40
3-hydroxybutanone (acetoin)	38, 41, 42	1, 25, 28, 31, 34, 37–42	1, 12, 31, 32, 34, 37–41	nd
1-phenyl-1-propanone (propiophenone)	24, 41	20*, 24	20*, 24	20*, 24
2-aminoacetophenone	1–6, 8–11, 15–21, 23,24, 28, 30, 33, 35–42	3, 11, 12, 17, 20, 23, 24, 30, 33, 35, 36, 38, 39, 41	1, 4, 5, 9, 10, 16, 20, 27, 30, 33, 34	3, 4, 16, 20*, 24, 30, 33
Indole	30, 33, 34, 36	15, 28*, 33	2–5, 8, 9–12, 20, 27, 30, 33, 34, 36, 41, 42	28, 30
1H-benzotriazole	1, 2, 7, 8, 11, 16–19, 21, 23, 26, 28, 35, 38–40, 42	1, 3, 6, 7, 13, 15, 20*, 28*, 33, 36, 39, 41	23, 28*	8, 11, 18, 24
2-methylquinoxaline	3–5, 9, 10, 16, 27, 34, 37, 38, 40	3–5, 9, 13, 15, 16, 20, 27, 34, 39	2–6, 8–10, 15, 16, 18–20, 27, 30, 34, 35, 39, 41	1–6, 8–13, 16, 20, 21, 23, 24, 26, 27, 29, 30, 34, 35, 39
Dimethyl disulfide	5, 11, 15, 18, 19, 20*, 21, 23, 26, 28*, 32, 33, 35–38, 40, 41	5, 17–19, 20*, 28*, 29, 32, 35–37, 41	nd	31
Dimethyl trisulfide	1, 3, 6–10, 12, 15, 20*, 26, 28*, 29, 36–38, 41	5, 6, 8–11, 14–16, 18, 19, 20*, 28*, 29, 32, 33, 36, 41, 42	nd	nd
1-undecene	2, 3, 6, 8–11, 15, 17–21, 23, 24, 27, 30, 33–36, 39, 40, 10	2, 5, 12, 16, 21, 24, 27, 28, 33, 35, 36	33, 36	3–5, 7, 8, 10, 15, 17, 30, 31, 33, 35
Dodecanoic acid	nd	2, 6, 15, 27, 29, 30, 31, 36	2, 9, 16, 25, 30	1, 3, 27, 29–32, 37, 39, 40
Tetradecanoic acid (myristic acid)	nd	nd	2, 12, 16, 20, 25, 34–36	nd
3-methylbutanoic acid (isovaleric acid)	nd	2–6, 8, 10–12, 16, 20, 27, 30, 39	2–6, 8–11, 16, 20, 27, 30, 34	2–6, 8–11, 16, 20, 26, 30, 34, 39
Benzyl acetate	2, 4, 5, 9, 13, 16, 22, 27, 28, 34, 35	3, 5, 13, 16, 17, 20, 21, 27, 30, 35	nd	nd
Octyl formate	2, 4–6, 8–11, 27, 30, 34, 39	2–4, 6, 16, 20, 23, 27, 34, 39	2, 4, 6, 8, 9, 11, 16, 20, 27, 30, 34, 39	2–4, 6, 10, 20, 27, 30, 34, 39

Significance was tested according to a Student's *t*-test compared with non-inoculated control plates ($P < 0.05$, $n = 3$–6), the numbers refer to the strains in Table 1, 'nd' indicates not detected. Strains marked with an asterisk (*) showed significant dose-dependent production of VOCs.

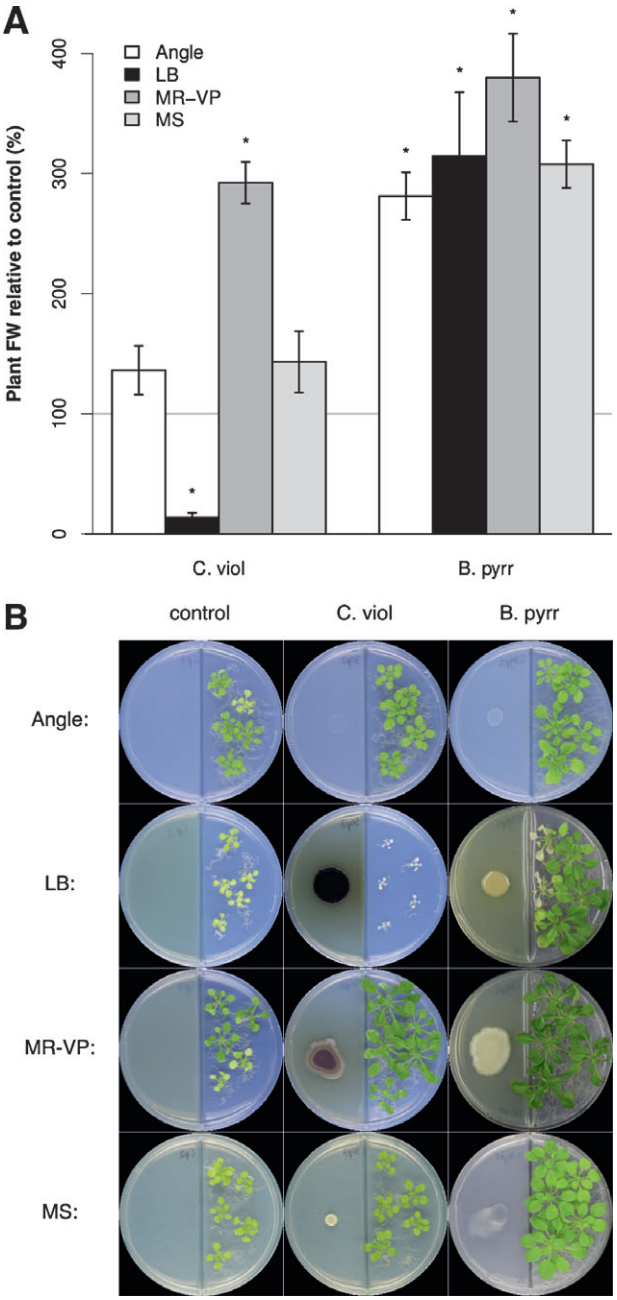

Fig. 1. The effect of the volatiles of *Chromobacterium violaceum* CV0 (C. viol) and *Burkholderia pyrrocinia* Bcc171 (B. pyrr) on the growth of *Arabidopsis thaliana* Col-0 with the bacteria cultivated on four different media. Error bars represent standard errors, results marked with an asterisk (*) are significantly different from the non-inoculated control according to a Student's *t*-test ($P < 0.05$, $n = 3–4$).

QS quenched strain of *B. pyrrocinia*. The killing effect of *C. violaceum* was no longer present with the *C. violaceum* mutant, whereas no difference could be observed between the wild-type and the quenched strain of *B. pyrrocinia* (Fig. 3).

Search for volatiles affecting plant growth

Headspace volatiles of the 42 bacterial strains grown in triplicate on each medium were sampled and analysed by GC-MS. The volatile profiles of the strains were compared with the volatiles retrieved from the respective control (non-inoculated medium). This resulted in 36 compounds of bacterial origin (Table 2), which were selected for further analysis. Detailed data about the GC-MS analysis of these compounds can be found in Table S2. Moreover, the dose-response experiment (see above) led to the identification of three additional compounds (1-butoxy-2-propanol, dimethyl trisulfide and 1-phenyl-1-propanone; see also Table 2 and Table S3). The volatile compounds were first tested as pure chemicals on the plants by applying 10 ng or 10 μg dissolved in dichloromethane (DCM) and lanolin on a filter paper in one of the halves of two compartment Petri dishes and growing the plants in the other. Three volatiles (1-hexanol, indole and pentadecane) yielded promising results in this first screen and were tested further in a range of 1 ng to 1 mg. 1-Hexanol showed only weak plant growth promotion when applied in moderate amounts, indole strongly promoted growth in low quantities, but killed the plants when 1 mg was used, and pentadecane stimulated plant growth best when applied in high concentrations (Fig. 4).

Effects of indole, 1-hexanol and pentadecane on basal and elicitor-induced ethylene production and oxidative burst

Indole, 1-hexanol and pentadecane were tested for their effect on two typical plant responses of microbial associated molecular patterns (MAMPs), namely ethylene production and burst of reactive oxygen species (ROS, Boller and Felix, 2009). None of the selected compounds induced ethylene production or an oxidative burst when supplied alone to the plants. However, when the elicitor flg-22, a fragment of bacterial flagellin (Felix *et al.*, 1999) was supplied in addition to the volatiles, we observed an alteration of the MAMP-response. 1-Hexanol had an inhibiting effect on flg-22-induced ethylene production, increasing with concentration (Fig. 5). Indole did not show significant effects on flg-22-induced ethylene production in low concentrations, but almost completely blocked ethylene production when added in the highest concentration. The ethylene response to pentadecane could not be tested due to interference of the compound with the gas chromatograph. Concerning the oxidative burst, we observed a slight but non-significant decrease in the flg-22-induced ROS production burst when indole was supplied in low concentrations, while high amounts of indole seemed to increase and prolong the oxidative burst

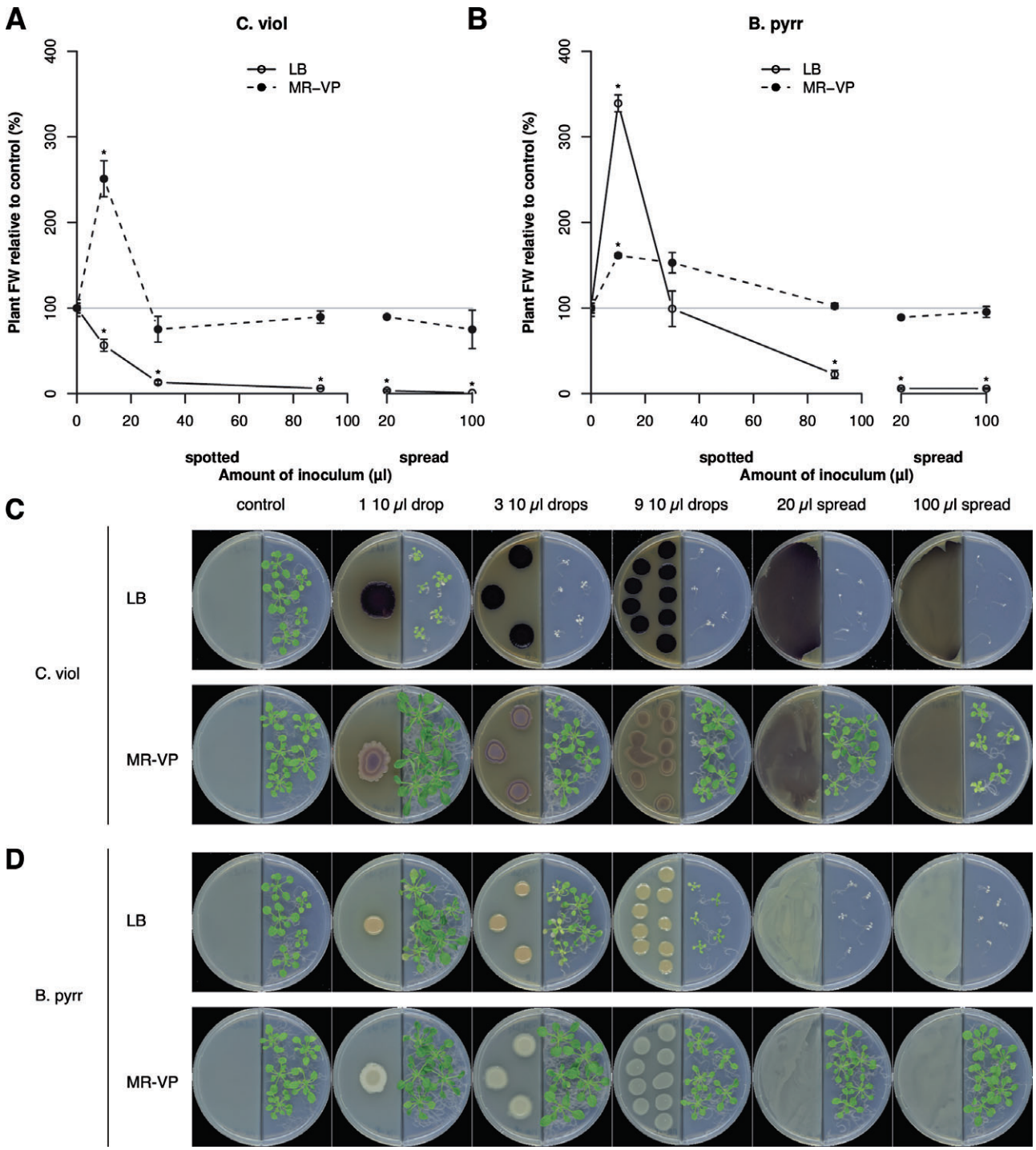

Fig. 2. The plant growth modulating effects of volatiles from *Chromobacterium violaceum* CV0 (C. viol, A and C) and *Burkholderia pyrrocinia* Bcc171 (B. pyrr, B and D) as a function of the amount and the method of inoculation. Error bars represent standard errors, results marked with an asterisk (*) are significantly different from the non-inoculated control according to a Student's *t*-test ($P < 0.05$, $n = 3$–4).

(Fig. 6). In contrast, 1-hexanol inhibited flg-22-induced ROS production, except in very high concentrations, where it not only increased ROS production, but also accelerated it. Additionally, the baseline of bioluminescence without flg-22 was slightly, but significantly, increased upon exposure to high amounts of indole, although this response did not show the typical dynamics of an oxidative burst (data not shown). For pentadecane, no significant changes in flg-22-induced ROS production could be detected (Fig. 6).

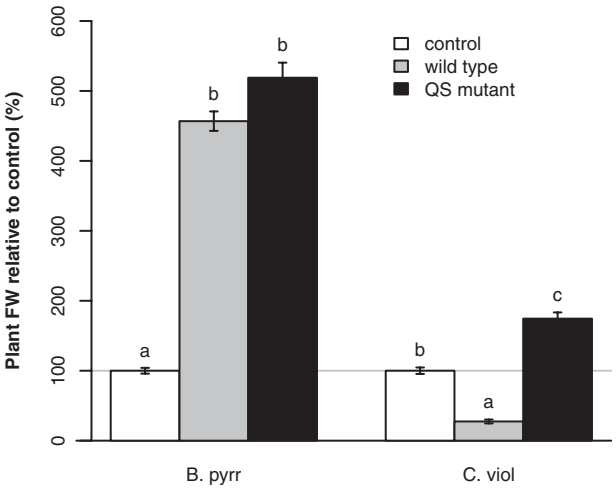

Fig. 3. The influence of quorum sensing (QS) on the production of plant growth modulating volatiles. The strains used were: *Burkholderia pyrrocinia* Bcc171 (B. pyrr) wild-type and quorum quenched (carrying the pMLBAD-*aiiA* plasmid, Wopperer *et al.*, 2006) and *Chromobacterium violaceum* CV0 (C. viol, wild-type) and the corresponding QS mutant CV026, both grown on LB. Error bars represent standard errors, results marked with different letters are significantly different from other bars from the same strain according to a Student's *t*-test ($P < 0.05$, $n = 3$–4).

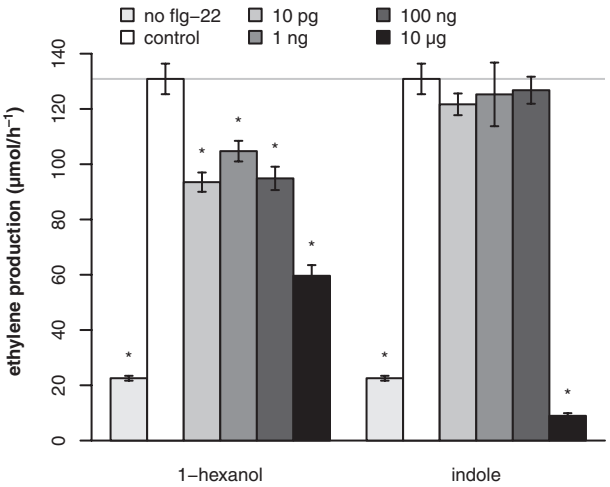

Fig. 5. The effect of 1-hexanol and indole on the flg-22-induced ethylene burst in *Arabidopsis thaliana*. Error bars represent standard errors, bars marked with an asterisk (*) are significantly different from a solvent control according to a Student's *t*-test ($P < 0.05$, $n = 12$).

Discussion

As a first hypothesis, we postulated that the volatile-mediated impact of bacteria on plant growth is a general phenomenon and is not limited to the few butanediol-producing *Bacillus* strains on which most research has focused before now (Ryu *et al.*, 2003; 2004; 2005a; Han *et al.*, 2006; Cortes-Barco *et al.*, 2010a,b). To test this hypothesis, we performed a large screening of soil-borne bacteria using a simple experimental set-up consisting of divided Petri dishes with bacteria growing on one side and plants on the other. We show that in this experimental set-up, every single strain tested significantly affected plant growth when cultured in at least one of the four media used. Moreover, the observed effects were very dramatic, ranging from plant killing (e.g. *C. violaceum* CV0 when grown on LB) to more than sixfold promotion of plant growth (e.g. *Burkholderia thailandensis* and *Pandoraea norimbergensis* grown on LB). It cannot be excluded that the closed Petri dish set-up led to higher accumulation of volatiles than would occur in a more natural situation, yet the porous structure of the soil and the rhizosphere seems a favourable environment for volatiles to accumulate as well. Among the 42 strains analysed, only one showed significant plant growth promoting effects on all four media tested. This strain, *Burkholderia pyrrocinia*, which belongs to the *Burkholderia cepacia* complex (Bcc, Coenye *et al.*, 2001), is a well-known biocontrol strain that employs a QS system to control the production of the fungicide pyrrolnitrin (Schmidt *et al.*, 2009). Many Bcc species are known as plant growth promoting strains and in our study, the Bcc species included (*B. anthina*, *B. cepacia*, *B. lata* and *B. pyrrocinia*) all promoted growth on either LB and/or MR-VP medium, and with the exception of *B. anthina* also on the soil-mimicking Angle medium. None of the Bcc strains showed growth inhibition or killing, while the non-Bcc strains *B. phenoliruptrix* and *B. phytofirmans* displayed plant killing activity when grown on LB (Table 1). Although *B. pyrrocinia* occurs mainly in soils (Coenye and Vandamme, 2003), many Bcc species also act as opportunistic pathogens in humans, which limit their possibilities for

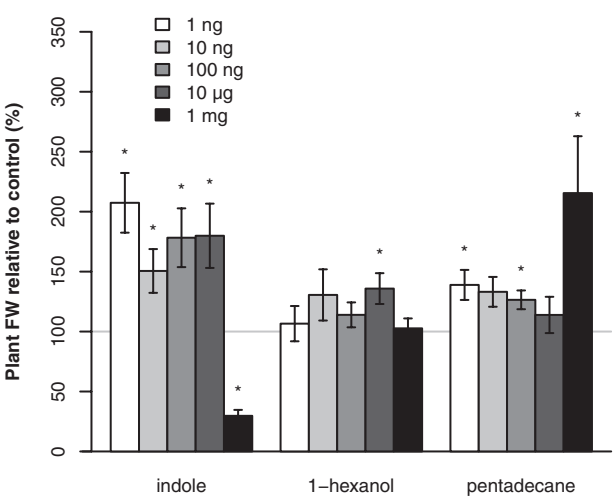

Fig. 4. The plant growth modulating effects of indole, 1-hexanol and pentadecane on *Arabidopsis thaliana* Col-0. Error bars represent standard errors, bars marked with an asterisk (*) are significantly different from a solvent control according to a Student's *t*-test ($P < 0.05$, $n = 4$–6).

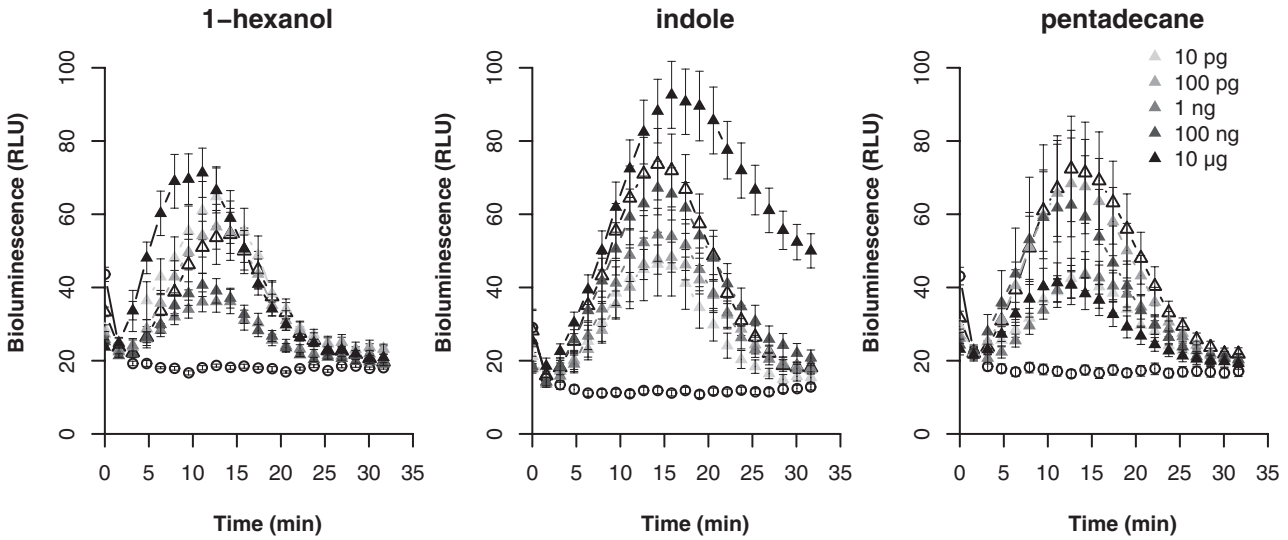

Fig. 6. The effect of 1-hexanol, indole and pentadecane on the flg-22-induced oxidative burst in *Arabidopsis thaliana*, measured as the occurrence of bioluminescence from the oxidation of luminol in the presence of peroxidase. Open circles: dichloromethane without flg-22; open triangles: dichloromethane with flg-22; closed triangles: five different amounts of 1-hexanol, indole and pentadecane with flg-22 (see keys). Error bars represent standard errors of 18 replicates.

field use (Mahenthiralingam *et al*., 2005). The strongest effects, both inhibitory and stimulating, were observed when bacteria were grown on LB, which suggested that the high cell density reached on such media might be needed to obtain the most drastic effects. Likewise, it appeared that although *B. pyrrocinia* grown on LB was promoting plant growth, it also induced chlorosis in the plants situated in closest proximity to the colony (see Fig. 1), suggesting that a blend of promoting volatiles could become plant-inhibiting when supplied in too high concentration. To assess the role of the inoculum size in the volatile-mediated effects of bacteria on plants, we selected two model strains, *B. pyrrocinia* (always promoting) and *C. violaceum* (killing on LB; promoting on MR-VP) and tested the effect of varying inoculum sizes on plant response. While *B. pyrrocinia* generally promoted plant growth it was found to kill plants when a larger inoculum were used (Fig. 2). This raised the question of whether this was owing to different quantities of volatiles produced, or whether specific volatiles were only produced at a high population density, i.e. when a large inoculum was used, possibly as a consequence of the induction of QS, or as part of a starvation response. To address this question, we performed GC-MS analyses of the volatiles produced by *C. violaceum* and *B. pyrrocinia* wild-type strains inoculated in different amounts, as well as of the volatiles emitted by the respective QS-deficient mutant or a quorum-quenched derivative. No new compound was detected in samples with the higher inoculation dose, but a few compounds were found to be present in significantly increased quantities (Table 2).

HCN might be involved in the killing effects observed in our study and by others (Vespermann *et al*., 2007) when plants are exposed to bacterial volatiles (Blom *et al*., 2011). The very strong plant growth promoting effects have yet to be explained. Interestingly, although most strains produced plant growth promoting volatiles when grown on MR-VP, this beneficial effect was obviously not due to the production of butanediol (Table 2). Although the release of CO_2 was considered likely to promote the growth of the C3 plant *A. thaliana* in a closed system such as the bipartite Petri dish we were using (Kai and Piechulla, 2009), it is highly unlikely that CO_2 would account for the sixfold increase in plant biomass that has been observed in the case of *P. norimbergensis*. The increase in plant biomass production due to elevated CO_2 levels has been reported to be at most 25% (Ward and Strain, 1997; Sun *et al*., 2002). Moreover, our measurements on bacterial CO_2 production during growth on different media suggested that on MS and Angle media the CO_2 release was very low and would not lead to a significant increase inside a system sealed with CO_2-permeable Parafilm (data not shown). It is thus to be expected that other compounds are responsible for the observed plant growth promotion. To search for such promoting volatiles, we performed GC-MS analyses of the headspace of our 42 bacterial strains grown on four different media. Spectral analysis revealed over 130 compounds, of which most could not be identified using the NIST library, suggesting high potential for the discovery of yet unknown, putative bioactive compounds. A subset of 36 compounds, which

were produced in large amounts and were commercially available, were used to build a quantitation library (database of mass spectra obtained from at least two different concentrations of each compound), which enabled us to calculate the quantity of the volatiles present in the original sample. Most compounds identified were known bacterial compounds (Schulz and Dickschat, 2007), some had been previously tested on plants, like 1-hexanol, which is also produced by truffles (Splivallo *et al*., 2007) and plants upon leaf damage (Mattiacci *et al*., 1995). Surprisingly, Splivallo and colleagues showed that 1-hexanol inhibited plant growth, while our data suggest a growth promoting effect. Only at very high quantities of 1-hexanol (1 mg) were slightly deleterious effects observed (Fig. 4). This is in line with the study of Splivallo and colleagues, who used much higher quantities (from approximately 3 mg to 30 mg) in their experiments.

Another interesting compound we found to be bioactive was indole, which gives rise to the typical smell of *E. coli* (Yu *et al*., 2000). Indole is reported to be involved in biofilm formation (Di Martino *et al*., 2003; Mueller *et al*., 2007), the expression of a type III secretion system (Hirakawa *et al*., 2009) and in antibiotic resistance in *E. coli* (Lee *et al*., 2010). It has also been reported to be produced by cotton and maize upon insect feeding (Alborn *et al*., 1997; Frey *et al*., 2000) and it was suggested to be involved in plant defence (Frey *et al*., 2000). The effect of indole was remarkable, in the sense that a clear dose-response was observed, with plant growth inhibition at high concentrations of indole and growth promotion at lower amounts. Pentadecane was the final compound selected as a bioactive volatile candidate, although it was not produced by many of the bacteria tested (Table 2). Pentadecane, in contrast to 1-hexanol and indole, showed a significant plant growth promoting effect at very high concentrations (Fig. 4). In addition to being produced by bacteria (e.g. *Pseudomonas* isolates, Fernando *et al*., 2005), pentadecane is known to be produced as an odour component in plants, e.g. as floral odour compound in *Pulsatilla rubra* (Jurgens and Dotterl, 2004).

During four independent experiments, we observed a clear plant growth promoting effect of indole, pentadecane and of 1-hexanol in at least some of the concentrations tested. While the inhibitory effect of indole was consistent, the positive effects of the bioactive volatiles could not be reproduced in later experiments. The observation that the lack of reproducibility correlated with better growth of control plants led us to speculate that some as yet undefined parameter(s) of the experimental set-up was imposing a stress on the plants and that the volatiles were only promoting growth under stressful conditions. This hypothesis could be corroborated by the modulating effect of 1-hexanol and indole on ethylene production (Fig. 5), a typical response of plants to MAMPs and 'stress' (see Boller and Felix, 2009). Moreover, 1-hexanol, indole and pentadecane also affected the oxidative burst induced by the MAMP flg-22 (Fig. 6), indicating that they may act as effectors of the defence response (Boller and He, 2009). Interestingly, as described above, the plant response-modulating volatiles 1-hexanol and indole were both reported to be released – at least by some plant species – upon leaf damage and might therefore be involved as endogenous factors in the plant's stress response. In addition to a specific activity of single compounds, synergistic effects of various volatiles present in the complex bouquet of a given strain are likely to occur, as it has been suggested recently for tritrophic interactions involving plants, herbivores and predatory mites (van Wijk *et al*., 2011). Testing artificial mixtures mimicking a particular strain's blend might represent a promising strategy for the future to elucidate the chemical basis of the volatile-mediated effect of bacteria on plants. Finally, the mechanisms of action of both promoting and inhibiting bacterial volatiles on plant metabolism deserve further attention in future studies, as does the question of whether the effects observed in controlled laboratory conditions would also hold true in the field.

Experimental procedures

Chemicals and culture media

Chemicals were purchased from Sigma-Aldrich, Buchs AG, Switzerland, unless specified otherwise. Bacteria were routinely cultured on LB [10 g of Bacto tryptone, 5 g of Bacto yeast extract, 4 g of NaCl (AppliChem/Axon), pH adjusted to 7.4 and supplemented with 16 g agar (European Bacteriological Agar, Chemie Brunschwig) when needed]. Half strength MS agar (Murashige and Skoog, 1962) contained (per litre) 2.2 g MS Basal Medium, 15 g sucrose and 8 g agar, pH was adjusted to 5.7. Methyl Red Vogues Proskauer agar (MR-VP, Voges and Proskauer, 1898) contained (per litre) 17 g Difco MR-VP medium and 16 g agar. Fe-EDTA solution (0.01 M) contained (per litre) 1.4 g Na_2EDTA, 5 g $FeSO_4{\cdot}7H_2O$, and 500 µl H_2SO_4 conc. SL6 solution (25 000 ×) contained (per litre) 250 mg $ZnSO_4{\cdot}7H_2O$, 325 mg $MnCl_2{\cdot}4H_2O$, 75 mg $Na_2MoO_4{\cdot}2H_2O$, 750 mg H_3Bo_3, 500 mg $CoCl_2{\cdot}6H_2O$, 25 mg $CuCl_2{\cdot}2H_2O$ and 50 mg $NiCl_2{\cdot}6H_2O$. TRIS-HCl solution (1 M) contained (per litre) 121 g tris(hydroxylmethyl)aminomethane (TRIS) and 25 ml 37% HCl. Angle Mix A (50 ×) contained (per litre) 100 g NH_4NO_3 and 203 g $MgCl_2{\cdot}6H_2O$. C-source mixture (10 000 ×) contained (per litre) 1.2 mg D-glucose, 1.2 mg D-fructose, 2.4 mg sucrose, 0.15 mg casamino acids, 1.3 mg citric acid, 0.89 mg malic acid and 0.79 mg succinic acid. Angle medium agar (modified from Angle *et al*., 1991) contained (per litre) 0.69 g $CaSO_4{\cdot}2H_2O$, 20 ml Angle Mix A, 100 µl FeEDTA, 20 ml TRIS-HCl, 40 µl SL6, 68 µl 1% KH_2PO_4 and 500 µl 1 M KOH, pH adjusted to 6.0 and supplemented with 16 g agar. One hundred microlitres of C-source mixture (Farrar *et al*., 2003) was added after autoclaving.

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table S1. They were stored at –80°C in LB broth containing 16% glycerol. Before use, the bacteria were streaked on LB agar plates and grown either 1–2 days at 30°C, or at least 3 days at room temperature. Single colonies were transferred to 5 ml LB broth in a 16 cm test tube and grown overnight (2 days for slow-growing strains) with shaking at 225 RPM.

Plant lines and growth conditions

Arabidopsis thaliana Col-0 was used for all plant experiments. Seeds were sterilized in 1.5 ml Eppendorf tubes by adding 1 ml of 70% ethanol and shaking for 2 min on an IKA Vortex Genius 3 with adapter at force 4. The supernatant was removed after centrifuging for 1 min at 3300 rcf and 1 ml of a 1% NaOCl (Fluka) solution containing 0.03% Triton X-100 (Fluka) was added and shaken for 20 min as described above. After centrifuging, the supernatant was removed and the pellet washed four times with sterile MiliQ water. The seeds were stratified in a sterile 0.15% agarose solution at 4°C overnight. To prepare seedlings, the seed solution was pipetted on 16 × 16 cm square Petri dishes (Greiner), the plates were sealed with parafilm and incubated in a climate chamber with a 12 h/12 h day/night rhythm at 20°C, 50% relative humidity and 100 μmol m^{-2} s^{-1} light for 6 days.

Plant-bacterial dual growth experiments

Experiments were performed in two-compartment dishes (Greiner) with five 6-day-old seedlings placed on one compartment containing half-strength MS agar and the second compartment containing LB, MR-VP, half-strength MS or Angle agar. In this compartment, 20 μl of a liquid culture (unless stated otherwise) of each strain to be investigated was spotted on the agar. Sterile LB broth was used as a control. The plates containing the plants and the bacterial inoculum (or a non-inoculated control) were sealed with parafilm and incubated in the plant growth chamber described above. Pictures were taken after 14 and 21 days and plants were harvested after 21 days. Shoots were cut and weighed. Results were expressed in percentage of plant biomass (fresh weight) relative to the biomass of control plants. All experiments were performed using at least three replicates.

GC-MS analysis of bacterial volatiles

For headspace volatile analysis, 20 μl bacterial culture was spotted on a 9 cm Petri dish containing LB, MR-VP, half-strength MS, or Angle agar. After four days of growth at 20°C, headspace air was trapped in glass Gerstel TDS tubes filled with c. 20 mg of Tenax TA 80–100 mesh by replacing the Petri dish lid with one in which a hole had been drilled and fitted with the TDS tube and sucking headspace air through at 0.1 l min^{-1} for 15 min using battery-operated pumps (PAS-500 personal air sampler Spectrex, Redwood city, CA, USA). The tubes were stored at –20°C after collection. For analysis of the bacterial volatiles, gas chromatography with mass selective detection (GC-MSD) was used. Samples were injected into a GC (Agilent 6890N) using a Gerstel thermodesorption system (TDS3, Gerstel Mühlheim, Germany) with cold injection system (CIS4; Gerstel). For thermodesorption, the TDS was heated from 30°C to 300°C at a rate of 60°C min^{-1} and held at the final temperature for 1 min. The CIS was set to –50°C during trapping of eluting compounds from the TDS. For injection, the CIS was heated to 150°C at a rate of 16°C s^{-1} and then to 250°C at a rate of 12°C s^{-1}, and the final temperature was held for 30 s. The GC was equipped with a HP-5 column (0.32 mm ID, 0.25 μm film thickness, 30 m length), helium was used as carrier gas at a flow rate of 2 ml min^{-1}. Compound determination and quantification was done using a mass selective detector (Agilent MSD 5975). Compounds were identified by comparison of spectra obtained from the bacterial samples, with those from a reference collection (NIST '05 library). In addition, retention times and spectra of synthetic standard compounds were compared with those of bacterial samples. To quantify individual compounds, synthetic standards were analysed in up to four different amounts (1 ng, 10 ng, 100 ng and 1 μg, depending on the response of the MSD to the compounds) on the GC-MSD system, and the resulting peak areas of the total ion chromatograms used to calculate a regression of amount and peak area. The linear regression coefficient was used to calculate the amounts in the bacterial samples from peak areas obtained in total ion chromatograms.

Plant exposure to pure volatile chemicals

Experiments were performed in two-compartment dishes (Greiner) with five 6-day-old seedlings placed on one compartment containing half-strength MS agar and the second compartment left empty. Dilutions of volatile chemicals were made in DCM with final concentrations of 1 ng, 10 ng, 100 ng, 10 μg and 1 mg per 10 μl. A solution of 1.6 g of lanolin in 10 ml DCM was also prepared for each experiment. Each compound was mixed in a 1:1 ration with the lanolin solution and 20 μl of this mixture was dropped on a sterilized piece of filter paper of about 1 by 1 cm. The filter paper was dried for 90 s in a glass Petri dish and then transferred to the empty side of the two-compartment dish, which was immediately sealed with Parafilm and incubated in the plant growth chamber described above. Plants were harvested after 21 days. Shoots were cut and weighed. Results were expressed in percentage of plant biomass (fresh weight) relative to the biomass of control plants. All experiments were performed using at least four replicates.

Ethylene production measurements

Ethylene production was measured essentially as described previously (Felix *et al.*, 1999), as follows: test tubes (8 × 50 mm) were supplied with 100 μl MiliQ water and two 7- to 10-day-old seedlings. After allowing the material to rest for one hour, 10 μl of a solution containing the volatile to be tested and/or the bacterial elicitor flg-22 (final concentration: 1 μM) was added and the tubes were closed airtight with a rubber stopper. After an incubation period of at least 2 h, 1 ml of headspace air was drawn from the tubes using a plastic syringe and injected in a Shimadzu GC-14A gas chromato-

graph fitted with an aluminium oxide column and a flame ionization detector (FID) coupled to a Shimadzu C-R4A CHROMATOPAC integrator, which was calibrated to read out the amount of ethylene produced in p.p.m.

Oxidative burst measurements

Oxidative burst was analysed luminometrically by measuring the oxidation of luminol by reactive oxygen species (ROS) in the presence of peroxidase, essentially as described previously (Felix *et al*., 1999). Discs of 5 mm diameter were punched out of leaves of 4-week-old *Arabidopsis thaliana* Col-0 plants and placed in a beaker containing MilliQ water for at least one hour to allow them to recover from the stress of cutting. The wells of a 96-well luminometer plate were filled with 100 µl of MilliQ water each, and one leaf piece was placed in each well. Twenty microlitres of a mix containing water, luminol solution (final amount: 1 µg per well), peroxidase solution (final concentration: 100 µM) and volatile solution was added to each well and measured for 30 min with an EG & G Berthold MicroLumat LB 96 P plate reader using WinGlow 1.25.00003 software (Berthold Technologies 1994).

Statistical analysis

Differences between treatments were compared using a two-sided Student's *t*-test ($n = 4$, $P < 0.05$) performed in Numbers '09 (Apple 2010) and R 2.12.1 for Mac (The R Foundation for Statistical Computing). Correlation and *t*-tests for significance on the GC-MS results were performed with SPSS 19.0.0 for Mac (IBM Corporation 2010).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Bacterial strains used in this study and their origin.
Table S2. The production of 50 volatile organic compounds by 42 bacterial strains grown on 4 different media.
Table S3. The production of 24 volatile organic compounds by 2 bacterial strains grown in 5 different amounts on 4 different media.