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Volatile-mediated interspecific plant interaction promotes root colonization by beneficial bacteria via induced shifts in root exudation

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Abstract

Background Volatile organic compounds (VOCs) released by plants can act as signaling molecules mediating ecological interactions. Therefore, the study of VOCs mediated intra- and interspecific interactions with downstream plant physiological responses is critical to advance our understanding of mechanisms underlying information exchange in plants. Here, we investigated how plant-emitted VOCs affect the performance of an interspecific neighboring plant via induced shifts in root exudate chemistry with implications for root-associated microbiota recruitment.

Results First, we showed that VOCs emitted by potato-onion plants stimulate the growth of adjacent tomato plants. Then, we demonstrated that this positive effect on tomato biomass was attributed to shifts in the tomato rhizosphere microbiota. Specifically, we found potato-onion VOCs to indirectly affect the recruitment of specific bacteria (e.g., *Pseudomonas* and *Bacillus* spp.) in the tomato rhizosphere. Second, we identified and validated the compound dipropyl disulfide as the active molecule within the blend of potato-onion VOCs mediating this interspecific plant communication. Third, we showed that the effect on the tomato rhizosphere microbiota occurs via induced changes in root exudates of tomato plants caused by exposure to dipropyl disulfide. Last, *Pseudomonas* and *Bacillus* spp. bacteria enriched in the tomato rhizosphere were shown to have plant growth-promoting activities.

Conclusions Potato-onion VOCs—specifically dipropyl disulfide—can induce shifts in the root exudate of adjacent tomato plants, which results in the recruitment of plant-beneficial bacteria in the rhizosphere. Taken together, this study elucidated a new mechanism of interspecific plant interaction mediated by VOCs resulting in alterations in the rhizosphere microbiota with beneficial outcomes for plant performance.

Keywords Volatile organic compounds, Interspecific plant interaction, Rhizosphere, Microbiota, Root colonization

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Background

Plants synthesize and release a diverse array of volatile organic compounds (VOCs) both constitutively and in response to biotic and abiotic stresses. These volatiles can function as chemical cues or signaling molecules mediating intra- and interspecific plant interactions [1–4]. For example, specific volatile compounds can inform the health status of a plant ("emitter plant"), resulting in physiological responses in a neighboring plant ("receiver plant") [5–8]. It is currently established that VOCs emitted by plants under biotic (e.g., pathogen or herbivore attacks) or abiotic stresses (e.g., drought, salt stress) can induce intraspecific neighbors to increase resistance [2, 9, 10]. In addition, plant-derived VOCs can mediate positive interspecific plant interactions, which can result in enhanced growth [8] and defense responses in receiver plants [11–13]. Recent studies have tried to elucidate the multiple mechanisms of plant chemical communication mediated via VOC signaling with implications for plant health and performance [2, 4–7].

The rhizosphere microbiota has direct effects on the growth and fitness of plants in natural and agricultural systems [14, 15]. A diverse array of beneficial bacteria can promote plant growth via multiple mechanisms, e.g., mobilization of nutrients and production of phytohormones [14, 16]. Terrestrial plants can actively manipulate root colonization by specific microbial taxa via shifts in root exudates, which act as substrates and signaling molecules [17–19]. Recent studies demonstrated that plant-derived VOCs can alter the plant rhizosphere microbiota either directly by affecting the microbial metabolism involved in root colonization or via shifts in root exudates mediated by interspecific plant interactions [20, 21]. For example, specific bacterial taxa in the rhizosphere of tomato (*Solanum lycopersicum* L.) plants were shown to affect the production of VOCs with direct implications for the modulation of the rhizosphere microbiota of intraspecific neighboring plants [21]. In addition, the modulation of the rhizosphere microbiota can occur in interspecific neighboring plant species [22–24]; however, the importance of specific VOCs in mediating interspecific plant interactions remains still largely unknown.

Plants belonging to the *Allium* species can emit unique volatile organosulfur compounds [25] commonly reported to alter the performance of interspecific neighbor plants [26, 27]. Here, we investigated the mechanism of interspecific plant interaction mediated by VOCs using potato-onion (*Allium cepa* var. *agrogatum* Don.) and tomato plants as a model system. Our overarching hypothesis is that VOCs released by potato-onion plants can alter the performance of tomato plants via modulation of the rhizosphere microbiota (Fig. 1A). To evaluate this hypothesis, we start by testing the effect of potato-onion VOCs on the growth and rhizosphere microbiota of tomato plants. After that, we partitioned the chemical composition of potato-onion VOCs to identify a specific molecule with a positive effect on tomato growth. Further experiments were used to validate the effect of this molecule and its mode of action, i.e., via induced shifts in tomato root exudates with implications for root colonization by specific bacterial taxa. Last, we performed a series of metabolic characterization and inoculation experiments to show the plant-growth promotion activities of these enriched plant-beneficial bacteria in the tomato rhizosphere.

Results

Effect of potato-onion VOCs on tomato plant growth

We used a twin-chamber system to test the effect of VOCs constitutively released from potato-onion plants on tomato plant growth (Fig. 1B). Since VOCs emitted by soil microbes are reported to be able to alter plant performance [28, 29], we also tested if microbial VOCs from the chamber containing potato-onion plants could exert an effect on tomato growth. For that, tomato plants were grown in natural soil and exposed to (*1*) VOCs from both potato-onion plants and soil microbes—this was achieved by growing potato-onion plants in pots containing natural soil, and (*2*) VOCs emitted only by potato-onion plants—this was achieved by growing potato-onion plants in pots containing sterile soil. The results showed that tomato plants exposed to both treatments had higher dry biomass when compared to controls (tomato plants not exposed to VOCs) (average increases of 0.75 g and 0.78 g per plant, respectively; Tukey's HSD test, $p < 0.05$; Fig. 1C). Most importantly, no statistically significant difference was detected when comparing these two treatments (Tukey's HSD test, $p > 0.05$), thus validating the importance of VOCs derived from potato-onion plants in positively affecting tomato plant growth.

The role of the rhizosphere microbiota in the VOC-mediated interspecific plant interaction

To evaluate whether potato-onion VOCs have a direct effect on tomato plants or act via modulation of the tomato rhizosphere microbiota, we performed a twin-chamber experiment by growing tomato and potato-onion plants in sterile soil. The results showed that exposure to potato-onion VOCs did not alter tomato plant dry biomass accumulation when tomato plants were grown in sterile soil (Fig. 1C). Then, the importance of the rhizosphere microbiota was further assessed in a rhizosphere transplant experiment (Fig. 1B). We used sterile field soil mixed with an inoculum of tomato rhizosphere samples (6% *w/w*). This inoculum was obtained

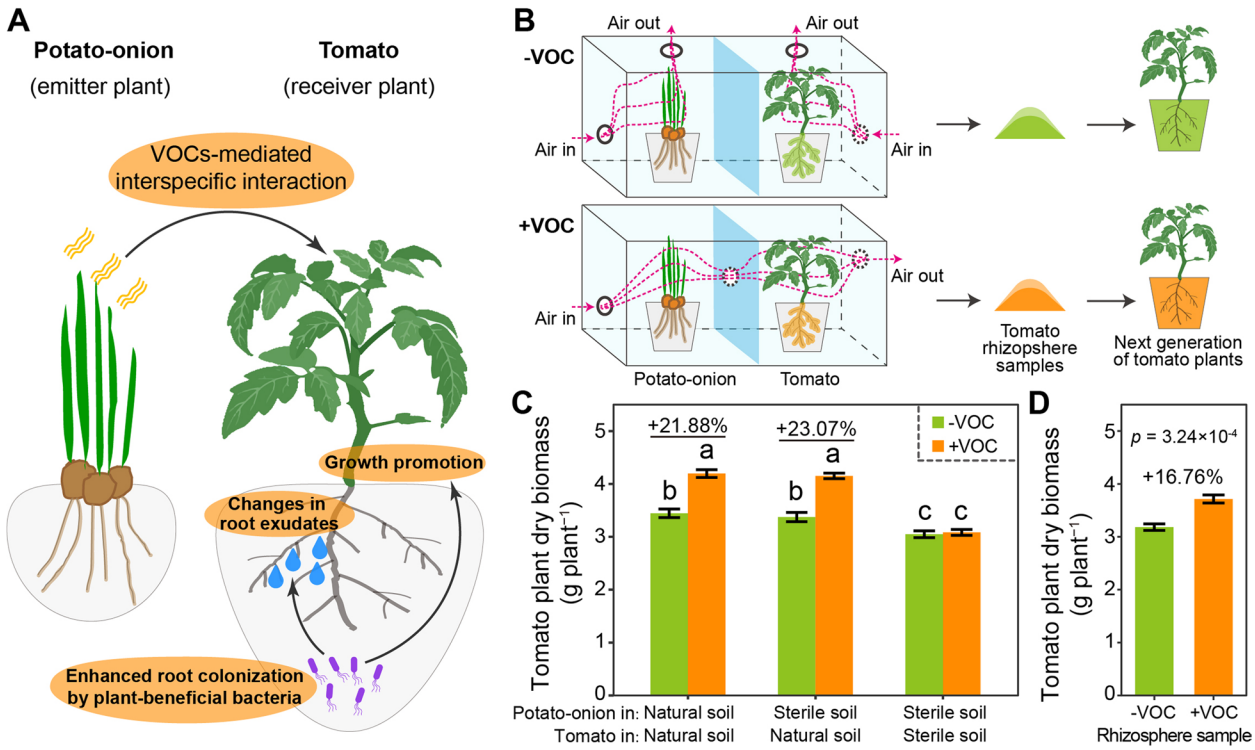

Fig. 1 Volatile-mediated interspecific plant interaction affects plant dry biomass accumulation. **A** Conceptual figure displaying the overarching hypothesis of this study. In brief, interspecific plant interaction mediated by VOCs alters root exudates with implications for root colonization by plant-beneficial bacteria. **B** Experimental setup of the twin-chamber system and the rhizosphere transplant experiment. In this system, tomato plants were exposed to potato-onion VOCs (+VOC) or not (−VOC). Potato-onion and tomato plants were grown under different soil conditions: (*1*) both plants were grown in natural soil, (*2*) potato-onion was grown in sterile soil and tomato in natural soil, and (*3*) both plants were grown in sterile soil. For the rhizosphere transplant experiment, tomato plants were grown in sterile soil mixed with a sample of the tomato rhizosphere grown in natural soil. **C** Values of tomato plant dry biomass from the twin-chamber experiment. **D** Values of tomato plant dry biomass from the rhizosphere transplant experiment. Values shown above bars indicate the increased (%) in plant dry biomass of treatments exposed to potato-onion VOCs. Data are shown as mean ± SEM ($n = 6$). *P* values were determined using Welch's *t* tests. Different letters indicate significant differences between treatments (Tukey's HSD test, $p < 0.05$)

from the twin-chamber experiment where potato-onion was grown in sterile soil and tomato plants in natural soil. The results showed that tomato dry biomass was higher when cultivated in the soil mixture containing the rhizosphere sample of tomato plants previously exposed to potato-onion VOCs (Welch's *t* test, $p < 0.05$; Fig. 1D). Collectively, these results support the effect of potato-onion VOCs on tomato biomass accumulation to act via modulation of the tomato rhizosphere microbiota.

Potato-onion VOCs alter the tomato rhizosphere bacterial community

We analyzed the structure of bacterial communities in the rhizosphere of tomato plants from the twin-chamber experiment (potato-onion grown in sterile soil and tomato in natural soil) using high-throughput sequencing of the 16S rRNA gene. The results showed exposure to potato-onion VOCs to decrease the α-diversity [i.e., number of observed operational taxonomic units (OTUs) and Shannon index] of bacterial communities in the rhizosphere of tomato plants (Welch's *t* test, $p < 0.05$; Figure S1A). Principal coordinate analysis (PCoA) showed a significant difference in the community structure between the treatments exposed or not to potato-onion VOCs [permutational multivariate analysis of variance (PERMANOVA), $R^2 = 0.172$, $p = 0.003$; Fig. 2A]. The treatment exposed to potato-onion VOCs had a higher relative abundance of taxa belonging to δ-Proteobacteria and a lower relative abundance of taxa belonging to Gemmatimonadetes in comparison to the treatment not exposed to potato-onion VOCs (Welch's *t* test, $p < 0.05$; Figure S1B).

Exposure to potato-onion VOCs resulted in increases in the relative abundance of 26 OTUs and decreases in the relative abundance of 114 OTUs in the tomato rhizosphere (Likelihood ratio test, false discovery rate-adjusted $p < 0.01$; $\log_2$ fold change > 1 or < -1, respectively; Fig. 2B). The OTUs with increases in relative

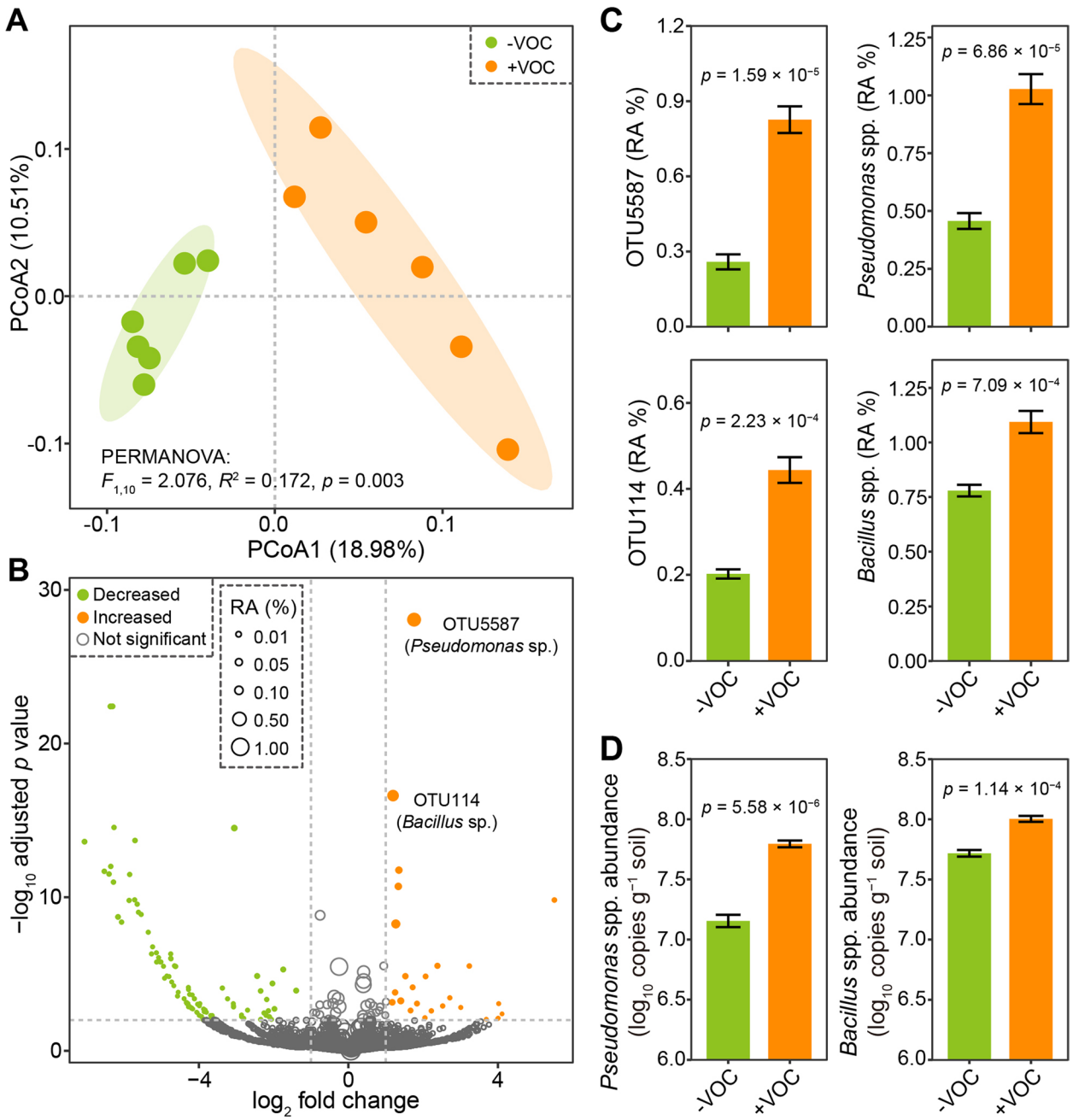

Fig. 2 Effect ofexposure topotato-onion VOCs on bacterial communities in the rhizosphere of tomato plants. **A** PCoA of bacterial communities based on the Bray–Curtis distances at the OTU level. **B** Volcano plot displaying the OTUs that significantly varied (in terms of relative abundance) due to exposure of tomato plants to potato-onion VOCs (false discovery rate-adjusted $p < 0.01$, $\log_2$ fold change > 1). The horizontal dashed line represents the false discovery rate-adjusted p value = 0.01. The vertical dashed lines represent $\log_2$ fold change value = − 1 and 1, respectively. **C** Relative abundance (RA) of OTU5587, OTU114, *Pseudomonas* and *Bacillus* spp. determined via amplicon sequencing. **D** Absolute abundance of *Pseudomonas* and *Bacillus* spp. determined via quantitative PCR. Data are shown as mean ± SEM ($n = 6$). *P* values were determined using Welch's *t* tests

abundance mainly affiliated with the phyla Proteobacteria (*Pseudomonas*, *Chujaibacter* spp.) and Actinobacteria (*Acidothermus* sp.), and the OTUs with decreases in relative abundance mainly affiliated with the phyla Proteobacteria, Actinobacteria, Acidobacteria, and Gemmatimonadetes (Figure S1C). Within these, two abundant OTUs (mean relative abundance > 0.1%)—*Pseudomonas* sp. OTU5587 and

Bacillus sp. OTU114—were strongly "stimulated" by exposure to potato-onion VOCs (Fig. 2B, C). Besides, potato-onion VOCs resulted in an overall increase in the relative abundance of the genera *Pseudomonas* and *Bacillus* (Welch's *t* test, $p < 0.05$; Fig. 2C). Quantitative PCR analysis further showed that the absolute abundance of *Pseudomonas* and *Bacillus* spp. in the tomato rhizosphere increased due to the exposure to potato-onion VOCs by 326% and 93%, respectively (Welch's *t* test, $p < 0.05$; Fig. 2D).

Dipropyl disulfide acts as an active molecule affecting tomato plant performance

We profiled the chemical composition of VOCs emitted by tomato and potato-onion plants using gas chromatography-mass spectrometry (GC–MS) analysis. The results showed tomato plants to emit compounds identified as β-phellandrene, (E)-2-hexenal, β-caryophyllene, β-ionone and 4-hexen-3-one, whereas dipropyl disulfide, (E)-2-hexenal and 4-hexen-3-one were mostly emitted by potato-onion plants (Fig. 3A). Specifically, dipropyl disulfide in the airflow from the chamber of tomato plants exposed to potato-onion VOCs (Fig. 1B) was detected at the concentration of 227.08 ± 9.78 ng per hour and not detected in the chamber of tomato plants not exposed to potato-onion VOCs. We further evaluated the potential effect of dipropyl disulfide on tomato plant growth and rhizosphere bacterial community. For that, tomato plants grown in natural soil were placed in a chamber with a single compartment (Fig. 3B) and exposed or not to dipropyl disulfide. This was carried out by placing a glass vessel containing dipropyl disulfide inside the chamber (optimized for a release rate of 220 ng per hour) (Fig. 3B). The results revealed that dipropyl disulfide increased tomato plant dry biomass by an average of 0.73 g per plant (Welch's *t* test, $p < 0.05$; Fig. 3C). Moreover, exposure to dipropyl disulfide also altered the bacterial community structure in the tomato rhizosphere (PERMANOVA, $R^2 = 0.236$, $p = 0.002$; Fig. 3D). Specifically, dipropyl disulfide caused an increase in the relative abundance of *Pseudomonas* and *Bacillus* spp. and OTUs belonging to these genera—i.e., OTU3304 and OTU4955, respectively (Welch's *t* test, $p < 0.05$; Fig. 3E and Figure S2). Quantitative PCR analysis validated the stimulatory effect of dipropyl disulfide on the absolute abundance of *Pseudomonas* and *Bacillus* spp. in the tomato rhizosphere (by 317% and 96%, respectively) (Welch's *t* test, $p < 0.05$; Fig. 3F). Together, these results provide evidence to support that dipropyl disulfide acts as the active molecule in potato-onion VOCs with a positive effect on tomato plant performance.

Potato-onion VOCs and dipropyl disulfide affect the colonization of the tomato rhizosphere by specific *bacteria*

To validate that both potato-onion VOCs and dipropyl disulfide have positive effects on the colonization of tomato roots by specific bacterial taxa, i.e., *Pseudomonas* and *Bacillus* spp., we performed inoculation experiments using representative bacterial isolates obtained from tomato plants exposed to potato-onion VOCs. After the removal of potential clonal duplicates (i.e., isolates with 100% similarity of the 16S rRNA gene sequence), a total of 17 *Pseudomonas* sp. and 21 *Bacillus* sp. isolates were obtained (Fig. 4A). We selected two representative isolates, i.e., *Pseudomonas frederiksbergensis* isolate RV33 and *Bacillus velezensis* isolate RV57, which displayed 100% sequence similarity with *Pseudomonas* sp. OTU5587 and *Bacillus* sp. OTU114, respectively (Fig. 4A) as the model organisms in the follow-up experiment. In brief, we tested the effects of potato-onion VOCs and dipropyl disulfide on the root colonization of tomato plants by RV33 and RV57 in a twin-chamber experiment using sterile soil inoculated with a mixture of these two bacteria (Fig. 4B). The results showed that exposure to potato-onion VOCs enhanced the abundance of RV33 and RV57 in the tomato rhizosphere by 321.30% and 169.39%, respectively (Welch's *t* test, $p < 0.05$; Fig. 4C). In line with that, dipropyl disulfide also increased the abundance of RV33 and RV57 in the tomato rhizosphere by 305.57% and 112.72%, respectively (Welch's *t* test, $p < 0.05$). Last, both potato-onion VOCs and dipropyl disulfide increased tomato plant dry biomass by an average value of 0.81 g (18.95%) and 0.73 g (17.47%), respectively (Welch's *t* test, $p < 0.05$). These results support a similar effect of potato-onion VOCs and dipropyl disulfide on tomato root colonization by specific bacterial isolates.

Potato-onion VOCs alter tomato root exudates to stimulate root colonization by Pseudomonas and *Bacillus* spp.

We experimentally evaluated whether the increased abundance of *Pseudomonas* and *Bacillus* spp. in the tomato rhizosphere was due to (*1*) the direct stimulating effect of potato-onion VOCs on these bacterial isolates or (*2*) the indirect effect of potato-onion VOCs on modulating tomato root exudates with implications for the recruitment of these bacterial isolates. First, we tested the effects of potato-onion VOCs on the abundance of *Pseudomonas* and *Bacillus* spp. in the soil in the absence of tomato plants. This was carried out using

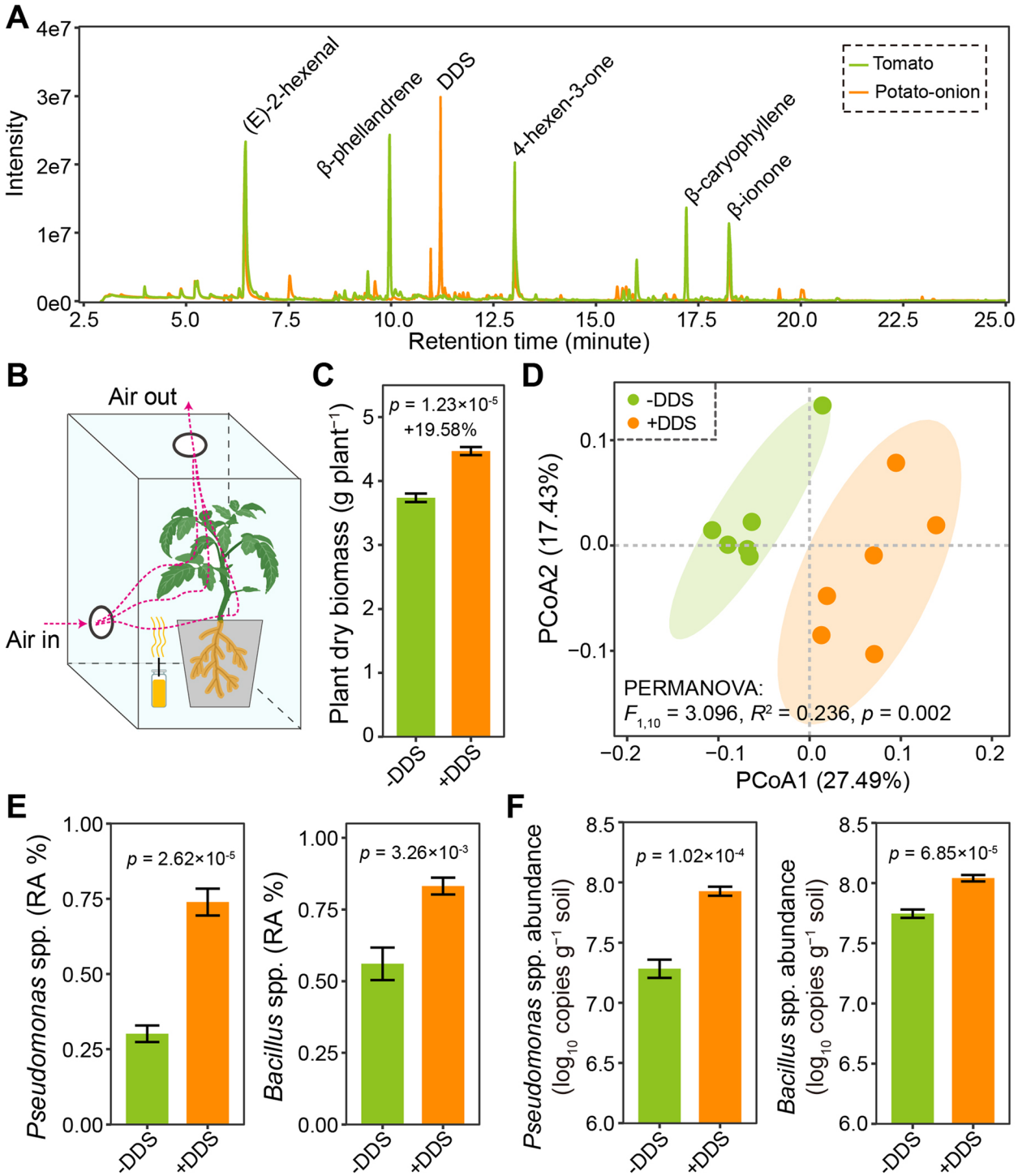

Fig. 3 Dipropyl disulfide mediates the interaction between potato-onionand tomato plants. **A** Gas chromatography–mass spectrometry chromatograms of VOCs emitted by tomato and potato-onion plants. **B** Schematic representation of the chamber system used to test the effect of dipropyl disulfide on tomato plant performance. Tomato plants were treated with dipropyl disulfide (+DDS) or not (−DDS). **C** Effect of dipropyl disulfide on tomato plant dry biomass. Values shown above the horizontal bars indicate the increased (%) in plant dry biomass of treatments exposed to dipropyl disulfide. **D** PCoA of bacterial communities based on the Bray–Curtis distances at the OTU level. **E** Relative abundance of *Pseudomonas* and *Bacillus* spp. determined via amplicon sequencing. **F** Absolute abundance of *Pseudomonas* spp. and *Bacillus* spp. determined via quantitative PCR. Data are shown as mean ± SEM ($n = 6$). *P* values were determined using Welch's *t* tests

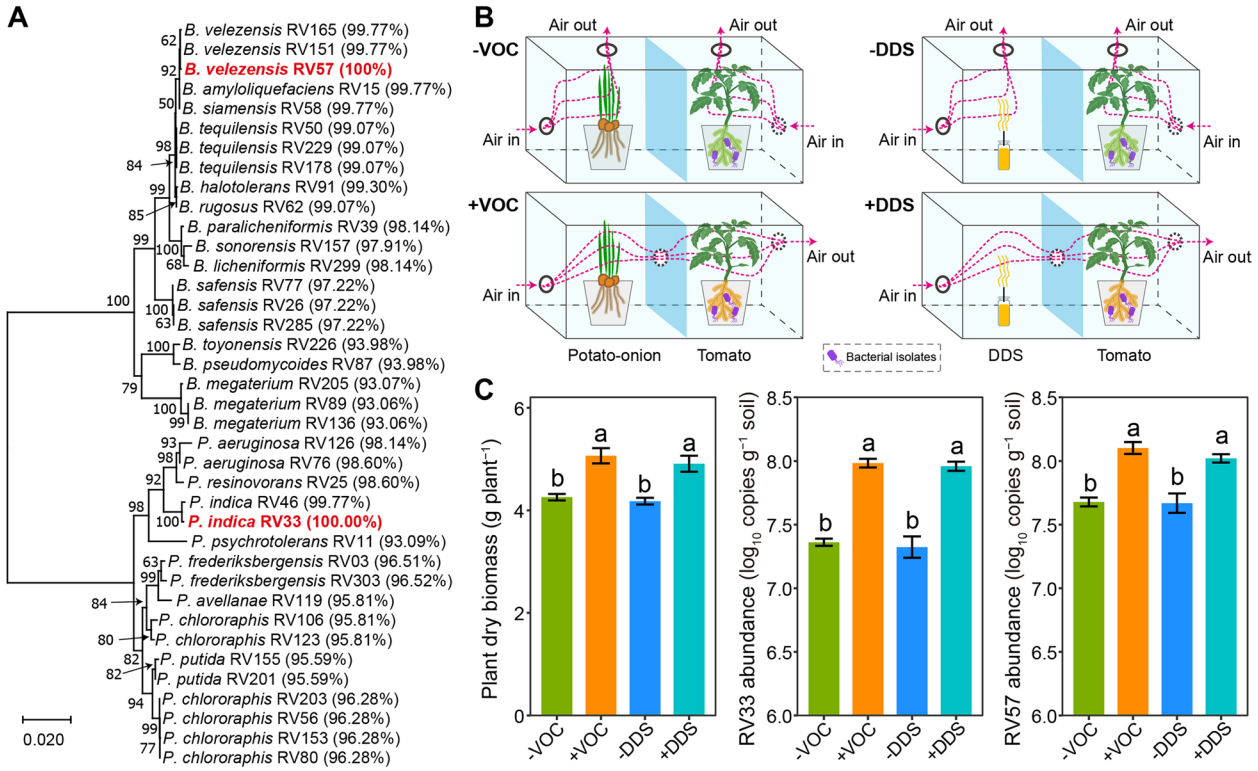

Fig. 4 Potato-onion VOCs and dipropyl disulfide stimulate the colonization of tomato roots by specific bacterial isolates. **A** Neighbor-joining tree based on bacterial 16S rRNA gene sequences of isolates taxonomically affiliated with *Pseudomonas* and *Bacillus* spp. Bootstrap was based on 1000 permutations and the obtained values are shown at the branching points. The sequence similarity of each *Pseudomonas* and *Bacillus* spp. isolates with OTU5587 and OTU114, respectively, is provided between parentheses. **B** Schematic representation of the experimental set-up and the twin-chamber system used to test the colonization of bacterial isolates on tomato roots. Tomato plants were grown in sterile soil inoculated with a mixture (1:1) of the bacterial isolates RV33 and RV57. These treatments were exposed to potato-onion VOCs (+VOC) or not (−VOC) or exposed to dipropyl disulfide (+DDS) or not (−DDS). **C** Effects of potato-onion VOCs and dipropyl disulfide on tomato plant growth and on the abundance of RV33 and RV57 in the tomato plant rhizosphere. Data are shown as mean ± SEM ($n = 6$). Different letters indicate significant differences between treatments (Tukey's HSD test, $p < 0.05$)

the twin-chamber system. For that, a pot containing potato-onion and a pot with natural soil without plants (termed "bare soil") were placed in each chamber (Figure S3A). The bare soil was exposed or not to potato-onion VOCs. Quantitative PCR analysis showed that the abundance of *Pseudomonas* and *Bacillus* spp. in the bare soil was not affected by potato-onion VOCs (Figure S3B). Then, the bare soil pots were removed from the chamber and planted with tomato plants (Figure S3A). The results showed that tomato plants had similar biomass when growing in the bare soil previously exposed or not to potato-onion VOCs (Figure S3C). We also evaluated the effects of dipropyl disulfide on the growth and biofilm formation (bacterial traits important for root colonization [19]) of RV33 and RV57. The results showed dipropyl disulfide at concentrations varying from 0.0001 to 1.0 μg ml^{-1} has no direct effect on the growth or biofilm formation of both tested isolates (Figure S3D).

We further compared the chemistry of root exudates of tomato plants exposed or not to potato-onion VOCs. The results revealed a significant effect of exposure to potato-onion VOCs on tomato root exudates (permutation test, $R^2X = 0.791$ and $Q^2 = 0.882$; Fig. 5A). Specifically, this exposure resulted in higher concentration of ten metabolites (i.e., glutaric acid, L-malic acid, D-fructose, riboflavin, rutin, *p*-hydroxyacetophenone, fumaric acid, L-lysine, L-phenylalanine, and L-glutamine) in tomato exudates (variable importance of projection (VIP) > 1.5, $\log_2$ fold change > 1, and false discovery rate-adjusted $p < 0.01$; Fig. 5B). Similarly, we also analyzed the effect of exposure to dipropyl disulfide on tomato root exudates. The results showed higher concentrations of glutaric acid, L-malic acid, D-fructose, and L-glutamine, thus constituting similar results (Welch's *t* test, $p < 0.05$; Figure S4A). Further in vitro experiments showed that L-malic acid, D-fructose, fumaric acid, L-lysine, and L-glutamine can stimulate the growth

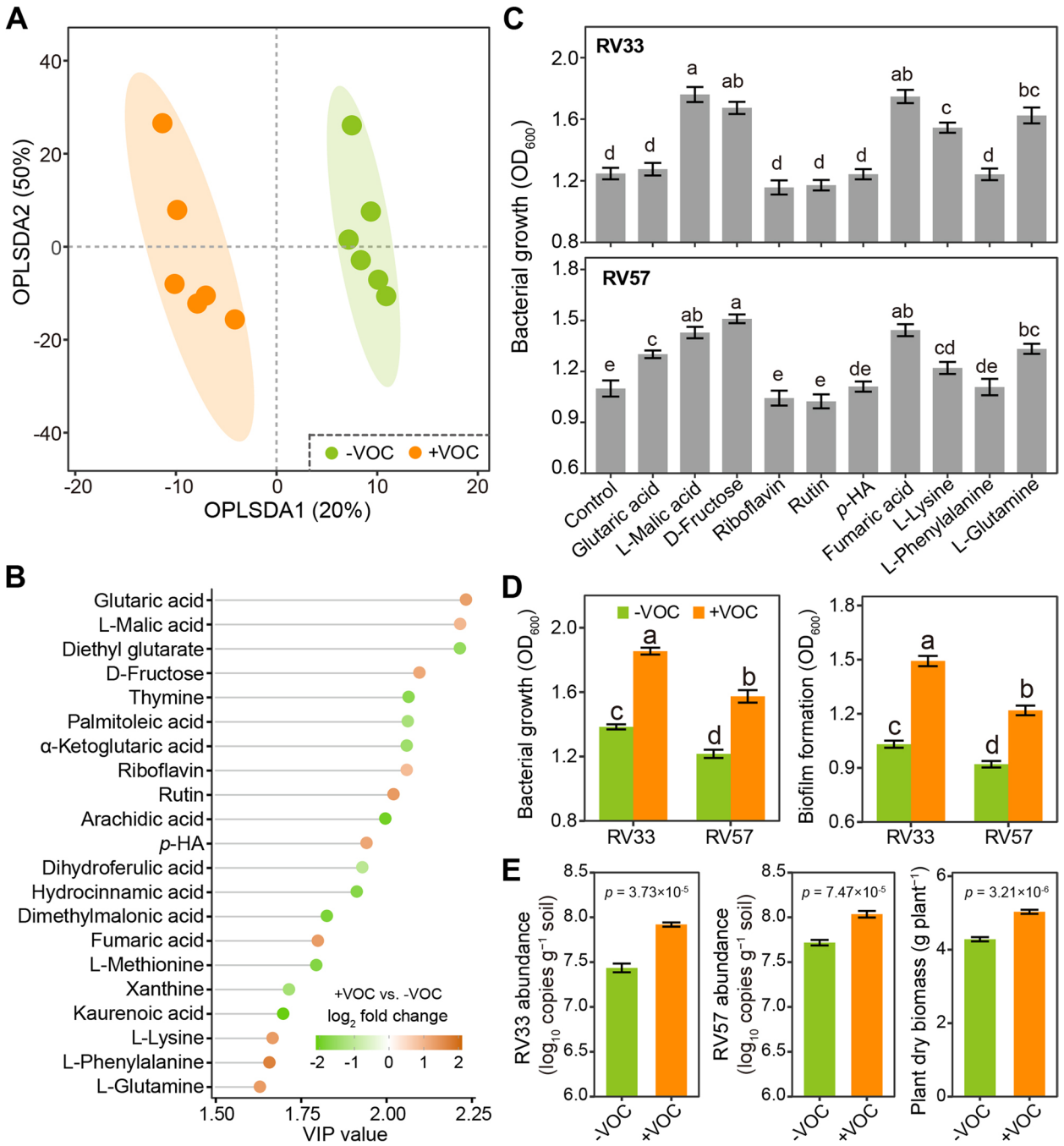

Fig. 5 Chemical changes in tomato root exudates alter the root colonization by RV33 and RV57. **A** Orthogonal partial least squares-discriminant analysis (OPLS-DA) displaying chemical changes in tomato root exudates due to exposure to potato-onion VOCs. **B** Tomato root exudate metabolites altered due to exposure to potato-onion VOCs. Metabolites with variable importance of projection (VIP) value > 1.5, $\log_2$ fold change > 1, and false discovery rate-adjusted $p < 0.01$ are shown. **C** Effects of selected root exudate compounds [glutaric acid, L-malic acid, D-fructose, riboflavin, rutin, *p*-HA (*p*-Hydroxyacetophenone), fumaric acid, L-lysine, L-phenylalanine, L-glutamine] on the growth of the bacterial isolates RV33 and RV57. **D** Effects of root exudates from tomato plants exposed to potato-onion VOCs (+VOC) or not (−VOC) on the growth and biofilm formation of the bacterial isolates RV33 and RV57. **E** Absolute abundance of RV33 and RV57 in the rhizosphere of tomato and tomato dry biomass of plants grown in soil amended with root exudates from tomato plants previously exposed to potato-onion VOCs (+VOC) or not (−VOC). Data are shown as mean ± SEM ($n = 6$). Different letters indicate significant differences between treatments (Tukey's HSD test, $p < 0.05$). *P* values were determined using Welch's *t* tests

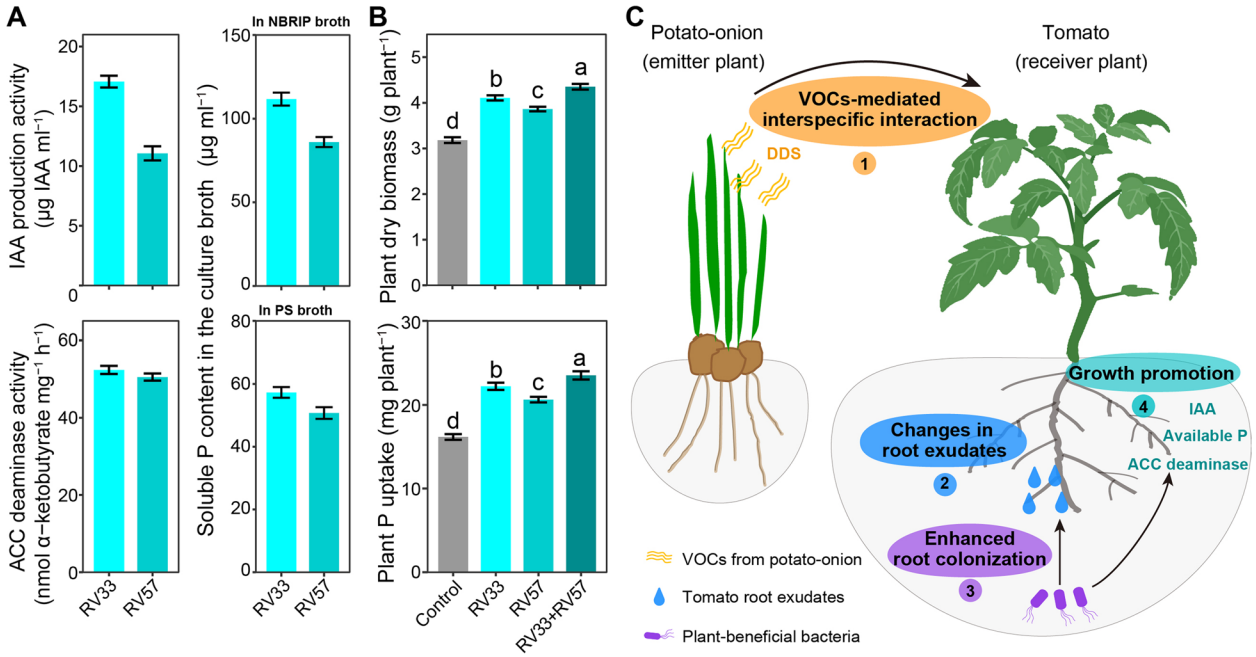

Fig. 6 Plant growth-promotion activities of the bacterial isolates RV33 and RV57. **A** Production of indole acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase and phosphate solubilization and phytate mineralization of bacterial isolates RV33 and RV57 (in vitro assays). NBRIP, National Botanical Research Institute Phosphate; PS, phytase screening. **B** Effects of single and mixed inoculation of the bacterial isolates RV33 and RV57 on plant dry biomass and plant P uptake (pot experiment). **C** Conceptual figure displaying the interspecific plant interactions mediated by VOCs, resulting in enhanced recruitment of plant-beneficial bacteria via modulation of root exudates of the receiver plant. Data are shown as mean ± SEM ($n = 6$). Different letters indicate significant differences between treatments (Tukey's HSD test, $p < 0.05$)

and biofilm formation of RV33 and RV57 (Tukey's HSD test, $p < 0.05$; Fig. 5C and Figure S4B). Glutaric acid was also found to stimulate the growth and biofilm formation of RV57 (Tukey's HSD test, $p < 0.05$). Similarly, we also evaluated the effects of tomato root exudates on the growth and biofilm formation of RV33 and RV57. The results showed that RV33 and RV57 had higher growth (+34.00% and +29.34%, respectively) and biofilm formation (+44.76% and +32.36%, respectively) when supplemented with root exudates of tomato plants exposed to potato-onion VOCs (controls consisted of tomato root exudates of plants not exposed to potato-onion VOCs) (Tukey's HSD test, $p < 0.05$; Fig. 5D). We performed a similar experiment using exudates from tomato plants treated with dipropyl disulfide and obtained equivalent results – i.e., positive effects on growth and biofilm formation of RV33 (+40.06% and +48.87%, respectively) and RV57 (+33.49% and +34.51%, respectively) when compared to controls (Tukey's HSD test, $p < 0.05$; Figure S5A).

Last, we tested the effects of tomato root exudate amendment on the root colonization by RV33 and RV57. This experiment was performed in pots containing sterile soil inoculated with a mixture of RV33 and RV57. The results showed that root exudates of tomato plants exposed to potato-onion VOCs increased the abundance of RV33 and RV57 in the tomato rhizosphere (by 198.43% and 108.83%, respectively; Welch's t test, $p < 0.05$; Fig. 5E). Similarly, the exogenous amendment of root exudates of tomato plants treated with dipropyl disulfide also increased the abundance of RV33 and RV57 in the tomato rhizosphere (by 208.42% and 137.59%, respectively; Welch's t test, $p < 0.05$; Figure S5B). We also found the exogenous amendment with root exudates of tomato plants exposed to potato-onion VOCs or dipropyl disulfide to result in greater tomato plant growth—average increases of 0.75 g per plant (17.28%) and 0.64 g per plant (14.77%), respectively (Welch's t test, $p < 0.05$; Fig. 5E and Figure S5B). Together, these results validate the effects of potato-onion VOCs and dipropyl disulfide in promoting plant growth by altering the recruitment of RV33 and RV57 in the rhizosphere via modulation of root exudates.

Plant growth-promoting activities of bacterial isolates stimulated by potato-onion VOCs

We evaluated the in vitro growth-promoting activities of the bacterial isolates RV33 and RV57. The results revealed these two isolates to be able to produce indole acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC)

deaminase (Fig. 6A). In addition, both isolates were able to (*1*) solubilize inorganic phosphate in NBRIP agar with tricalcium phosphate [$Ca_3(PO_4)_2$, a poorly soluble inorganic phosphate] as the sole phosphate source [30], and (*2*) mineralize organic phosphate on the phytase screening agar with phytate (a predominant type of organic phosphate present in soils) as the sole phosphate source [31] (Figure S6A) (see the "Methods" section for details). Further experiments in NBRIP broth and phytase screening broth validated the phosphorous mobilization activities of these isolates (Fig. 6A). The analysis of exometabolites produced by these isolates via high-pressure liquid chromatography (HPLC) revealed that both isolates produce organic acids (e.g., gluconic, acetic, malonic, and quinic acids by RV33; and isobutyric, oxalic, and gluconic acids by RV57) when cultured in NBRIP broth (Figure S6B). Moreover, both isolates decreased the pH of the NBRIP broth and showed phytase enzyme activities in the phytase screening broth (Figure S6C).

We validated the plant growth-promotion of these isolates on tomato plants in a sterile soil pot inoculation experiment. The inoculation of RV33 and RV57 separately resulted in an average increase in tomato plant dry biomass by 0.93 g per plant (29.13%) and 0.68 g per plant (21.43%), respectively (Tukey's HSD test, $p < 0.05$; Fig. 6B). The inoculation of a mixture of RV33 and RV57 (at the ratio of 1:1) resulted in an average increase in tomato plant dry biomass by 1.17 g per plant (36.77%) (Tukey's HSD test, $p < 0.05$). The single or mixture inoculation of these isolates resulted in increased plant P uptake and greater available P in the plant rhizosphere (Tukey's HSD test, $p < 0.05$; Fig. 6B and Figure S6D). We also tested whether RV33 and RV57 enhance tomato growth in the presence of insoluble forms of phosphate. For that, tomato plants were grown in a soilless medium (i.e., vermiculite and perlite) and supplemented with one-half strength of modified Hoagland's solution [32] containing a limited concentration (10 μM) of soluble potassium dihydrogen phosphate (KH_2PO_4) [33]. The results revealed that the plant growth-promoting activities of these isolates were greater when insoluble forms of phosphate [$Ca_3(PO_4)_2$ or Na-phytate] were amended in the system (Figure S6E).

Last, we evaluated the P uptake by tomato plants from the experiment used to test the effects of potato-onion VOCs and dipropyl disulfide on tomato plant growth (Figs. 1B and 3B). The results showed that potato-onion VOCs increased the P uptake and the availability of P in the rhizosphere of tomato plants grown in natural soil (Tukey's HSD test, $p < 0.05$; Figure S7A). Similarly, exposure to dipropyl disulfide also resulted in a positive effect on P uptake by tomato plants (Welch's *t* test, $p < 0.05$; Figure S7B).

Discussion

The importance of VOCs in mediating interspecific plant interactions

Recent studies have focused on elucidating the mechanisms by which VOCs mediate intra- and interspecific plant interactions [2, 3]. For example, one study showed that VOCs emitted by *Centaurea stoebe* (known as spotted knapweed) can positively affect the growth of diverse other interspecific neighboring plant species [8]. In line with that, our study found that VOCs constitutively released from potato-onion plants have a positive effect on the growth of tomato plants. The identified compound dipropyl disulfide—an organosulfur volatile in the blend of potato-onion VOCs—was validated as an effective molecule with an indirect and positive effect on tomato plant biomass. This finding is interesting because dipropyl disulfide has also been described to be produced by several *Allium* species [25]. In addition, we also tested whether potato-onion VOCs and dipropyl disulfide act directly by altering the tomato plant physiology or via induced changes in the root microbiota. The results revealed that both (potato-onion VOCs and dipropyl disulfide) induce changes in tomato root exudates with implications for the recruitment of plant-beneficial bacteria. Last, we validated the plant-growth promotion activities of specific bacterial isolates recruited in the tomato rhizosphere when plants are exposed to potato-onion VOCs or dipropyl disulfide, thus elucidating this new mechanism of interspecific plant interaction with positive effects on tomato plant performance (Fig. 6C). Collectively, our results explain and corroborate the positive effects of this intercropping system previously observed in field settings [34].

Previous studies demonstrated that plant diversity can enhance ecosystem productivity [35, 36]. In both natural and agricultural systems, positive interaction between plant species can lead to increases in ecosystem productivity, especially under biotic/abiotic stress conditions, e.g., nutrient deficiency [35, 36]. Particularly in intercropping systems, enhanced nutrient acquisition is often attributed to belowground root-root interactions [36–38]. For example, in a maize (*Zea mays* L.) and faba bean (*Vicia faba* L.) intercropping system, faba bean was reported to acidify the rhizosphere via exudation of organic acids, which stimulates the mobilization of P in the soil with positive effects for P uptake by maize plants [37]. In line with that, we have also previously demonstrated that potato-onion plants can enhance P uptake by tomato plants in an intercropping system—under controlled experimental conditions and in field settings [26, 34]. Here, we elucidated the mechanism by which this positive interspecific interaction operates. That is by enhancing the recruitment of plant-beneficial

bacteria in the tomato rhizosphere with growth-promotion activities.

VOC-mediated interspecific plant interactions integrated with rhizosphere ecology

The status of the rhizosphere microbiota has a strong effect on plant fitness. This occurs via diverse mechanisms, including the abundance and activities of plant-growth promotion microbes acting via facilitating nutrient uptake and producing phytohormones [14, 15]. Here, we show that the positive effect of potato-onion VOCs on tomato growth was attributed to shifts in the tomato rhizosphere microbiota. We initially validated this finding using a treatment in which tomato plants exposed to VOCs were grown in sterile soil. The results revealed that the absence of the soil microbiota leads to no significant effect of potato-onion VOCs on tomato growth (Fig. 1C). To further validate this finding, we performed a rhizosphere transplant experiment that recovered the positive effect on tomato growth (Figure S1D) when the rhizosphere sample of a previous tomato plant exposed to potato-onion VOCs was transplanted into the system. Later, we found that exposure to potato-onion VOCs increased the abundance of specific bacterial taxa (e.g., *Pseudomonas* and *Bacillus* spp.) in the tomato rhizosphere. These genera are well-known to contain species with plant-growth promotion traits [39]. Subsequent isolation and culturing of some of these bacteria allowed us to validate their plant growth-promoting traits, such as P mobilization, and the biosyntheses of IAA and ACC deaminase. The plant-growth-promoting abilities of the isolates RV33 and RV57 were further validated using pot inoculation experiments (Fig. 6B).

The study of intra- and interspecific plant interactions integrated with general aspects of rhizosphere ecology is relatively recent [21–24]. For example, it was shown that the production of cyanide by neighboring cassava (*Manihot esculenta* Crantz) plants can trigger the biosynthesis of ethylene in peanut (*Arachis hypogaea* L.) roots and alter the microbiota with positive effects on nutrient availability and peanut seed production [21–24]. In another study, Kong et al. [21] showed that the inoculation of tomato plants with the plant growth-promoting bacterium *Bacillus amyloliquefaciens* strain GB03 triggers the release of VOCs with consequences for the microbiota assembly in neighboring intraspecific plants. Here, we elucidated a new mechanism of interspecific plant communication integrated with chemical and biological shifts in the receiver plant rhizosphere. Specifically, we validated the molecule dipropyl disulfide emitted as a VOC by potato-onion plants to modulate the chemical exudation of a neighboring tomato plant. This chemical interaction resulted in a shift in the tomato rhizosphere microbiota with a positive effect on tomato growth.

Changes in root exudates alter the root colonization by plant-beneficial *bacteria*

Interspecific plant interactions can lead to morphological and physiological responses (phenotypic plasticity) in receiver plants, which may confer a fitness advantage [1, 11, 40]. Plant-root exudates can serve as nutrient sources for microbes in the rhizosphere and act as chemical cues for the recruitment of plant-beneficial bacteria [17–19, 41]. Corroborating our findings, other studies have shown how plant intra- and interspecific interactions can lead to alterations in plant root exudates modulating the microbiota assembly and functioning [21, 22, 42]. Here, we showed that exposure to potato-onion VOCs or dipropyl disulfide alters tomato root exudates, for example, by increasing the exudation of glutaric acid, L-malic acid, D-fructose, L-glutamine. Moreover, using in vitro assays, we showed some of these compounds to stimulate the growth and biofilm formation—i.e., traits associated with rhizosphere competence—in the bacterial isolates RV33 and RV57 retrieved from the tomato rhizosphere samples and identified as plant-growth promotion taxa. Therefore, these observed changes in root exudates can be seen as a plastic phenotypic response of tomato plants to VOCs emitted by the neighboring potato-onion plants (Fig. 6C). Interestingly, in the root colonization experiment, the potato-onion VOCs and dipropyl disulfide were applied to tomato leaves and not directly into the soil (Fig. 4C). Thus, it is possible to assume that the observed effect of dipropyl disulfide on modulating the tomato root exudates is likely systemic (i.e., from shoot to root).

In natural plant communities, neighboring plants generally compete for limited resources. The ability of plants to efficiently perceive and respond to chemical cues can have consequences for plant fitness and confer competitive advantage [1, 5, 43]. Evidence is mounting that plant VOCs carry specific information about the genetic identity of the emitter plants and that nearby plants can use this information to detect competitive neighbors [1, 7]. Specifically important for this study, we have previously reported an asymmetric benefit in terms of growth and biomass accumulation of tomato and potato-onion species cultivated in an intercropping system. That is, whereas tomato plant growth is greater in the intercropping, the potato-onion biomass is reduced [34]. The present study, however, complements these findings by showing that tomato plants respond to VOCs emitted by potato-onion plants by altering the root exudate chemistry and recruiting plant-beneficial bacteria in the rhizosphere. This response has a direct positive effect

on tomato plants' performance. Therefore, this response can be seen as a mechanism promoting selective advantage in receiver plants. In this context, it is important to notice, however, that these two plant species do not share an evolutionary history of coexistence that might explain this interspecific interaction. That is, this interaction is a result of crop management choice rather than derived from a long-term co-existence between these two species within an evolutionary context.

Conclusions

This study elucidates a new mechanism of interspecific plant communication via VOCs resulting in induced shifts in rhizosphere microbiota assembly (Fig. 6C). Specifically, we identified the compound dipropyl disulfide as the active molecule within the blend of potato-onion VOCs mediating this interspecific plant communication. Exposure to either potato-onion VOCs or dipropyl disulfide induced trackable changes in tomato root exudates. We showed this modulation in tomato root exudate to stimulate the growth and biofilm formation of plant-beneficial bacteria belonging to the genera *Pseudomonas* and *Bacillus* spp. Using representative isolates, we characterized the plant-growth promotion traits of these bacteria and validated their activities using pot inoculation experiments. Together, this study unraveled new information on the ecology of plant interspecific interactions and rhizosphere biology with consequences for the modulation of the rhizosphere microbiota affecting plant growth and performance. Our findings open new avenues for the investigation of VOC-mediated rhizosphere recruitment with beneficial outcomes for crop systems.

Methods

Soil and plant materials

The soil used in this study was collected (top 0–30 cm) from a field with a history of more than 15 years of maize (*Zea mays* L.) monoculture cultivation in Xiangyang farm, Harbin, China (45° 46′ N, 126° 56′ E). After removing plant debris and rocks, the soil was sieved (< 2 mm) and homogenized. The soil was classified as sandy loam, containing 26.35 g kg^{-1} of organic matter, 76.56 mg kg^{-1} of inorganic nitrogen, 38.36 mg kg^{-1} of available phosphorous (Olsen P), 113.26 mg kg^{-1} of available potassium, EC (1:2.5, w/v) of 0.32 mS cm^{-1}, and pH (1:2.5, *w/v*) 7.41. This soil was used in the experiment under two conditions: (*1*) natural soil, i.e., the soil collected from the field, and (*2*) sterile soil, the natural soil autoclaved twice (121 ℃ for 30 min).

Tomato (cv. Jinfen) seeds and potato-onion bulbs (cv. SH) were surface sterilized with sodium hypochlorite solution (3%) for 10 min and rinsed five times with sterile water. Sterilized potato-onion bulbs were planted into plastic pots (16 cm × 14 cm) filled with 1 kg of natural or sterile soil. Surface-sterilized tomato seeds were germinated on cotton gauze at 28 ℃ in a growth chamber and planted into plastic pots (16 cm × 14 cm) filled with 1 kg of natural or sterile soil. To avoid the potential interaction between tomato and potato-onion plants via VOCs, each plant species was maintained in a separate greenhouse (light/dark cycle of 16/8 h, mean day/night temperature of 30 ℃/22 ℃, and 70% relative humidity) before the start of the experiments.

Twin-chamber experiment used to test the influence of potato-onion VOCs on tomato performance

The twin-chamber system was implemented as previously described [40]. This system consists of a series of clear glass cages divided into two chambers (each chamber with a dimension of 0.3 m × 0.3 m × 0.6 m, length × width × height) (Fig. 1B). Thirty-day-old tomato (four-leaf stage) and potato-onion (three-leaf stage) plants were placed into the twin-chamber system, each chamber containing one pot of one species (i.e., tomato or potato-onion). The experiment consisted of two treatments: (*1*) tomato plants exposed to potato-onion VOCs (+ VOC treatment) and (*2*) tomato plants not exposed to potato-onion VOCs (− VOC treatment). For the + VOC treatment, a circular opening (5 cm diameter) in the middle of the separating wall was used for air flow between chambers. The air was vented via an air-delivery system into the potato-onion chamber, then into the tomato chamber, and finally out of the greenhouse via a polyvinyl chloride tube (air flow rate of 0.3 L min^{-1}). For the − VOC treatment, there was no opening in the separating, and the air vented in and out of each chamber.

This experiment was performed with potato-onion and tomato plants grown under distinct soil conditions: (*1*) both plant species cultivated in natural soil, (*2*) potato-onion plants cultivated in sterile soil and tomato plants in natural soil, and (*3*) both plant species cultivated in sterile soil. The experiment consisted of six treatments (i.e., − VOC and + VOC treatments in each of the three soil conditions). Each treatment was replicated six times, and each replicate contained 30 sets of the twin-chamber system for potato-onion in sterile soil and tomato in natural soil and 5 sets of the twin-chamber system for the other soil conditions. The twin-chamber system was maintained in a greenhouse, and plants were regularly irrigated with sterile water. After 20 days, tomato plants were harvested to measure plant dry biomass and P uptake. For treatments with potato-onion grown in sterile soil and tomato in natural soil, tomato plant root exudates were also collected. Rhizosphere samples from tomato plants were collected to measure available

P content, obtain material for the rhizosphere transplant experiment, and isolate culturable bacteria. These rhizosphere samples were also subjected to total DNA extraction for microbiome profiling.

Plant dry weight was measured after drying plant materials to constant weight in an oven at 70 ℃. Dry plant materials containing shoots and roots were ground and digested with sulfuric acid and hydrogen peroxide to measure total P content using the molybdenum blue spectrophotometry method [44]. Available P in the soil was extracted with sodium bicarbonate and measured using the molybdenum blue spectrophotometry method [44].

Rhizosphere transplant experiment

The rhizosphere transplant experiment was performed as previously described [45, 46]. In brief, rhizosphere samples from tomato plants obtained in the twin-chamber experiment (i.e., the treatment containing tomato plants grown in natural soil and potato-onion in sterile soil) were mixed with sterile soil at a ratio of 6% (*w/w*). After that, 30-day-old tomato plants were transplanted into plastic pots (16 cm × 14 cm) filled with 1 kg of this soil mixture (Fig. 1B). This experiment consisted of two treatments: tomato plants grown in sterile soil mixed with tomato rhizosphere of (*1*) the − VOC treatment and (*2*) the + VOC treatment. A single tomato plant was planted in each pot. Each treatment was replicated six times, and each replicate contained a total of 10 plants. All plants were maintained in a greenhouse and regularly irrigated with sterile water. Twenty days after the transplantation, tomato plants were harvested to measure plant dry biomass.

Chemical analysis of VOCs released by tomato and potato-onion plants

The VOC profiles from tomato and potato-onion plants were analyzed by headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC–MS). To sample plant VOCs, tomato or potato-onion plants grown in pots were placed in a 20-l vacuum desiccator for two hours with SPME fibers (100 μm polydimethylsiloxane; Supelco, Bellefonte, USA) used to absorb VOCs. To avoid the collection of VOCs emitted from the soil, the entire pot was covered with two layers of tinfoil paper, and the connection between the tinfoil paper and the plants was sealed using tape. This procedure was performed without causing any damage to the plants. GC–MS analysis was carried out using a 7890A gas chromatograph connected to a 5975C mass spectrometry system (Agilent Technologies, Santa Clara, USA). A DB-Wax column (30 m long × 0.25 mm internal diameter × 0.25 μm film thickness) was used with helium as the carrier gas at the flow rate of 1 ml min^{-1}. The initial oven temperature was set at 40 ℃ for 2 min, raised to 170 ℃ at a rate of 6 ℃ min^{-1}, then raised to 250 ℃ at a rate of 10 ℃ min^{-1}, and finally kept at 260 ℃ for 4 min. The mass spectrometer was operated with electron ionization at 70 eV (source temperature of 250 ℃) and scanning an m/z range of 40–450. Metabolite identification was performed by comparing the mass spectra of the samples with the data system (NIST 08 and WILEY 05) and using an *in-house* library of metabolites (Metware Biotechnology Co., Ltd. Wuhan, China). The concentration of dipropyl disulfide in the twin-chamber experiment was quantified as previously described [29]. Volatiles from the chamber containing tomato plants were trapped with Super Q divinyl benzene polymer adsorbent (Alltech, Deerfield, USA) for 1 h and released by rinsing with methylene chloride. The extract was analyzed using GC–MS and the quantification of dipropyl disulfide was performed using an external standard method.

Evaluation of the effect of dipropyl disulfide on tomato performance

An experiment using a single-compartment chamber was performed to test the effect of dipropyl disulfide on tomato plant performance (Fig. 3B). Thirty-day-old tomato plants grown in natural soil were placed into the single chamber system. Only a single pot was incubated per chamber. The exposure of tomato plants to dipropyl disulfide was carried out by placing a glass vessel containing dipropyl disulfide dissolved in Tween-80 (1:2, *w:w*) inside each chamber. The control consisted of a glass vessel containing only Tween-80. The distance from the tomato plants to the vessel was 10 cm. We adjusted the size of the hole in the vessel to achieve a release rate of dipropyl disulfide of ~ 220 ng per hour. These glass vessels were replaced in each chamber every two days. The air was vented in and out of the chamber (air flow rate of 0.3 L min^{-1}). This experiment consisted of two treatments (i.e., dipropyl disulfide treatment and non-treated control) with six replicates, and each replicate contained 10 independent chambers. These systems were maintained in a greenhouse, and plants were regularly irrigated with sterile water. After 20 days, tomato plants were harvested to measure dry biomass and P uptake. These plants were also subjected to rhizosphere and root exudate sample collection.

Isolation and characterization of culturable *bacteria* from the rhizosphere of tomato plants

Culturable bacteria were isolated from the tomato rhizosphere samples collected in the twin-chamber experiment (i.e., tomato grown in natural soil and potato-onion in sterile soil). Bacterial isolation was performed using the

plate culturing method on 1/10 tryptic soy agar, R2A agar, and King's B agar media. The taxonomic affiliation of the obtained isolates was performed by sequencing the 16S rRNA gene with the primer set 27F/1492R [47]. Within the obtained isolates, we selected RV33 and RV57—displaying 100% sequence similarity with OTU5587 and OTU114, respectively—for further experiments.

The in vitro production of IAA by RV33 and RV57 was measured by growing each isolate in Luria–Bertani broth supplemented with 500 mg L^{-1} of L-tryptophan at 28 ℃ for 2 days. The IAA content in the culture filtrate was measured using the Salkowski reagent [48]. The ACC deaminase activity was determined by monitoring the amount of α-ketobutyrate generated by the enzymatic hydrolysis of ACC [49]. The phosphate solubilization potential was determined using the NBRIP agar plate containing tricalcium phosphate as the sole phosphate source [30]. The phytate mineralization potential was determined using the phytate screening agar plate containing Na-phytate as the sole phosphate source [31]. Quantitative estimation of P-mobilizing potential was performed in NBRIP broth and phytate screening broth, respectively. In brief, each isolate was cultured in Luria–Bertani broth at 28 ℃ with shaking at 120 rpm for 24 h, followed by culture dilution to an optical density—OD_{600} of 1.0 using fresh Luria–Bertani broth. A volume of 100 µl of the diluted culture was transferred into 50 ml of NBRIP broth or phytate screening broth. After incubation at 28 ℃ with shaking at 120 rpm for 48 h, bacterial cells were removed from the culture medium via membrane filtering (0.22 µm). The culture filtrates of the NBRIP broth were analyzed for soluble phosphate content, pH, and organic acid content. The culture filtrates of the phytate screening broth were analyzed for soluble phosphate content and phytase activities. Additional details of these methods are described in Supplementary Methods.

A pot experiment using 20-day-old tomato plants grown in sterile soil was performed to evaluate the effects of RV33 and RV57 on tomato growth and P uptake. This experiment consisted of four treatments: tomato plants inoculated with (*1*) RV33, (*2*) RV57, (3) a mixture of both isolates (1:1), and (4) a non-inoculated control. Bacterial isolates were inoculated as a soil drench at a final density of 1.0×10^6 CFU g^{-1} soil. Each treatment was replicated six times with 15 plants in each replicate. All plants were maintained in a greenhouse and regularly irrigated with sterile water. Twenty days after the start of the experiment, tomato plants were harvested to measure plant dry biomass and P uptake. These plants were also subjected to rhizosphere sample collection to quantify available P content.

A hydroponic experiment was performed to test the effects of RV33 and RV57 on the performance of tomato plants grown in the presence of insoluble forms of inorganic and organic phosphate. In brief, 20-day-old tomato plants were transferred into pots containing a mixture of vermiculite and perlite (1:1, *w:w*) supplemented with one-half strength of modified Hoagland's solution [32]. The system was supplemented with potassium dihydrogen phosphate (KH_2PO_4) added at 10 µM (this constitutes a P-limited concentration for tomato growth [33]). The growth medium was supplemented or not with an insoluble form of phosphate – $Ca_3(PO_4)_2$ or Na-phytate (at the concentration of 15 mg g^{-1} soil). Bacterial isolates RV33 or RV57 were inoculated into the solution. The experiment consisted of nine treatments: (*1–3*) RV33 or (*4–6*) RV57 inoculation in each phosphate condition, and (*7–9*) non-inoculated controls in each phosphate condition. Each treatment was replicated six times with a total of ten plants in each replicate. After growing tomato plants for 20 days under these conditions, the plants were harvested to measure dry biomass and P uptake.

Evaluation of the effects of potato-onion VOCs and dipropyl disulfide on the root colonization by bacterial isolates

Thirty-day-old tomato plants grown in sterile soil were inoculated with a 1:1 mixture of the bacterial isolates RV33 and RV57 at a final density of 1.0×10^6 CFU g^{-1} soil (Fig. 4B). These plants were placed in one chamber of a twin-chamber system (described above). The other chamber consisted of one potato-onion plant grown in sterile soil or a glass vessel containing dipropyl disulfide dissolved in Tween-80 (1:2, *w:w*). The experiment consisted of four treatments: (*1*) tomato plants exposed to potato-onion VOCs (+VOC treatment), (*2*) tomato plants not exposed to potato-onion VOCs (−VOC treatment), (*3*) tomato plants exposed to dipropyl disulfide (+DDS treatment), and (*4*) tomato plants not exposed to dipropyl disulfide (-DDS treatment). The release rate of dipropyl disulfide was set at ~ 220 ng $hour^{-1}$. Each treatment was replicated six times, and each replicate contained eight sets of the twin-chamber system. The air was vented in and out of the chamber (air flow rate of 0.3 L min^{-1}). To avoid the direct effect of potato-onion VOCs and dipropyl disulfide on the bacterial isolates, the entire pot was covered with two layers of tinfoil paper, and the connection between the tinfoil paper and the plants was sealed using tape. The twin-chamber systems were maintained in a greenhouse, and plants were regularly irrigated with sterile water. Twenty days after the start of the experiment, tomato plants were harvested to measure dry biomass. These plants were also subjected to rhizosphere

sample collection to quantify the abundances of RV33 and RV57 via quantitative PCR.

Evaluation of effects of potato-onion VOCs on *Bacillus* and Pseudomonas spp. abundances in bare soil

This experiment was performed in the twin-chamber system. One chamber contained 30-day-old potato-onion plants grown in sterile soil and the other chamber contained a pot with natural soil without plants (termed "bare soil") (Figure S3A). The bare soil chamber was exposed or not to potato-onion VOCs. For the bare soil treatment exposed to potato-onion VOCs, the air was vented into the chamber containing the potato-onion plant, then into the chamber containing the bare soil, and finally vented outside the greenhouse via a polyvinyl chloride tube (air flow rate of 0.3 L min^{-1}). For the control treatment, the air vented in and out of each chamber independently. Each treatment was replicated six times, and each replicate consisted of five sets of the twin-chamber system. These systems were maintained in a greenhouse, and plants were regularly irrigated with sterile water. After 20 days, all bare soils were harvested, and the abundances of *Bacillus* and *Pseudomonas* spp. were determined using quantitative PCR. In a follow-up experiment, all pots containing the bare soil were taken out of the twin-chamber system and planted with 30-day-old tomato plants (Figure S3A). These tomato plants were maintained in a greenhouse and regularly irrigated with sterile water. Twenty days after the transplant, tomato plants were harvested to measure dry biomass.

Analysis of bacterial community composition and abundances in the tomato rhizosphere

Soil genomic DNA was extracted from 0.25 g of tomato rhizosphere samples using the Power Soil DNA Isolation Kit (QIAGEN, Maryland, USA), following the manufacturer's instructions. The quality and concentration of the obtained DNAs were checked with 1.2% (*w:v*) agarose gel electrophoresis and using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Wilmington, USA).

Bacterial community composition was profiled via high-throughput amplicon sequencing. The V4 – V5 regions of the bacterial 16S rRNA gene were amplified with the primer set F515/R907 containing specific overhang Illumina adapters. A second eight-cycle PCR was performed to add dual index and Illumina sequencing adapters. PCR products were purified, quantified, and normalized before pooling. The DNA library pool was paired-end sequenced (2 × 300) on an Illumina Miseq PE300 platform (Illumina Inc., San Diego, USA). Raw sequence reads were processed using QIIME [50]. Chimeras were screened and removed using USEARCH with the UCHIME algorithm [51]. Sequences were clustered into OTU at 97% nucleotide similarity with UPARSE [51]. One representative sequence per OTU was taxonomically assigned using the SILVA database (v132) [52]. Sequences belonging to chloroplasts, mitochondria, and archaea were removed, and samples were rarefied to the minimum number of sequences per sample before statistical analyses.

The absolute abundances of *Bacillus* and *Pseudomonas* spp. in the tomato rhizosphere were determined using quantitative PCR with the primer sets BacF/BacR [53] and Pse435F/Pse686R [54] targeting the partial 16S rRNA gene of each genus. Primer sequences and details of these methods are described in Supplementary Table 1 and Supplementary Methods.

Collection and analysis of tomato root exudates

Tomato root exudates were collected following a previously described method [22]. In brief, tomato plants were carefully removed from the pots, and roots were washed with sterile deionized water. Then, tomato plants were transferred into a beaker with their roots immersed in sterile deionized water supplemented with calcium chloride (0.5 mM). The beaker was wrapped with black paper to prevent light and placed in a growth chamber at 28 ℃ under light for 5 h. The collected solutions were filtered through 0.45 μm (Merck, Darmstadt, Germany), frozen at – 20 ℃, and lyophilized for further analysis.

Root exudate samples were analyzed using a Nexera X2 Ultra High-Performance Liquid Chromatography system (SHIMADZU, Kyoto, Japan) coupled with a 4500 QTRAP mass spectrometer (AB Sciex, Foster City, USA). For HPLC analysis, a gradient-elution program was used based on the mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile): 0 to 1 min, 95% A plus 5% B; 1 to 10 min, 65% A plus 35% B; 10 to 20 min, 50% A plus 50% B; 20 to 22 min, 10% A plus 90% B; and 22 to 25 min, 90% A plus 10% B. The parameters consisted of a column temperature of 40 ℃, injection volume of 5 μl, and flow rate of 0.35 ml min^{-1}. The mass spectrometer was operated with an ion source, turbo spray; ion temperature at 550 ℃; ion spray voltage at 5500 V; ion source gas I and II at 50 and 60 psi; curtain gas at 25 psi; and scanning an m/z range of 50–1000. Metabolite identification was performed using exact mass and retention time compared with an *in-house* library of metabolites (Metware Biotechnology Co., Ltd. Wuhan, China), corresponding to the second level of putative identification. These data were normalized using the respective root dry weight.

Bacterial isolate growth and biofilm assays

For the bacterial growth assay, each bacterial isolate was pre-grown in Luria–Bertani broth at 28 °C with

shaking at 120 rpm for 24 h, and the culture was diluted to $OD_{600}=1.0$ with fresh Luria–Bertani broth. A volume of 250 µl of the diluted cell suspension was inoculated in 25 ml of fresh Luria–Bertani broth supplemented with dipropyl disulfide solution, tomato root exudates, or its component solutions (see below). After incubation at 28 °C with shaking at 120 rpm for 24 h, bacterial growth was determined by measuring OD_{600}. For the biofilm assay, a volume of 200 µl of the diluted cell suspensions was transferred into each well of a sterile 48-well polystyrene microtiter plate. After that, 20 µl of dipropyl disulfide solution, root exudates, or its component solutions (see below) were added to each well. The system was incubated at 28 °C for 24 h, and cells adhered to the wells were stained with 0.1% crystal violet for 30 min. Biofilm formation was quantified by measuring the OD_{600} of each well. For these experiments described above, the effect of dipropyl disulfide was tested at varying concentrations of 0.0001, 0.001, 0.01, 0.1, and 1.0 µg ml^{-1}. These dilutions were made using a 50-mM stock solution of dipropyl disulfide in 80% methanol. The control treatments consisted of equal amounts of methanol without dipropyl disulfide. Likewise, the treatments containing tomato root exudates consisted of exudates collected from the experiment set to evaluate the effects of potato-onion VOCs and dipropyl disulfide on tomato plant performance (see above). Last, the treatments consisting of exudate components were characterized by each compound at a final concentration of 10 µM (i.e., glutaric acid, L-malic acid, D-fructose, riboflavin, rutin, *p*-hydroxyacetophenone, fumaric acid, L-lysine, L-phenylalanine, and L-glutamine). The bacterial growth and biofilm assays consisted of six replicates per treatment.

Evaluation of the effects of tomato root exudates on root colonization by specific bacterial isolates

Thirty-day-old tomato plants grown in sterile soil were inoculated with a 1:1 mixture of the bacterial isolates RV33 and RV57 at a final density of 1.0×10^6 CFU g^{-1} soil. Then, 20 ml of different tomato root exudates (see below) was applied to the soil surface as a soil drench every 3 days. The treatments consisted of root exudates of tomato plants exposed or not to potato-onion VOCs, and root exudates of tomato plants treated or not with dipropyl disulfide. Each treatment was replicated six times with five pots in each replicate. All tomato plants were maintained in a greenhouse and regularly irrigated with sterile water. Twenty days after the start of the experiment, tomato plants were harvested to determine dry biomass. These plants were also subjected to rhizosphere sample collection to quantify the absolute abundances of RV33 and RV57 using quantitative PCR.

Statistical analyses

Statistical analyses were performed in R (v4.3.0, http://www.r-project.org/). Data distributions and homogeneity of variances of the data were checked using the Shapiro–Wilk test and Levene's test, respectively. Significant differences in the variance of parameters were evaluated using Welch's *t* test (for two groups) or one-way analysis of variance (ANOVA) followed by Tukey's HSD test (for more than two groups). PCoA and PERMANOVA based on Bray–Curtis dissimilarities were performed to analyze bacterial community β-diversity with the "vegan" package [55]. Differences in the relative abundance of bacterial OTUs between treatments were determined using the likelihood ratio test implemented in the "edgeR" package [56]. Differences in tomato root exudate profiles were analyzed using the OPLS-DA combined with the likelihood ratio test. The *p* values were corrected for multiple comparisons using the false discovery rate method.

Supplementary Information

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Additional file 1: Supplementary Methods: Quantitative PCR analysis of Bacillus and Pseudomonas spp. Evaluation of plant growth-promoting traits in bacterial isolates. Fig. S1. Effects of potato-onion VOCs on the α-diversity and taxonomic composition of the tomato rhizosphere bacterial community. A Bacterial community α-diversity from the tomato rhizosphere. The number (No.) of observed OTUs and the Shannon index are shown. B Relative abundance (RA) of the main bacterial phyla/Proteobacteria classes (mean relative abundances > 1%) in each treatment. Tomato plants were exposed to potato-onion VOCs (+VOC) or not (-VOC). Each bar represents the mean value of six replicates. C Manhattan plot displaying the taxonomic assignments of OTUs that increased or decreased (in terms of relative abundance) after exposure to potato-onion VOCs (false discovery rate-adjusted $p < 0.01$, log 2 fold change > 1). * and ** indicate significant difference at $p < 0.05$ and $p < 0.01$, respectively (Welch's t -test). Fig.S2. Effect of dipropyl disulfide on bacterial OTUs in the tomato rhizosphere. The Manhattan plot displays the taxonomic assignments of OTUs that increased in relative abundance after exposure to dipropyl disulfide (false discovery rate-adjusted $p < 0.01$, log 2 fold change > 1). The bar plot within the panel shows the relative abundances (RA) of OTU3304 and OTU4955. Tomato plants were treated with dipropyl disulfide (+DDS) or not (-DDS). Data are shown as mean ± SEM ($n = 6$). *P* -values were determined using Welch's t -tests. Fig. S3. Effects of potato-onion VOCs on Pseudomonas and Bacillus spp. growth and biofilm formation.A The experimental set-up of the twin-chamber system was used to test the effect of potato-onion VOCs on the abundance of Pseudomonas and Bacillus spp. in bare soil. In this system, the bare soil was exposed to potato-onion VOCs (+VOC) or not (-VOC). In the following pot experiment, tomato plants were grown in bare soil from the twin-chamber system. B Pseudomonas and Bacillus spp. abundances in bare soil from the twin-chamber system. C Tomato plant dry biomass in the following pot experiment. D Effects of dipropyl disulfide on the in vitro growth and biofilm formation of RV33 and RV57. Data are shown as mean ± SEM (n = 6). *P* -values were determined using Welch's t -tests. Different letters indicate significant differences between treatments (Tukey's HSD test, $p < 0.05$). Fig. S4. Effect of dipropyl disulfide on tomato root exudates and the effects of selected metabolites on the biofilm formation of bacterial isolates.A Concentration of selected metabolites in the tomato root exudate that were stimulated by exposure to dipropyl disulfide. B Effects of selected root exudate compounds (glutaric acid, L-malic acid, D-fructose, riboflavin, rutin, p -HA, fumaric acid, L-lysine, L-phenylalanine, L-glutamine)

on the biofilm formation of the bacterial isolates RV33 and RV57. p -HA: p -Hydroxyacetophenone. Data are shown as mean ± SEM ($n = 6$). Different letters indicate significant differences between treatments (Tukey's HSD test, $p < 0.05$). *P* -values were determined using Welch's t -tests. Fig.S5. Root exudates of tomato plants treated with dipropyl disulfide stimulate root colonization by specific bacteria that positively affect plant growth.A Effects of root exudates of tomato plants treated with dipropyl disulfide (+ DDS) or not (-DDS) on the growth and biofilm formation of the bacterial isolates RV33 and RV57. B Abundances of RV33 and RV57 in the rhizosphere and dry biomass of tomato plants grown in soil amended with root exudate of tomato plants treated with dipropyl disulfide (+ DDS) or not (-DDS). Data are shown as mean ± SEM (n = 6). *P* -values were determined using Welch's t -tests. Fig.S6. Phosphorous mobilization activity of the bacterial isolates RV33 and RV57.A Photographs showing the abilities of RV33 and RV57 to mobilize P on National Botanical Research Institute Phosphate (NBRIP) agar and phytase screening (PS) agar. B Organic acids produced by RV33 and RV57 in NBRIP broth determined by high-performance liquid chromatography analysis. C Changes in the pH of the culture medium caused by bacteria grown in NBRIP broth, and the bacterial phytase activity in PS broth. D Effects of the bacterial isolates RV33 and RV57 on the rhizosphere Olsen P content in the pot experiment. E Effects of RV33 and RV57 on the dry biomass and P uptake of tomato plants grown in soilless medium supplemented with different forms of phosphates. KP, soluble phosphate potassium dihydrogen phosphate (KH 2 PO 4); CaP, poorly soluble inorganic phosphate tricalcium phosphate [Ca 3 (PO 4) 2]; OP, organic phosphate Na-phytate. The values above bars are the increased percentages in each bacteria treatment vs. control. Data are shown as mean ± SEM ($n = 6$). Different letters indicate significant differences between treatments (Tukey's HSD test, $p < 0.05$). Fig.S7. Potato-onion VOCs and dipropyl disulfide enhance tomato P uptake.A Tomato P uptake and rhizosphere Olsen P content data from the twin-chamber system used to test the effects of potato-onion VOCs on tomato growth. B Tomato plant P uptake and rhizosphere Olsen P content in the experiment used to test the effects of dipropyl disulfide on tomato growth in natural soil. Values above bars are the increased percentages in the + VOC treatment vs. the -VOC treatment (or in the + DDS treatment vs. the -DDS treatment). Data are shown as mean ± SEM (n = 6). *P* -values were determined using Welch's t -tests. Different letters indicate significant differences between treatments (Tukey's HSD test, $p < 0.05$). Table S1. PCR primers used in this study.

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Not applicable.

Authors' contributions
X.Z., Z.W., and F.W. conceived and designed the experiments; X.Z., J.Z., and J.S. performed the experiments and analyzed the data; X.Z., M.K.u.R., H.L., F.W., F.D-A., and Z.W. wrote the manuscript. All authors edited the manuscript and approved the final version.

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Data availability
The partial 16S rRNA gene sequences for isolated bacteria were submitted to NCBI GenBank with the accession numbers PP212036-PP212056 and PP212833-PP212849. Amplicon sequencing data was deposited in the Sequence Read Archive at NCBI with the accession number PRJNA985392. The *R* scripts used for the statistical analysis and plotting of the figures are available at https://github.com/xingangzhouneau/VOCs.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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