Molecules and Cells

Elicitation of Innate Immunity by a Bacterial Volatile 2-Nonanone at Levels below Detection Limit in Tomato Rhizosphere

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https://doi.org/10.14348/molcells.2022.2009
www.molcells.org

Bacterial volatile compounds (BVCs) exert beneficial effects on plant protection both directly and indirectly. Although BVCs have been detected *in vitro*, their detection *in situ* remains challenging. The purpose of this study was to investigate the possibility of BVCs detection under *in situ* condition and estimate the potentials of *in situ* BVC to plants at below detection limit. We developed a method for detecting BVCs released by the soil bacteria *Bacillus velezensis* strain GB03 and *Streptomyces griseus* strain S4-7 *in situ* using solid-phase microextraction coupled with gas chromatography-mass spectrometry (SPME-GC-MS). Additionally, we evaluated the BVC detection limit in the rhizosphere and induction of systemic immune response in tomato plants grown in the greenhouse. Two signature BVCs, 2-nonanone and caryolan-1-ol, of GB03 and S4-7 respectively were successfully detected using the soil-vial system. However, these BVCs could not be detected in the rhizosphere pretreated with strains GB03 and S4-7. The detection limit of 2-nonanone in the tomato rhizosphere was 1 μM. Unexpectedly, drench application of 2-nonanone at 10 nM concentration, which is below its detection limit, protected tomato seedlings against *Pseudomonas syringae* pv. *tomato*. Our finding highlights that BVCs, including 2-nonanone, released by a soil bacterium are functional even when present at a concentration below the detection limit of SPME-GC-MS.

Keywords: 2-nonanone, *Bacillus velezensis*, bacterial volatile compounds, caryolan-1-ol, plant growth-promoting rhizobacteria, solid-phase microextraction/gas chromatography mass spectrometry

INTRODUCTION

Bacterial volatile compounds (BVCs) are characterized by low molecular mass (100-500 Da), low polarity, and high vapor pressure (Chung et al., 2016; Schmidt et al., 2015; Vespermann et al., 2007). BVCs released by plant-associated bacteria (phytobacteria) exhibit beneficial or harmful effects on the growth and physiology of other organisms such as plants, fungi, and other bacteria (Chung et al., 2016; Garbeva and Weisskopf, 2020; Sharifi and Ryu, 2018b; Weisskopf et al., 2021). For nearly two decades, diverse BVCs have been identified and classified through computational data base referred to as "mVOC DB" (Lemfack et al., 2014). The detection and identification of BVCs was challenged in earlier studies that employed the gas chromatography-mass spectrometry (GC-

Received 26 October, 2021; revised 24 February, 2022; accepted 24 February, 2022; published online 4 July, 2022

eISSN: 0219-1032

MS) method (Weisskopf et al., 2021). Since its introduction in 2004, headspace solid-phase microextraction (HS-SPME) combined with GC-MS has become a standard method for the detection and identification of BVCs produced by phytobacteria (Farag et al., 2006; Tait et al., 2014). Compared with the traditional extraction method, SPME-GC-MS does not require a pretreatment step, is solvent-free, and involves rapid and simple procedures (Aulakh et al., 2005; Merkle et al., 2015; Weisskopf et al., 2021). However, the detection of BVCs *in situ* (e.g., in soil) remains challenging. Most of the previous studies involving BVC identification were performed *in vitro* using nutrient-rich media (Farag et al., 2013; Kai, 2020; Wu et al., 2019). To understand the nature of BVCs *in situ*, the BVCs synthesized *de novo*, for instance, in the plant root by rhizosphere bacteria (rhizosphere), need to be evaluated (Insam and Seewald, 2010). This task is challenged by the incompatibility between the *in vitro* emission of BVCs on artificial growth media and their *in situ* production in the natural environment (Garbeva et al., 2014a; 2014b; Schmidt et al., 2015). It is well known that amount and composition of BVCs are dependent on nutrient availability, pH, temperature, humidity, and biotic interactions (Claeson et al., 2007; Garbeva et al., 2014a; Schmidt et al., 2016).

To improve the sensitivity of detection tools, alternative direct injection mass spectrometry techniques can be employed (Kai, 2020). A commonly used direct injection technique for evaluating BVCs is proton transfer reaction mass spectrometry (PTR-MS) (Kai et al., 2010). PTR-MS is an optimized BVC detection method used to identify diverse classes of volatile compounds including ketones, aldehydes, and alcohols at very low concentrations, from parts per million (ppm) to parts per trillion (ppt) (Lindinger et al., 1998). Another potent and sensitive technique is ion mobility mass spectrometry (IMS), which can detect and analyze BVCs at concentrations ranging from parts per billion (ppb) to ppt (Baumbach, 2006). Recently, another ambient volatile analysis technique, atmosphere press chemical ionization mass spectrometry (APCI-MS), was developed (Liang et al., 2014). Compared with PTR-MS, the APCI-MS method can conduct the fragmentation (tandem mass spectrometry [MS/MS]) of BVCs for facilitated identification. The APCI-MS method was successfully used to identify 1-pyrroline, a signature BVC of *Pseudomonas aeruginosa*, from the breath of an infected patient (Hu et al., 2016). However, the BVC detection limit of GC-MS is as low as 0.1 ppt, which is similar to or lower than that of other sensitive techniques (Blake et al., 2009). Additionally, the SPME protocol has the advantage of being a solvent-free technique that can simplify the detection of BVCs *in situ* (Zhang et al., 1994).

In this study, we detected BVCs *in situ* rather than on complex artificial media. Among the diverse *in situ* conditions, plant rhizosphere containing many phytobacterial species was used with the sterilized soil vial system and the SPME-GC-MS platform. Two representative rhizosphere bacteria, *Bacillus velezensis* GB03 (syn. *Bacillus amyloliquefaciens* subsp. *plantarum* strain) and *Streptomyces griseus* S4-7, were used for optimizing the BVC detection method on bacterial concentration and their BVC production time points (Cha et al., 2016; Kloepper et al., 2004). The BVC 2-nonanone produced by *B. velezensis* GB03 was successfully quantified in the soil. The *in planta* experiment revealed that 2-nonanone synthesized *de novo* by *B. velezensis* GB03 elicited systemic immune response against *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 in tomato seedlings at levels below its detection limit.

MATERIALS AND METHODS

Preparation of bacterial inoculum

Wild-type and mutant strains of *B.velezensis* (GB03 and GB03 *ΔyneP*::cmr, respectively) were cultured on tryptic soy agar (TSA; Difco Laboratories, USA) at 30°C for 24 h. To re-isolate bacterial strains from soil, a rifampicin resistant spontaneous mutant of strain GB03 was generated (Nicholson and Maughan, 2002). Colonies of the mutant strain GB03 *ΔyneP*::cmr were selected on TSA containing 100 μg/ml rifampicin and 5 μg/ml chloramphenicol. Inocula of *B. velezensis* strain GB03 and *S.griseus* strain S4-7 were prepared as described previously (Cho et al., 2017; Kong et al., 2021). Concentrations of GB03 and S4-7 inocula were adjusted to 2×10^7 and 5×10^7 colony forming units (CFU/ml), respectively, using sterile distilled water (SDW).

Construction of the mutant strain GB03 *ΔyneP::cmr*

Primers used for contruction of *yneP* mutant are listed in Supplementary Table S1. The schematic presentation to construct the series of plasmids was shown in Supplementary Fig. S1. In detail, to remove the type IIS *Bsa*I restriction sites from the pUC19 plasmid, two fragments were amplified from pUC19 using two primer pairs mPAD-F1/pUC-R and pUC-F/mPAD-R4. The resulting polymerase chain reaction (PCR) products were ligated using the Cold Fusion Cloning kit (System Biosciences, USA) to construct the pSGC1 plasmid. To insert the Golden Gate assembly site into pSGC1, the pMGold-sCBE4 plasmid (Kim et al., 2021a) was digested with *Eco*RI and *Sac*I restriction endonucleases, and the cleaved fragment was cloned into the *Eco*RI- and *Sac*I-digested pSGC1 plasmid to construct the pSGC2 vector. To insert an additional selectable marker gene, the neomycin resistance gene amplified from pHCas9 (So et al., 2017) was cloned into the *Sac*I- and *Nsi*I-digested pSGC2 plasmid using the pSGC2-neo-F/pSGC2-neo-R primer pair, resulting in the pSGC2N plasmid. To construct plasmid for deleting the *yneP* gene, two DNA fragments corresponding to upstream and downstream regions of *yneP* were amplified from the genome of *B. velezensis* GB03 using the primer pairs 1G-GByneP-FF/1G-GByneP-FR and 3GX-GByneP-BF/3GX-GByneP-BR, respectively. The chloramphenicol-resistance gene was amplified from the pMGold-sCBE4 vector using the primer pair GGC-cat-F/cat-R. The pSGC2N-yneP plasmid was constructed by cloning the above three fragments into pSGC2N using Golden Gate assembly, as described previously (Kim et al., 2021a). The pSGC2N-yneP plasmid was introduced into *B. velezensis* GB03 by *Escherichia coli* S17-1 conjugation, as described previously (Teng et al., 1998). The transconjugants showing resistance to chloramphenicol and sensitivity to neomycin were selected. The resulting *yneP* mutants were confirmed by colony PCR.

BVC detection in sterilized soil vials

To investigate BVCs in the soil, sterilized soil vials were prepared using field soil (Fig. 1A) collected from a pepper field located in Nonsan, South Korea (36°12'07.9"N and 127°09'39.9"E). Soil samples were collected as described previously (Lee et al., 2021), with slight modifications. The collected soil samples were homogenized using a 2 mm sieve and stored at 4°C until needed for further analysis. Then, 2 g of the homogenized soil was transferred to 20 ml SPME vials. All materials were sterilized before conducting the experiments.

The vials containing strains GB03 and S4-7 in the soil vials were incubated at 30°C for 0, 1, 3, and 7 days. BVCs produced by GB03 and S4-7 on artificial media were detected as described previously (Farag et al., 2017). The equipment condition of SPME-GC-MS was modified using the method described by Song et al. (2019).

Plant preparation for BVC detection in the greenhouse

The rhizosphere of tomato (*Solanum lycopersicum* L. cv. Juiken) (Sakatakorea, Korea) plants was used for the GB03 application experiment, while that of strawberry (*Fragaria* × *ananassa* cv. Seolhyang) (Pcf Farming Association Corporation, Korea) plants was used for the S4-7 application experiment. The tomato seeds were sown on autoclaved soilless potting medium (Punong Horticulture Nursery Medium Low; Punong, Korea) containing zeolite, perlite, color dust, and lime (pH = 4.5 to 7.5) (Song et al., 2016). Two-week-old tomato plants and 5-week-old strawberry plants were cultivated at 28°C and 25°C, respectively, under a 14 h/10 h light/dark cycle and fluorescent lamps with approximately 7,000 lux light intensity. A single tomato or strawberry seedling was planted in a pot (60 mm × 55 mm × 55 mm) filled with 400 g mixture of sterilized-dried field soil and soil-less potting medium (1:1). Pots containing tomato and strawberry seedlings were moved to the greenhouse. Seedlings were grown at 25°C-30°C and watered with SDW. Then, pots containing 3-week-old tomato seedlings and 6-week-old strawberry seedlings were drenched with 10 ml of *B. velezensis* GB03 suspension and *S. griseus* S4-7 suspension, respectively. The rhizosphere of tomato and strawberry seedlings was sampled at 0, 3, and 7 days post-drench application (pda) for BVC detection. Watering of pots was stopped 24 h before each sampling.

Soil treatments

The soil pots in the greenhouse were treated with 50 ml of three different concentrations of 2-nonanone and caryolan-1-ol standard solutions (100 μM, 1 μM, and 10 nM) by drench application. Then, 2 g of soil was sampled from each pot at 0, 1, and 2 days after treatment. The soil samples were transferred to SPME vials for analysis.

Pathogen inoculation

Pathogen inoculation assays were performed as described previously (Uppalapati et al., 2007). *Pto* DC3000 was grown at 30°C for 2 days on King's B (KB) medium containing 100 μg/ml rifampicin. Three-week-old tomato plants were pretreated with 50 ml of GB03 suspension (optical density at 600 nm [OD_{600}] = 1) and S4-7 suspension (OD_{600} = 1). After 1 week, the 4-week-old tomato plants were spray inoculated with *Pto* DC3000 suspension (OD_{600} = 0.1). The inoculated plants were grown at 28°C under under a 14 h/10 h light/dark cycle period (Song et al., 2019). Disease symptoms were evaluated at 7 days post-inoculation (dpi) by determining the average number of bacterial specks on six leaves of 5-week-old tomato plants. Additionally, the number of *Pto* DC3000 cells on spray-inoculated leaves was determined (Kim et al., 2021b). Briefly, discs were excised from the leaves of 5-week-old tomato plants and homogenized in 10 mM $MgSO_4$. Then, a dilution series of suspension of leaf discs was plated on KB medium containing 100 μg/ml rifampicin.

Statistical analysis

The experimental data were subjected to ANOVA using the JMP software (ver. 5.0) (SAS Institute, USA; https://www.sas.com/). The normality and homogeneity of variance of the data were assessed. Non-normal and heterogenous data were transformed using Box-Cox. Significant treatment effects were determined based on the magnitude of the *F*-value (P = 0.05). When a significant *F*-value was obtained, separation of means was accomplished by the protected Fisher's least significant difference (LSD) test at P = 0.05.

RESULTS

Comparison of BVC emission between soil-vial system and artificial medium

To identify species-specific signature BVCs *in situ*, we compared the BVCs produced by *B. velezensis* strain GB03 and *S. griseus* strain S4-7 on TSA and in soil. The results revealed 2-nonanone and caryolan-1-ol as signature BVCs of strains GB03 and S4-7, respectively (Figs. 1B and 1C). The number of BVCs detected in the soil was significantly less than that detected on artificial media (TSA) (Figs. 1B and 1C). In the strain GB03 treatment, the 9 BVCs, acetic acid, 3-methyl-1-butanol, 3-methyl butanoic acid, 2-methyl butanoic acid, 2-nonanone, 2-methoxyphenol, 2-undecanone and 2-undecanol were detected (Fig. 1B). The three BVCs, 2-nonanone, 2-methylbutanoic acid, and 3-methylbutanoic acid from strain GB03 were detected in both TSA and soil vial (Fig. 1B). Meanwhile, in the strain S4-7 treatment, 36 BVCs were detected including dimethyl disulfide, S-methyl butanethioate, α-copaene, (-)-β-elemene, geosmin, caryophyllene, β-gurjunene, eremophilene, ginsenol, γ-muurolene, germacrene D, cis-calamenene, caryolan-1-ol and unknown C15 sesquiterpene (Fig. 1C, Supplementary Table S2). Among 36 BVCs, three BVCs, geosmin, caryolan-1-ol, and unknown C15 sesquiterpene were detected in both media and soil vial (Fig. 1C). Further experiments revealed that 2-nonanone and caryolan-1-ol were detected in all 3 replicates. However, the detection of 2-methylbutanoic acid, 3-methylbutanoic acid, 2-methoxyphenol and geosmin was inconsistent during conducting experiments. Also, unknown sesquiterpene (C15) was not clearly identified. Therefore, 2-nonanone and caryolan-1-ol were selected as markers BVC of strain GB03 and S4-7, respectively, in soil at following experiments. Together, we set up the method with two BVC markers for further studies that

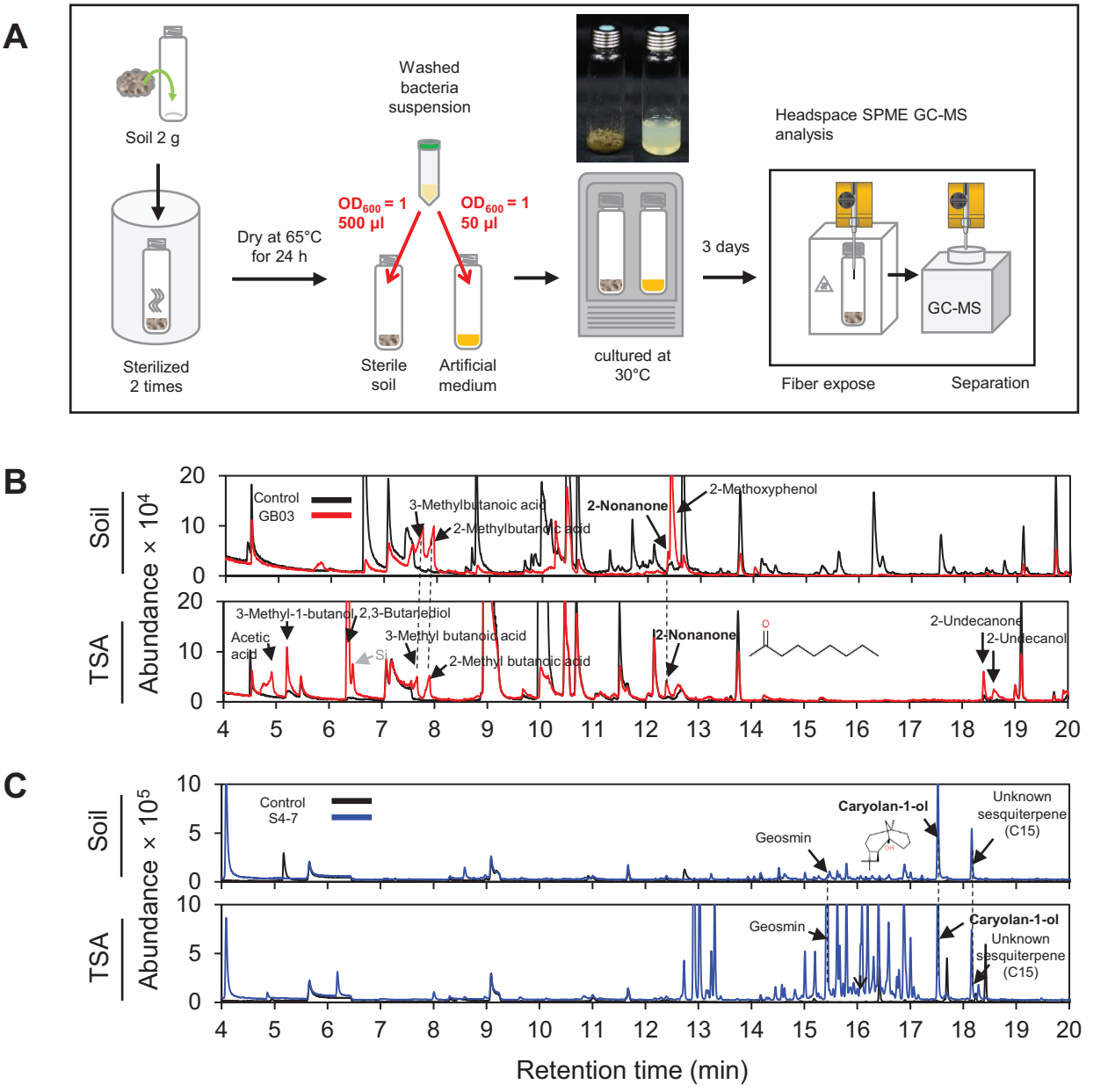

Fig. 1. Profiling BVCs produced by bacteria in the soil and on agar media. (A) Schematic representation of the experimental design using the sterilized soil vial system. (B) *In situ* and *in vitro* BVC detection following inoculation of sterilized soil and TSA media, respectively, with *B. velezensis* GB03. Red and black peaks indicate GB03 and control treatments, respectively. (C) *In situ* and *in vitro* BVC detection following inoculation of sterilized soil and TSA agar media, respectively, with *S. griseus* S4-7. Blue and black peaks indicate S4-7 and control treatments, respectively.

detect the strain GB03 and strain S4-7 in soil at greenhouse.

Optimizing the detection of two signature BVCs in soil-vial system

Next, to optimize the concentration of the bacterial inoculum, we examined the detection time of BVCs, 2-nonanone and caryolan-1-ol in the soil treated with different titers of GB03 and S4-7. The BVC 2-nonanone showed peak areas of 4,660,964 (coefficient of variation [CV], 6.04%), 4,660,922 (CV, 9.51%), and 3,804,399 (CV, 16.5%) when the soil was treated with 10^7, 10^5, and 10^3 CFU/g soil strain GB03, respectively, while caryolan-1-ol showed peak areas of 3,530,777 (CV, 14.86%), 2,599,363 (CV, 15.8%), and 1,964,842 (CV, 8.36%) when the soil was treated with 10^5, 10^3, and 10^1 CFU/g soil strain S4-7, respectively (Figs. 2A and 2B). In the 10^7 CFU/g soil GB03 treatment, the peak areas of 2-nonanone showed no significant differences among the three sampling time points (day 1, 3, and 7 pda); however, in the

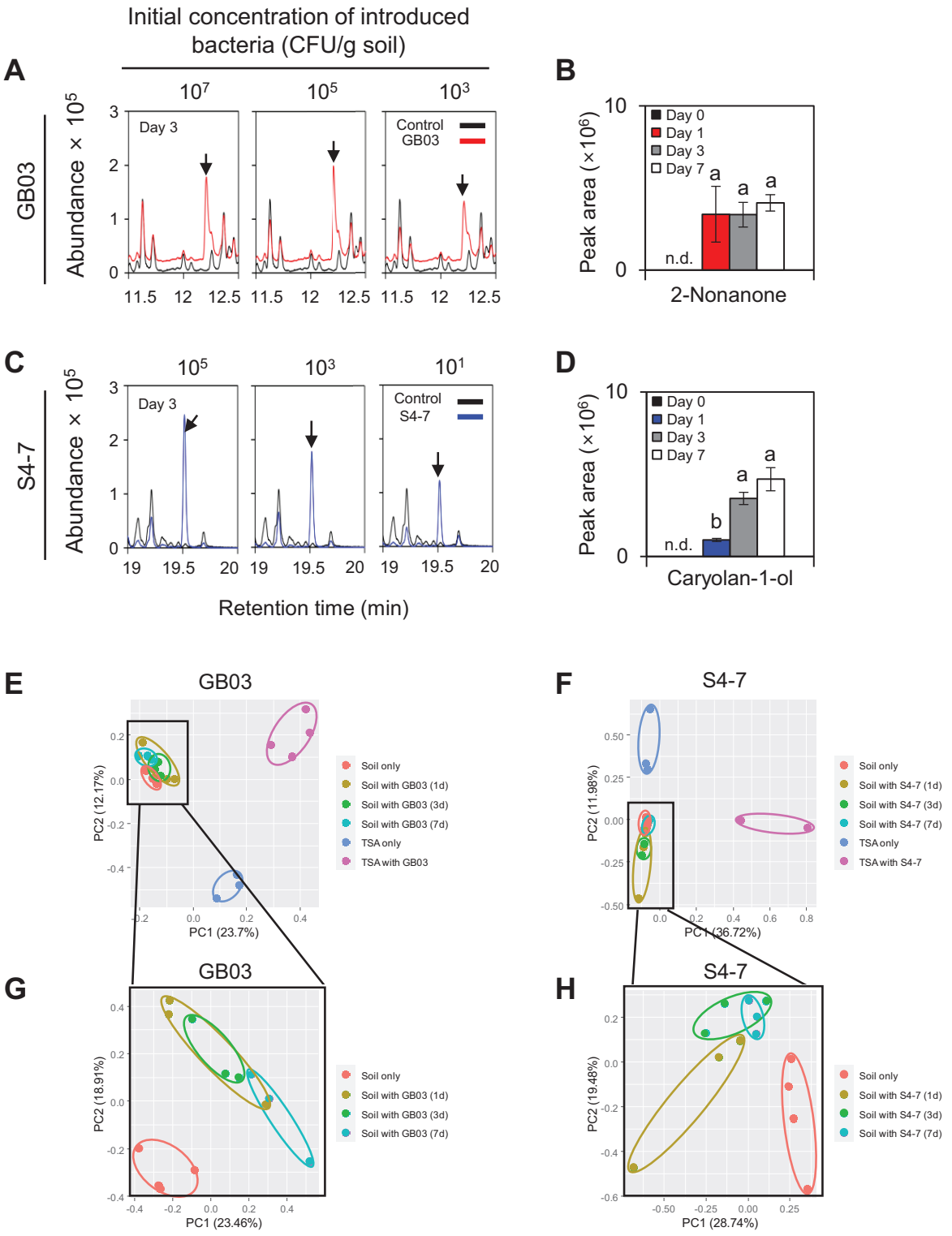

Fig. 2. Volatile detection in serial dilutions of *B. velezensis* GB03 and *S. griseus* S4-7 cultures. (A and C) Chromatograms of *B. velezensis* GB03 (A) and *S. griseus* S4-7 (C). Different concentrations of GB03 (10^7, 10^5, and 10^3 CFU/g soil) and S4-7 (10^5, 10^3, and 10^1 CFU/g soil) were used to inoculate the sterilized soil, and cultured for 3 days at 30°C. Red, blue, and black peaks indicate the GB03, S4-7, and respective control treatments, respectively. (B and D) Quantitation and comparison of peak areas of 2-nonanone (B) and caryolan-1-ol (D). n.d., not detectable. (E-H) PCA (principal component analysis) performed using peak areas of BVCs patterns. (E and F) Significant differences in BVCs patterns were detected between soil-vials and TSA medium samples. (G and H) The BVCs patterns of bacteria inoculated soil were detected differently with controls. Different letters indicate significant differences at $P = 0.05$.

10^5 CFU/g soil S4-7 treatment, the 2-nonanone peak area on day 1 was significantly different from those on day 3 and day 7 pda (Figs. 2C and 2D). The number of bacteria in the soil was saturated at approximately 10^4 to 10^5 CFU/g soil on day 0, 3, and 7 pda (Supplementary Fig. S2).

To determine whether the BVCs detected in inoculated soil, media, and control samples were statistically different, we performed PCA (principal component analysis). The results revealed no differences among the three different soil samples (day 1, 3, and 7 pda); however, differences were detected between the soil samples and media samples (Figs. 2E and 2F). Three soil samples (day 1, 3, and 7 pda) of strains GB03 and S4-7 inoculated, respectively, were statistically different from controls (Figs. 2G and 2H).

Limitation of BVCs detection from rhizosphere in the greenhouse

To expand the *in situ* BVC detection protocol, we employed the previously optimized procedures. Neither 2-nonanone nor caryolan-1-ol were detected in the rhizosphere of tomato and strawberry (Figs. 3A and 3B), even though two rhizospheres of tomato and strawberry were treated with 10^7 CFU/g soil GB03 and 10^7 CFU/g soil S4-7 (Figs. 3C and 3D). This led us to test whether the BVCs synthesized *de novo* following inoculation with such high bacterial titer could be detected by SPME-GC-MS analysis. When tomato seedling roots were treated with 100 μM and 1 μM 2-nonanone, the BVCs could be detected on day 0 but not on day 1 (Figs. 3E and 3F). When tomato seedling roots were treated with 100 μM caryolan-1-ol, BVCs could be detected at all three time points (day 0, 1, and 2); however, upon treatment with 1 μM caryolan-1-ol, BVCs could be detected only on day 0 and day 1 (Figs. 3G and 3H). Treatment with 10 nM 2-nonanone or caryolan-1-ol resulted in no peak at the three time points.

The biological function of 2-nonanone at levels below the detection limit

The pretreatment of soil with strain GB03 significantly (P = 0.05) reduced the *Pto* DC3000 speck numbers and bacterial cell numbers on tomato leaves at 7 days post-inoculation to levels similar to those obtained in the positive control, in which leaves were treated with 1 mM benzothiadiazole (BTH), a chemical trigger of systemic acquired resistance (Figs. 4A and 4B). Meanwhile, the pretreatment of soil with strain S4-7 failed to reduce the numbers of bacterial speck caused by *Pto* DC3000 and CFUs per leaf disc at 7 days post-inoculation (Supplementary Fig. S3A). Statistical data analysis revealed that treatment with all different concentrations of 2-nonanone resulted in significant differences in symptoms (i.e., bacterial speck number) and bacterial growth on the leaves compared to control (Figs. 4C and 4D). Even 10 nM 2-nonanone application successfully elicited systemic resistance against *Pto* DC3000 (Figs. 4C and 4D). On the other hand, all concentrations of caryolan-1-ol tested in this experiment failed to reduce the bacterial speck number (Supplementary Fig. S3A). Only 100 nM and 10 μM caryolan-1-ol application reduced CFU/leaf disc (Supplementary Fig. S3B). The caryolan-1-ol application did not show consistent results through repeated experiments (data not shown). The GB03 *yneP* mutant failed to induce systemic resistance, as indicated by the two abovementioned parameters (i.e., speck number and bacterial cell number) (Figs. 4F-4H). The peak of GB03 *yneP* mutant was detected at the same retention time as that of the wild-type GB03 as a different chemical, n-hexyl acrylate by the National Institute of Standards and Technology (NIST) library (Fig. 4E).

DISCUSSION

Since the role of BVCs in plant–microbe interactions has been reported previously, it is important to understand the mechanism of their production *in situ* (Garbeva et al., 2014a; Ryu et al., 2004; Schenkel et al., 2018; Schmidt et al., 2015). Interactions between the rhizosphere bacteria and the plant root system have been the focus of recent studies since the next generation sequencing-based metagenomics and advanced chemical analysis technology led us to understand on the communication between plant and microbes (Berg et al., 2020). However, BVC detection in the soil is challenging because of our limited understanding of the physical and chemical properties of BVCs and of soil under *in situ* conditions, and because of technological limitations affecting the identification of BVCs below their detection limit. Therefore, most studies on BVC detection are performed using bacterial culture grown on artificial media *in vitro* (Kai, 2020; Weisskopf et al., 2021).

In this study, our primary objective was to establish a method for detecting BVCs in the soil directly by SPME-GC-MS (Figs. 1B and 1C). Despite the importance of BVC detection, factors limiting BVC detection directly in the soil have been reported recently (Martín-Sánchez et al., 2020). Variations in the physicochemical characteristics of soil (i.e., humidity levels, soil temperature, and nutrient availability) can lead to the rapid evaporation and dilution of volatile compounds (Insam and Seewald, 2010; Ryu, 2015). Here, we successfully identified 2-nonanone and caryolan-1-ol as the signature BVCs of strains GB03 and S4-7, respectively (Figs. 1B and 1C), in soil and on artificial media, indicating that the two BVCs are produced in the soil (Figs. 1B and 1C). We failed to re-isolate strain GB03 from synthetic bacterial community in soil vial system (data not shown). We speculate that the rhizosphere competence of rhizobacteria plays a critical role on the survival of strain GB03 (Yi et al., 2016). According to the mVOC database, 86 bacterial and 18 fungal species produce 2-nonanone (Lemfack et al., 2018). This is the first report of *B. velezensis* producing 2-nonanone; only three *Bacillus* species including *Bacillus simplex, Bacillus subtilis,* and *Bacillus weihenstenphanensis* have been previously identified as 2-nonanone producers (Supplementary Table S3). To understand the characteristics of BVCs in the soil, it is important to investigate the bacterial biochemical process involved in the *de novo* synthesis of 2-nonanone.

Notably, 2,3-butanediol, a well-characterized and -studied model BVC involved in plant–bacterial volatile interactions (Fincheira and Quiroz, 2018; Kong et al., 2018; Sharifi and Ryu, 2018a; Song et al., 2019; Wu et al., 2018; Yi et al., 2016), was not detected in the soil treated with strain GB03 (Fig. 1B). Three possibilities could explain this result: 1) un-

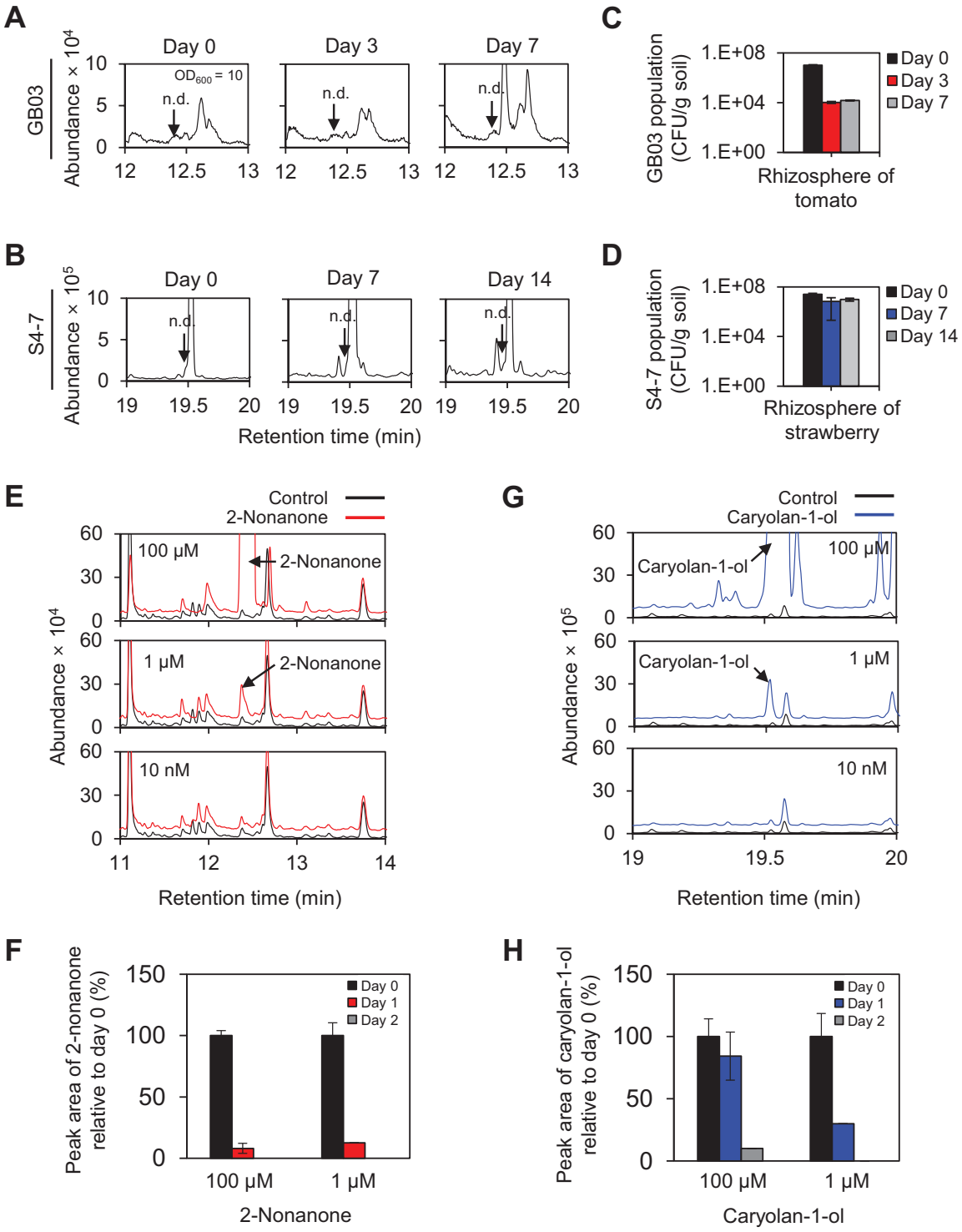

Fig. 3. BVC analysis and colonization monitoring of model strains, and time-course analysis of BVC production in soil post-drench application under greenhouse conditions. (A) Chromatograms of the rhizosphere of tomato seedlings pretreated with strain GB03. BVC analysis was conducted at 0, 3, and 7 days post-inoculation with GB03 (10^7 CFU/g soil). (B) Chromatograms of rhizosphere of strawberry seedlings pretreated with strain S4-7. BVC analysis was conducted at 0, 7, and 14 days post-inoculation with S4-7 (10^7 CFU/g soil). n.d., not detectable. (C) Population of GB03 in the rhizosphere of tomato seedlings at 0, 3, and 7 days post-inoculation. (D) Population of S4-7 in the rhizosphere of strawberry seedlings at 0, 7, and 14 days post-inoculation. (E and G) Chromatograms of 2-nonanone-drenched (E) and caryolan-1-ol-drenched (G) soil at day 0. The soil was treated with three concentrations of 2-nonanone and caryolan-1-ol (100 μM, 1 μM, and 10 nM). Then, 2 g of soil from each treatment was analyzed by SPME-GC-MS. (F and H) Peak area of 2-nonanone (F) and caryolan-1-ol (H) at 0, 1, and 2 days after drenching standard solutions of 2-nonanone and caryolan-1-ol, respectively, into soil.

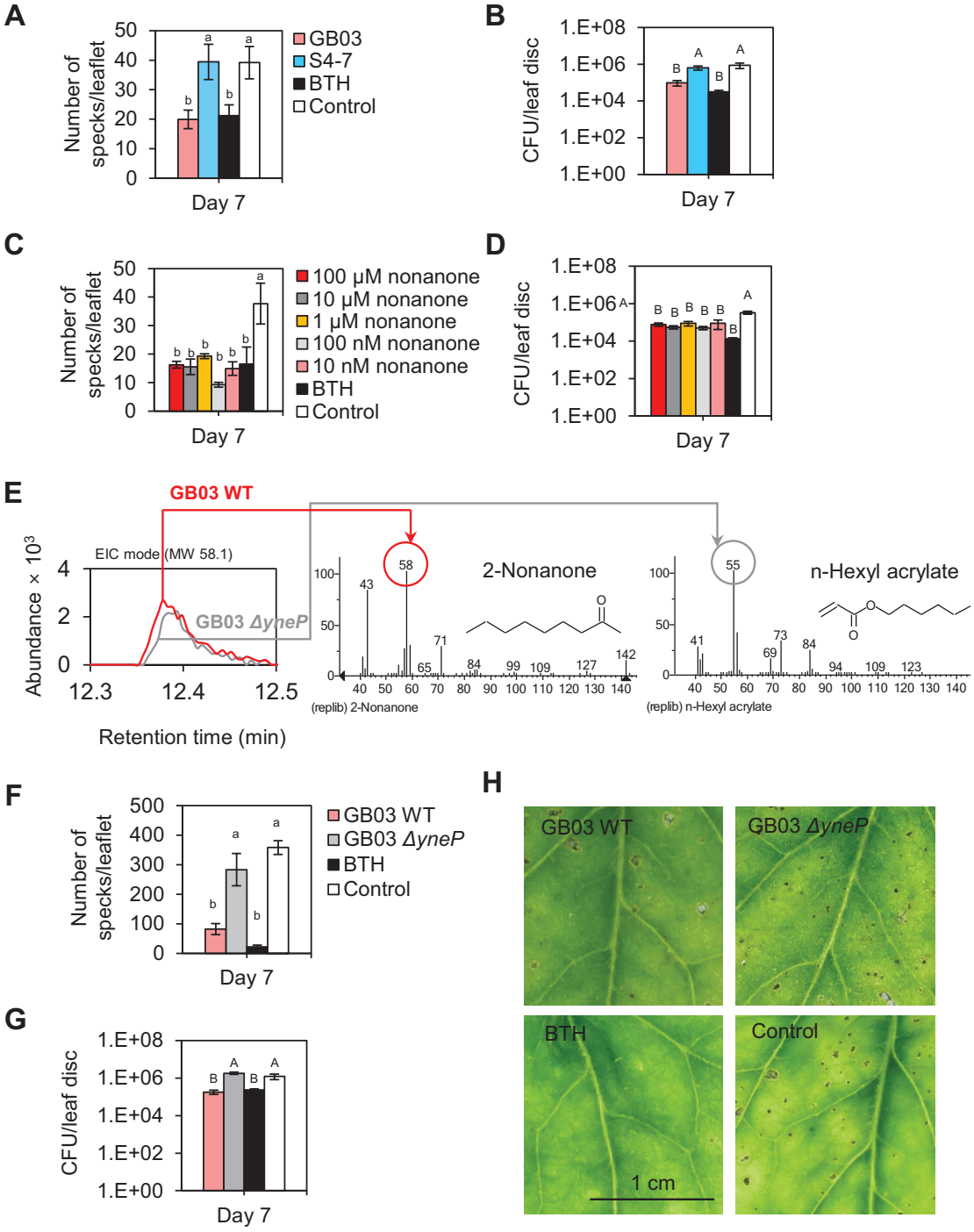

Fig. 4. Pretreatment with *B. velezensis* GB03 and 2-nonanone standard solution enhances the resistance of tomato seedlings against *P. syringae* pv. *tomato* (*Pto*) DC3000. (A) Number of specks of *Pto* DC3000 on the leaves of GB03- and S4-7-treated tomato seedlings at 7 days post-inoculation. (B) Population of *Pto* DC3000 on the leaves of GB03- and S4-7-pretreated tomato seedlings at 7 days post-inoculation. (C) Number of *Pto* DC3000 specks on the leaves of 2-nonanone-treated tomato seedlings at 7 days post-inoculation. (D) Pathogen population of *Pto* DC3000 on the leaves of 2-nonanone standard solution-pretreated tomato seedlings at 7 days post-inoculation. Treatment with 1 mM BTH was used as a positive control. (E) Mass spectrometry analysis of wild-type (WT) and mutant (*Δyne*P) GB03. (F) Number of *Pto* DC3000 specks on the leaves of wild-type and mutant GB03-treated tomato seedlings at 7 days post-inoculation. EIC, extracted ion chromatogram; MW, molecular weight. (G) Pathogen population on the leaves of wild-type and mutant GB03-pretreated tomato leaves at 7 days post-inoculation. Treatment with 1 mM BTH was used as a positive control. Different letters indicate significant differences among treatments ($P = 0.05$; LSD test). Error bars indicate SE (n = 4). Experiments were repeated twice with four biological replications. (H) Pictures of tomato seedlings 7 days after inoculation of *Pto* DC3000.

availability or insufficient availability of the substrate in the rhizosphere required for the production of 2,3-butanediol by *B. velezensis* GB03; 2) unfavorable environmental conditions (oxygen, pH, nutrient states, and temperature) inhibiting the production of 2,3-butanediol in *B. velezensis* GB03 cells; and 3) 2,3-butanediol present in the soil at a concentration lower than the detection limit of SPME-GC-MS.

The third possibility led us to evaluate whether 2-nonanone was still functional at levels below its detection limit. To test the possibility, we applied 2-nonanone at a concentration below its detection limit, and examined the 2-nonanone biosynthesis ability of the GB03 null mutant. Because strain GB03, as a model plant growth-promoting rhizobacteria, was previously reported to elicit induced systemic resistance (ISR) when applied to plant roots (Kloepper et al., 2004), we evaluated the ability of 2-nonanone to activate ISR against *Pto* DC3000 in tomato (Figs. 4C and 4D). At a concentration undetectable by SPME-GC-MS, 10 nM 2-nonanone successfully protected tomato leaves from *Pto* DC3000 (Figs. 4C and 4D), whereas the GB03 null mutant failed to trigger ISR. We also failed to detect any differentially expressed genes when applied with 10 nM 2-nonanone and strain GB03 treatments (Supplementary Fig. S4). For further evaluation, RNA-sequencing can be employed to identify specific genes that is specifically induced by strain GB03 and 2-nonanone. These results demonstrate that BVCs are functional below their detection level under *in situ* conditions.

In conclusion, initially we attempted to develop a simple method for the *in situ* detection of BVCs using soil vials with the SPME-GC-MS method. However, unexpectedly, we discovered technological limitations affecting the detection of BVCs in the soil. Our findings highlight that BVCs synthesized *de novo* by soil bacteria can modulate the plant physiology and activate plant immunity. These results broaden our knowledge of the role of BVCs under real-life conditions such as in the agricultural field.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

We thank Dr. Youn-Sig Kwak (Gyeongsang National University, South Korea) for providing *S. griseus* S4-7. This research was supported by grants from the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (NRF-2020M3E9A1111636), Cooperative Research Program for Agriculture Science and Technology Development (PJ014790022021) from Rural Development Administration, and the KRIBB Initiative Program, South Korea.

AUTHOR CONTRIBUTIONS

M.R. carried out the *in situ* BVCs analysis studies, plant assay, and wrote the manuscript. M.S.K. carried out the generation of bacteria mutant and partially participated in drafted the manuscript. S.-K.C. participated the generation of bacteria mutant. S.-K.O. reviewed the manuscript. C.-M.R. conceived of the study, and participated in its design and coordination and wrote the manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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