Enhanced performance of the microalga *Chlorella sorokiniana* remotely induced by the plant growth-promoting bacteria *Azospirillum brasilense* and *Bacillus pumilus*

Edgar Amavizca¹, Yoav Bashan¹,²,³, Choong-Min Ryu⁴, Mohamed A. Farag⁵, Brad M. Bebout⁶ & Luz E. de-Bashan¹,²,³

Remote effects (occurring without physical contact) of two plant growth-promoting bacteria (PGPB) *Azospirillum brasilense* Cd and *Bacillus pumilus* ES4 on growth of the green microalga *Chlorella sorokiniana* UTEX 2714 were studied. The two PGPB remotely enhanced the growth of the microalga, up to six-fold, and its cell volume by about three-fold. In addition to phenotypic changes, both bacteria remotely induced increases in the amounts of total lipids, total carbohydrates, and chlorophyll *a* in the cells of the microalga, indicating an alteration of the microalga’s physiology. The two bacteria produced large amounts of volatile compounds, including CO₂, and the known plant growth-promoting volatile 2,3-butanediol and acetoin. Several other volatiles having biological functions in other organisms, as well as numerous volatile compounds with undefined biological roles, were detected. Together, these bacteria-derived volatiles can positively affect growth and metabolic parameters in green microalgae without physical attachment of the bacteria to the microalgae. This is a new paradigm on how PGPB promote growth of microalgae which may serve to improve performance of *Chlorella* spp. for biotechnological applications.

*In vitro* culturing at laboratory or mass industrial scales is the most common way by which the biotechnological industry is producing products from microalgae¹,² and inoculants of plant growth-promoting bacteria (PGPB) for agricultural and environmental applications¹. The current paradigm of how PGPB enhance plant growth is through attachment of the bacteria to plant roots. PGPB first establish a stable physical interaction with its host and subsequently colonize the root system, which results in beneficial effects on plants⁴,⁵ When the PGPB move toward its host before attachment, the chances of successful colonization improve⁶. In the absence of attachment and colonization to plant surface, no effect of PGPB on higher plants are recorded⁷.

Attachment of the PGPB *Azospirillum* spp. to roots of many plant species has been demonstrated since the emergence of the field some 40 years ago⁴,⁸–¹². *A. brasilense* has also been observed to attach to, and form stable aggregates with, the microalga *Chlorella vulgaris*¹³. Similar interactions have also been proposed for Gram-positive bacteria, such as *Bacillus* spp.¹⁴. While attachment to roots has been documented as the major requirement for a beneficial association of PGPB with plants⁷, it is also well documented that the PGPB

¹Environmental Microbiology Group, Northwestern Center for Biological Research (CIBNOR), Av IPN 195, La Paz, B.C.S. 23096, Mexico. ²The Bashan Institute of Science, 1730 Post Oak Court, Auburn AL 36830, USA. ³Dept. of Entomology and Plant Pathology, 301 Funchess Hall, Auburn Univ., Auburn, AL 36849, USA. ⁴Molecular Phytophacteriology Laboratory, Korean Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-600, South Korea. ⁵Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt. ⁶Exobiology Branch, NASA Ames Research Center, Moffett Field, CA 94035, USA. Correspondence and requests for materials should be addressed to L.E.d.-B. (email: luz@bashanfoundation.org)
Azospirillum spp. can enhance many biotechnological processes of interest through association with the microalgae Chlorella spp. Processes enhanced by the association of Azospirillum with Chlorella spp. include: production of carbohydrates15,16, lipids and fatty acids17, photosynthetic pigments18, algal biomass19,20, C and N transport21. Several of the metabolic processes enhanced by this association have been applied to wastewater treatment22. All of these processes were demonstrated to be enhanced under the conditions in which Azospirillum was kept in close proximity or actually attached to the Chlorella spp.23 and they are attributed to transfer of metabolites, mainly the phytohormone indole-3-acetic acid (IAA) from the bacteria to the microalgae22,42,25. However, the requirement of a firm attachment between the two microorganisms has not been demonstrated as it has in higher plants.

Production of volatile compounds by microorganisms commonly occurs as part of normal metabolism, and plays a role in the communication within, and between, organisms. Plants respond to these volatiles both by increasing and reducing growth26,27. Volatiles emitted by PGPB28,29, such as 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol, produced by Bacillus spp. and Burkholderia spp., have been shown to stimulate the growth of Arabidopsis thaliana without physical contact30,31. Beneficial microbial volatile organic compounds (mVOCs) have been reported in several model plants, including peppermint32, alfalfa (lucerne)33, tobacco34, and Physcomitrella moss35. In contrast, several bacterial volatiles, such as ammonia36, ethylene37, and hydrogen cyanide28 can harm plants. While more than 300 potential molecules with the capacity to affect plants have been identified29,30,39,40, GC-MS analyses of bacterial volatiles continue to reveal many compounds that have yet to be identified as serving a function in plants. Positive effects, well-demonstrated in assays using miniature laboratory plant models grown in Petri dishes, are significantly more challenging to demonstrate with full-sized plants41.

Although Chlorella spp. are found in natural bodies of water, these microalgae are commonly cultured for biotechnological purposes2. Cultures of Chlorella spp. provide a convenient tool to determine whether volatiles produced by PGPB can affect plant cells in the absence of physical attachment. Azospirillum brasilense is a common diazotrophic PGPB that fixes nitrogen only under microaerophilic conditions. It affects the growth of plants26,27. Volatiles emitted by PGPB28,29, such as 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol, produced by Bacillus spp. and Burkholderia spp., have been shown to stimulate the growth of Arabidopsis thaliana without physical contact30,31. Beneficial microbial volatile organic compounds (mVOCs) have been reported in several model plants, including peppermint32, alfalfa (lucerne)33, tobacco34, and Physcomitrella moss35. In contrast, several bacterial volatiles, such as ammonia36, ethylene37, and hydrogen cyanide28 can harm plants. While more than 300 potential molecules with the capacity to affect plants have been identified29,30,39,40, GC-MS analyses of bacterial volatiles continue to reveal many compounds that have yet to be identified as serving a function in plants. Positive effects, well-demonstrated in assays using miniature laboratory plant models grown in Petri dishes, are significantly more challenging to demonstrate with full-sized plants41.

Production of CO2 and mVOC by PGPB. Both PGPB grew well in Brunner's medium (Fig. 1e), and both bacteria increased the pH of their growth medium in a similar manner (Fig. 1f). After two days in Brunner's medium, both PGPB produced significant amounts of CO2, as expected, which accumulated in the headspace at concentrations significantly higher than atmospheric CO2 (Table 1). B. pumilus produced a larger amount of CO2 with fewer cells than A. brasilense (Table 1). E. coli is well known to produce significant amounts of CO2 under these conditions44. SPME coupled to GC-MS analysis identified 47 volatiles produced by PGPB (Table 2). A representative gas chromatogram is shown in Suppl. Fig. 1, showing the differences in volatile emission patterns between strains of A. brasilense, B. pumilus, and E. coli. GC-MS analysis of mVOCs revealed consistent differences in the volatile blends released by specific strains of A. brasilense and B. pumilus, and to E. coli (Table 2, mass spectra of major labeled peaks are presented as Supplementary Material, Fig. S4). In all cases, reported values represent the relative percentile of each percentage of total volatile to the total amount of volatiles detected with volatile peak area being first normalized to the amount of the spiked internal standard hexnyl acetate. No absolute measurements of individual of volatiles were made considering that no standard calibration curve was made for each target volatile with its standard. Such an approach has been reported from our work reporting plant volatiles analysis using headspace SPME45. Growth-promoting volatiles 2,3-butanediol and 3-hydroxy-2-butanoate (also referred to as acetoin) were consistently major volatile components (75% and 62% of total volatile blend) produced by A. brasilense and B. pumilus, respectively, whereas these volatiles were released at much lower levels by E. coli (4%). 3-methyl-1-butanoic acid (isovaleric acid), 2-methyl-butyric acid, and 3-methyl-1-butanol (also referred to as fusel alcohol) were found only in A. brasilense, and B. pumilus. Several short-chain fatty acyl esters (octanoic, decanoic, and dodecanoic acid ethyl esters) were identified, though at much lower levels compared to short-chain alcohols in A. brasilense and B. pumilus volatile blends.
A remote effect induced by the PGPB and *E. coli* on accumulation of total lipids, carbohydrates, and chlorophyll *a* in *C. sorokiniana*. Total lipids were significantly enhanced by a remote effect from all three species of bacteria (two PGPB and the control *E. coli*), with *B. pumilus* inducing the highest effect. Lipids produced by microalgae growing alone were below the detection limit of the method used (Fig. 2a). Similar results occurred for carbohydrate production by the microalgae, but in this case, *A. brasilense* induced the highest effect (Fig. 2b). All bacterial species enhanced the quantity of chlorophyll *a*, relative to the level of chlorophyll *a* in microalgae growing alone (Fig. 2c).

**Discussion**

Culturing microalgae in the presence of PGPB is an artificially created experimental situation. For biotechnological purposes, any beneficial interaction occurring under these artificial conditions would result in an economic gain and therefore be worth exploring. The main purpose of this proof-of-concept study was to show that the enhancement of performance of microalgae, by volatiles of PGPB, is an effective strategy for common biotechnological applications involving cellular growth and metabolite production. As our results demonstrate,
| Name                        | Kovat index | Azospirillum brasilense | Bacillus pumilus | Escherichia coli | Biological function                                                                                                                                                                                                 | Reference |
|-----------------------------|-------------|------------------------|------------------|-----------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Acetoin*                    | 669         | 67.8                   | 5.9              | 56.7            | 7.3                                                                 | Average  SD | Average  SD | Average  SD | Induce growth promotion (leaf surface area), systemic resistance (ISR) and regulate auxin homeostasis in Arabidopsis thaliana. It is an attractant to Anastrepha ludens (Diptera). Acetoin as a pheromone synergist for R. palmarum. | 29,65,66  |
| 2,3-Butanediol*             | 682         | 7.7                    | 0.7              | 4.6             | 0.3                                                                 | Average  SD | Average  SD | Average  SD | Induce plant growth promotion (leaf surface area), systemic resistance (ISR) and regulate auxin homeostasis in Arabidopsis thaliana. | 28,29,31  |
| α-Terpineol                 | 1190        | 0.0                    | 0.0              | 0.2             | 0.1                                                                 | Average  SD | Average  SD | Average  SD | Antibacterial, antifungal activities, anticancer | 67        |
| (Z)-3-Hexen-1-ol, acetate (IS) | 979         | 9.1                    | 2.5              | 11.8            | 1.5                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| p-methyl acetophenone       | 1032        | 0.1                    | 0.0              | 0.4             | 0.3                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Indole*                     | 1272        | 2.1                    | 1.1              | 1.6             | 2.4                                                                 | Average  SD | Average  SD | Average  SD | Able to regulate biofilm formation. It also induces the formation of myxospores in Stigmatella aurantiaca. Regulation of expression of multi-drug exporter genes and inhibition of biofilm formation of Escherichia coli, Pseudomonas fluorescens and Pseudomonas aeruginosa. Intercellular signal in microbial communities. | 65,68,69  |
| α-Nonanoic acid             | 1245        | 0.1                    | 0.0              | 0.1             | 0.0                                                                 | Average  SD | Average  SD | Average  SD | Stimulation of oviposition, directing egg laying to favorable habitat of Aedes aegypti. | 70        |
| p-Dimethylbenzene           | 866         | 0.1                    | 0.0              | 1.0             | 0.8                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Trimethyl benzene           | 938         | 0.4                    | 0.1              | 2.3             | 1.7                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Unknown                     | 970         | 0.3                    | 0.1              | 2.4             | 1.9                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Nonanal*                    | 1077        | 0.0                    | 0.0              | 0.1             | 0.1                                                                 | Average  SD | Average  SD | Average  SD | Active against the phytopathogenic fungus Sclerotinia sclerotiorum. | 65        |
| Unknown                     | 907         | 0.0                    | 0.0              | 0.3             | 0.3                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| 3-Carene                    | 987         | 0.0                    | 0.0              | 0.2             | 0.1                                                                 | Average  SD | Average  SD | Average  SD | Alarm pheromone in some termite species | 71        |
| Phenylethyl alcohol         | 1092        | 1.0                    | 0.9              | 0.8             | 0.2                                                                 | Average  SD | Average  SD | Average  SD | Autoantibiotics produced by the fungus Candida albicans | 72        |
| Ethyl decanoate             | 1359        | 0.5                    | 0.5              | 0.0             | 0.0                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Ethyl dodecanoate           | 1563        | 0.2                    | 0.2              | 0.0             | 0.0                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Ethyl octanoate             | 1167        | 0.2                    | 0.2              | 0.0             | 0.0                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| α-Heptanoic acid            | 1049        | 0.0                    | 0.0              | 0.1             | 0.0                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Undecane*                   | 1071        | 0.0                    | 0.0              | 0.2             | 0.1                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Dodecane*                   | 1193        | 0.1                    | 0.0              | 0.3             | 0.2                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Tridecane*                  | 1289        | 0.1                    | 0.0              | 0.3             | 0.1                                                                 | Average  SD | Average  SD | Average  SD | 61.5 12.4 Defensive against predators by the stink bug Cosmopepla bimaculata | 73        |
| Pentadecane*                | 1479        | 0.1                    | 0.0              | 1.3             | 1.1                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Octadecane*                 | 1787        | 0.4                    | 0.3              | 0.7             | 0.6                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| n-Dodecanal                 | 1377        | 0.1                    | 0.0              | 0.2             | 0.1                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| 2-Methylbutyric acid        | 840         | 0.6                    | 0.2              | 0.8             | 0.3                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Butanol 3-methyl-acetate    | 942         | 0.1                    | 0.0              | 0.1             | 0.0                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| 2-Decanol                   | 1145        | 0.2                    | 0.0              | 0.3             | 0.1                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Isovaleric acid             | 833         | 4.3                    | 1.1              | 2.5             | 1.5                                                                 | Average  SD | Average  SD | Average  SD | Stimulation of spore germination of Agaricus bisporus Inhibition of proliferation and cytokine production in lymphocyte cells Reduction of heat resistant spores, prevention of spore formation | 74        |
| Acetic acid*                | 610         | 0.4                    | 0.4              | 3.0             | 1.0                                                                 | Average  SD | Average  SD | Average  SD | Highly attractive to Mexican fruit flies Reduction of heat resistant spores, prevention of spore formation | 65        |
| α-Caprylic acid             | 1140        | 0.8                    | 0.1              | 0.6             | 0.2                                                                 | Average  SD | Average  SD | Average  SD | Decrease yeast viability | 75        |
| Acetone*                    | 775         | 0.1                    | 0.0              | 0.0             | 0.0                                                                 | Average  SD | Average  SD | Average  SD | Inhibited growth of fungi. Has no effect on bacteria | 74        |
| 2-Methylpentanal            | 1128        | 0.1                    | 0.0              | 0.1             | 0.0                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| 2-Ethylhexanol              | 859         | 0.1                    | 0.0              | 0.1             | 0.1                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Unknown alcohol             | 1003        | 0.3                    | 0.0              | 1.3             | 0.9                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| 1-Hexene, 4methyl-          | 999         | 0.1                    | 0.0              | 0.4             | 0.3                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Caproic acid                | 961         | 0.5                    | 0.2              | 1.4             | 0.8                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Ethyl caprylate             | 1167        | *                      |                   |                 |                                                                     |          |          |          | n/a                                                                                |           |
| Unknown furfural            | 997         | 0.8                    | 0.2              | 0.4             | 0.1                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Unknown terpene             | 1224        | 0.2                    | 0.2              | 0.8             | 0.3                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Unknown hydrocarbon         | 1278        | 0.3                    | 0.3              | 0.2             | 0.1                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Unknown hydrocarbon         | 1360        | 0.2                    | 0.1              | 0.5             | 0.2                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Unknown                     | 1020        | 0.2                    | 0.1              | 0.2             | 0.1                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |
| Unknown                     | 1337        | 0.2                    | 0.1              | 1.8             | 1.5                                                                 | Average  SD | Average  SD | Average  SD | n/a                                                                                |           |

Table 2. Relative quantification of volatiles expressed as relative percentile and biological function of the volatile compounds produced by the three bacterial species along with their reported biological functions. Compounds denoted by an asterisk were confirmed with authentic standard.
The enhancement of algal production by PGPB can be accomplished without attachment of the PGPB to plant surfaces (as normally occurs when PGPB is inoculated on plants).

The significant positive effects of both *A. brasilense* and *B. pumilus* on general growth and metabolism of *Chlorella* spp. when the microorganisms are in forced close proximity inside an alginate bead is well documented (see Introduction for references). At least one genus of PGPB, *Bacillus*, was known to produce a variety of volatiles. Both PGPB are expected to produce CO2 during normal aerobic respiration, the growth conditions used in this study. Consequently, it is possible that the effects of volatiles will affect performance of microalgae.

The design of the experiments presented here ensures a lack of physical contact between any PGPB and the microalgae. Supplying cultures of microalgae and other plants with CO2 to increase growth is a commonly used laboratory and even industrial technique. Growth promotion of *Arabidopsis thaliana* by the PGPB *Serratia odorifera* (produces quantities of volatiles) was partly attributed to enrichment with CO2. Consequently, the significant improvement in growth from exposure of *C. sorokiniana* to volatiles of both PGPB and *E. coli* could also reasonably be attributed to the effects of CO2 produced by the bacteria.

The relative importance of CO2 in growth promotion in the experiments reported here was evaluated by reducing the quantity of CO2 in the headspaces of the experimental flasks using lithium hydroxide. Removal of CO2 in the headspace completely inhibited autotrophic growth of *C. sorokiniana* growing alone, but not when a PGPB was present in the adjacent flask. Under conditions of physical separation, growth of the microalgae was initially delayed but resumed after a few days. We suggest that this happened as the concentrations of CO2 produced by the bacteria increased and the filter could not absorb all the CO2 that was continuously produced by the PGPB. Alternatively, this was also assisted by the organic volatiles produced by the PGPB. Finally, CO2 can affect the pH of the microalgae substrate. CO2 that accumulated in the headspace of the culture can be incorporated as carbonate in the medium and consequently raise the pH to more neutral values than the initial pH, as shown.

![Figure 2.](image_url)

**Figure 2.** Remote effects of emissions on accumulation of lipid, carbohydrate, and chlorophyll a content in *Chlorella sorokiniana* by the PGPB *Azospirillum brasilense* Cd and *Bacillus pumilus* ES4 after 96 h of incubation. In each subfigure, columns denoted by different letters are significantly different. Analyses were made by one-way ANOVA and LSD post-hoc analysis at *P* < 0.05.
in this study. Extremely low pH negatively affects growth of *Chlorella* spp.\(^\text{19}\). Reduction of O\(_2\) partial pressure in the enclosed flasks is less likely. Although theoretically this can inhibit photosynthesis and growth of microalgae, these parameters were enhanced in our system.

We have also found that the enteric bacteria *E. coli* had similar growth promotion effects on *C. sorokiniana* as both PGBP. While co-culturing *E. coli* with *C. minutissima* yielded faster growth and cell density in the microalgae\(^\text{47}\) and a surprising phytostimulation of maize by *E. coli* was also reported\(^\text{48}\), so far, no solid explanation for these effects have been provided. Consequently, we assumed that the growth effect can be partly attributed to production of large quantities of CO\(_2\), as well known for *E. coli*. It seems unlikely that the results of these experiments can be explained as simply due to the effects of CO\(_2\) enhancement, however, as *B. pumilus* had the highest CO\(_2\) production, but did not produce the highest growth enhancement of the microalgae during the first 72 h.

The literature on organic volatile-mediated bacterial-plant interactions (see Introduction for references) suggests that production of growth-promoting volatiles is a widespread phenomenon among rhizosphere bacteria. The presumptive effect of volatiles of *A. brasilense* was predicted\(^\text{34}\), but neither determined nor quantified. In contrast, the presence of *Bacillus* sp. volatiles and their effect on plant growth are well documented\(^\text{28,29,49,50}\). Both PGBP tested in our study produced a large array of volatiles, some of which are known for growth promotion, such as 2,3-butanediol and acetoin. Their role as plant growth-promoting volatile metabolites has been determined even through exogenous application to plants\(^\text{25}\). The abundance of these two key volatiles in *A. brasilense* and *B. pumilus* volatile blends was far higher than what was previously reported for *B. subtilis* and *B. amyloliquefaciens*\(^\text{28}\). This comparison requires that a reservation be added. A direct comparison cannot be made because different volatile collection methods were used in these studies, namely dynamic versus static SPME headspace sampling. Additionally, using SPME fiber coatings in our study may have limited sensitivity by preferentially absorbing or excluding particular volatiles, based on polarity or size. For example, divinylbenzene/carboxen/PDMS fibers favor short-chain polar compounds such as 2,3-butanediol\(^\text{51}\).

In this study, as in others involving plant–volatile interactions, the biological functions for several identified volatiles were not established\(^\text{26,52}\). Some volatiles (nonanoic acid, indole, nonanal, isovaleric acid, and pentanoic acid) affect other organisms, whereas other detected mVOCs have no currently known function. In view of the chemical complexity and diversity of mVOCs, assessment of many of these compounds as relevant growth enhancers remains to be done\(^\text{27,52}\).

Both CO\(_2\) and organic volatiles produced by PGBP were measured in this study. CO\(_2\) serves as a natural substrate for growth of photosynthetic microalgae and organic volatiles have been shown to promote the growth of several plants. Our experimental design, while demonstrating that these volatiles did affect growth and metabolism of *C. sorokiniana*, could not distinguish between the relative contribution of the compounds. The extremely rapid multiplication of microalgae (seen after removal of CO\(_2\) by the lithium oxide filter followed by replenishment of CO\(_2\) by the remote presence of the PGBP) could be attributed to a starvation effect for carbon in the microalgae. Similar responses in microalgae growth rate are known after being deprived of nitrogen and phosphorus\(^\text{47}\).

Cells of *A. brasilense* attached to *C. vulgaris* and *C. sorokiniana* significantly promote accumulation of starch\(^\text{51,56}\), lipids\(^\text{18}\), fatty acids\(^\text{17}\), and pigments\(^\text{18}\) in the microalgae. Volatiles of *B. subtilis* and *E. coli* promote accumulation of starch in *Arabidopsis*\(^\text{41}\). As a result of the minimal mineral culture medium we used, the absolute quantity of these metabolites is lower than quantities produced when the microalgae are growing in rich medium. Still, our study clearly demonstrated that an enhancement of the production of key metabolites can be observed in the absence of any physical attachment of the PGBP to the microalgal cells. This provides insights into a novel mechanism in microalgal–bacteria interactions. This phenomenon may be complementary to the established paradigm of attachment of PGBP to plant surfaces, but can only be investigated under special culturing conditions.

The biotechnological ramifications of this study need to be explored further. Both PGBP used here are produced on a large industrial scale as agricultural inoculants\(^\text{54}\). The biotechnological potential of *C. sorokiniana* without physical contact.

**Materials and Methods**

**Microorganisms and initial growth conditions.** The unicellular microalga *Chlorella sorokiniana* Shih et Krauss (UTEX 2714, University of Texas, Austin, TX; formerly *C. vulgaris* UTEX 2714\(^\text{45}\)), and two strains of PGBP were used: *Azospirillum brasilense* Cd (DSMZ 1843; Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and *Bacillus pumilus* ES4\(^\text{46}\). This microalgal strain can grow at pH 5 and higher. In experiments that measured the potential effect of CO\(_2\), *Escherichia coli* DH5\(_\alpha\) (Invitrogen, Carlsbad, CA) served as the negative control for microalgal growth and promoting metabolites because it does not have any plant growth-promoting effects; it also served as a positive control in the CO\(_2\) experiment because it produces CO\(_2\), as any *E. coli*. All cultures were tested under sterile conditions.

For initial culturing of the microalga, 10 mL axenic *C. sorokiniana* culture, cultivated in sterile mineral medium (C30), was added to a sterile flask containing 90 mL sterile C30 medium, composed of (in g L\(^{-1}\)):\n
\[\begin{align*}
\text{KNO}_3 & \quad 3, \\
\text{KH}_2\text{PO}_4 & \quad 4, \\
\text{K}_2\text{HPO}_4 & \quad 1, \\
\text{FeSO}_4 \cdot 7\text{H}_2\text{O} & \quad 1, \\
\text{H}_3\text{BO}_3 & \quad 2.86, \\
\text{MnCl}_2 \cdot 4\text{H}_2\text{O} & \quad 0.11, \\
\text{ZnSO}_4 \cdot 7\text{H}_2\text{O} & \quad 0.09, \\
\text{CuSO}_4 \cdot 5\text{H}_2\text{O} & \quad 0.09 \\
\text{NaNO}_3 & \quad 0.021, \\
\end{align*}\]

pH 5.25 and incubated at 27 ± 2 °C on a shaker at 150 rpm for 72 h. The literature on organic volatile-mediated bacterial-plant interactions (see Introduction for references) suggests that production of growth-promoting volatiles is a widespread phenomenon among rhizosphere bacteria. The presumptive effect of volatiles of *A. brasilense* was predicted\(^\text{34}\), but neither determined nor quantified.
a rotary shaker at 120 rpm under 60 μmol photon·m$^{-2}$·s$^{-1}$ continuous light intensity for 6 days$^{37}$. Initial culturing of *A. brasilense* was done in BTB medium and *B. pumilus* and *E. coli* were both cultivated in TYG medium$^{54}$ in 125 mL flasks and incubated at 28±2°C for 16–18 h on a rotary shaker at 120 rpm. Bacteria were then harvested by centrifugation at 3720 × g for 7 min, rinsed twice in 0.85% saline solution, and subsequently transferred to minimal mineral Brunner’s medium (DSMZ medium 457) composed of (in g L$^{-1}$): Na$_2$HPO$_4$ (2.44), KH$_2$PO$_4$ (1.52), (NH$_4$)$_2$SO$_4$ (0.50), MgSO$_4$·7H$_2$O (0.20), CaCl$_2$·2H$_2$O (0.05), EDTA (0.50), FeSO$_4$·7H$_2$O (0.20), and (in μ g L$^{-1}$): ZnSO$_4$·7H$_2$O (0.10), MnCl$_2$·4H$_2$O (0.03), H$_2$BO$_3$ (0.30), CoCl$_2$·6H$_2$O (0.20), CuCl$_2$·2H$_2$O (0.01), NiCl$_2$·6H$_2$O (0.02), Na$_2$MoO$_4$·2H$_2$O (0.03). The mineral medium was supplemented with 5 g L$^{-1}$ glucose (for *B. pumilus* and *E. coli*) or 5 g L$^{-1}$ glucan (for *A. brasilense*). Cells were grown for 48 h at 32 ± 2°C and 120 rpm. After incubation, the bacterial cultures were rinsed, as described above, diluted in saline solution, and subsequently served as the inoculum for 250 mL flask, each containing 2 × 10$^6$ CFU·mL$^{-1}$ inoculum concentration. *C. sorokiniana* was inoculated into the same flasks using 10 mL of a suspension containing 8 × 10$^6$ cells·mL$^{-1}$.

**Experimental design and culturing conditions.** All experiments were run in batch cultures in pairs of 250 mL sterile Kitasato (Büchner) flasks (Corning, Corning, NY) containing 100 mL medium in each flask, with each pair of flasks serving as a single experimental unit (Fig. 3). Contents of the flasks were inoculated with the respective microorganism, in the above mentioned media, hermetically sealed with a new rubber stopper, and incubated at 28 ± 1°C for 96 h under illumination of 90 μmol photon·m$^{-2}$·s$^{-1}$ on a rotary shaker at 120 rpm. Some experiments were incubated for up to 216 h. A lithium hydroxide filter (described below) was used in experiments designed to remove CO$_2$. In all other experiments, the filter was not used.

Each experiment was performed with five replicates per treatment, where a pair of Kitasato flasks served as a single replicate. Each experiment contained the following treatments: *A. brasilense* and *C. sorokiniana*; *B. pumilus* and *C. sorokiniana*; *C. sorokiniana* and distilled water (as a control) and, in experiments involving potential production of CO$_2$, *C. sorokiniana* and *E. coli* (as a positive control).

**Counting microorganisms.** In each experiment, five samples from each flask and from each treatment were counted at each sampling period. *C. sorokiniana* cells were counted under a light microscope, using a Neubauer hemocytometer (bright line counting chamber, Hauser Scientific, Horsham, PA) connected to an image analyzer (Image ProPlus 6.3, Media Cybernetics, Silver Spring, MD). *A. brasilense* Cd, *B. pumilus* ES4, and *E. coli* DH5α were counted after serial dilution by the plate count method on nutrient agar medium (M7519, Sigma-Aldrich, St. Louis, MO). Cell volume of *C. sorokiniana* was measured by the same image analyzer. Five samples per treatment per each sampling time were analyzed and each was analyzed by five microscopic fields (n = 50 individual analyses). The volume of spherical cells was calculated.

**Analytical methods.** Determination of total carbohydrate content. Microalgal cells extracted by centrifugation were hydrolyzed with acid for 60 min at 100 °C$^{36}$ to release carbohydrates. Quantification of carbohydrates was by the phenol-sulphuric acid method$^{60}$, adapted to a microplate using glucose as standard.

Determination of total lipid content. Extraction of lipids followed the standard method$^{59}$ with small modifications to adapt it to microalgae, which involved sonication to disrupt cell walls$^{37}$. Lipids were quantified in the range of 70 μg to 1.33 mg permitted by this analytical method$^{60}$.

Determination of chlorophyll a content. Extraction of chlorophyll *a* from cells used the method described by Youngman$^{54}$, with minor modifications, where 5 mL freshly harvested culture were centrifuged for 10 min at 6000 × g. The supernatant was discarded and 5 mL 90% methanol was added to the pellet and heated in a water bath for 10 min at 60°C. After cooling, the samples were incubated in the dark for 24 h at 4°C. Then, the samples were centrifuged for 10 min at 4°C at 6000 × g. Absorbance in the supernatant was recorded at 655 and 750 nm.
and Wessjohann63 and peaks were first deconvoluted using AMDIS software (www.amdis.net) and identified by 40–500 m/z (mass-to-charge ratio). Volatile components were identified using the procedure described in Farag MS, Agilent Technologies) was operated in the electron ionization mode at 70 eV. The scan range was set at 40–500 m/z. Injections were made in the split-less mode for 30 s. The gas chromatograph was calibrated using CO2 standards (Matheson Tri-Gas, Basking Ridge, NJ). Reduction of quantities of CO2 in the headspace of the flasks was accomplished by incorporating a UV-sterilized (lithium hydroxide plus water) filter, which strongly absorbs CO2 (12) at quantities of 0.3 or 0.5 g per filter into culture flasks.

Analysis of microbial volatile organic compounds (mVOCs). Bacteria cultures were inoculated by pipetting 10 μL glycerol stock prepared in tryptic soy agar (TSA) containing 20% glycerol in 50 mL MS liquid medium62 containing 1.5% (w/v) sucrose, 0.4% (w/v) TSA and kept for 24 h at 37°C. For volatile capture, 5 mL aliquots of the broth were placed in a 20 mL solid-phase microextraction (SPME) vials in a laminar flow hood. Then, 10 μL of ultra-pure (Resistivity: 18.2 MΩ-cm at 25°C) water containing 1 μg cis-3-hexenyl acetate (Sigma-Aldrich, St. Louis, MO) as the internal standard was added and the vials sealed with Teflon®-lined magnetic caps using a hand crimper to prevent the escape of volatiles. SPME and gas chromatography–mass spectrometry analysis of the volatiles were performed as detailed below49. Earlier studies, in which the volatile composition of media alone was determined using the same analytical approach, showed a very negligible background (i.e., Fig. 2 in Ryu et al.29).

To capture the collected volatiles, a 50/30 μm DVB-CAR-PDMS SPME fiber (57328-U, Supelco, Bellefonte, PA) was inserted into the headspace above the bacterial culture and the vials were placed in a temperature-controlled oven at 50°C. Heating is essential and used in all SPME methods to ensure equilibration and saturation of volatiles in the headspace. Adsorption of volatiles was performed for 30 min, and fibers were desorbed at 210°C for 1 min in the injection port of a gas chromatograph interfaced with a mass spectrometer (GC-17A GC and QP-5000 MS, Shimadzu, Kyoto, Japan).

Volatile compounds were identified using the procedure described in Farag and Wessjohann63 and peaks were first deconvoluted using AMDIS software (www.amdis.net) and identified by its Kovat retention indices (RI) relative to n-alkanes (C8–C20) in the NIST/EPA/NIH mass spectral library database and with volatile standards, when available. The Kovat index refers to relative retention time measurements comparing hydrocarbon stand mixture C8–C20 to allow comparisons among databases.

Statistical analysis. Each experiment was repeated at least twice. Results presented are the average of two or three experiments, in each case. All data was analyzed by ANOVA, employing Fisher’s post-hoc analysis at p < 0.05 in Statistica 8.0 software (StatSoft, Tulsa, OK).

Considering the complexity of GC-MS data, multivariate data analyses are commonly used to detect compositional differences between species and help identify potential chemical markers for discrimination in an untargeted manner. Principal component analysis (PCA) is the most commonly used unsupervised multivariate data analysis method. Models allow clustering of samples according to intrinsic variance between them and without being biased by desired outcomes64. Multivariate data analysis of mVOCs was done by PCA and orthogonal partial least squares–discriminant analysis (OPLS-DA), performed using the program SIMCA-P 13.0 (MKS Umetrics, Malmö, Sweden). The PCA was run to obtain a general overview of the variance of volatile metabolites, and OPLS-DA was performed to obtain information on differences in the composition of volatiles between A. brasilense and B. pumilus strains. The Distance to the Model (DModX) test was used to verify the presence of outliers and evaluate whether a sample fell within the model applicability domain.

References
1. de-Bashan, L. E. & Bashan, Y. Immobilized microalgae for removing pollutants: Review of practical aspects. Bioresource Technol. 101, 1611–1627 (2010).
2. Perez-Garcia, O. & Bashan Y. Microalgal heterotrophic and mixotrophic culturing for bio-refining: From metabolic routes to techno-economics in Algal Biorefineries. Vol. 2: Products and Refinery Design (ed. Prokop, A., Bajpai, R. & Zappi, M.) 61–131 (Springer International Publishing, Cham, Switzerland, 2015).
3. Bashan, Y., de-Bashan, L. E., Prabhuj, S. R. & Hernandez, J-P. Advances in plant growth-promoting inoculant technology: formulations and practical perspectives (1998–2013). Plant Soil 378, 1–33 (2014).
4. Levanony, H., Bashan, Y., Romano, B. & Klein, E. Ultrastructural localization and identification of Azospirillum brasilense Cd on and within wheat root by immuno-gold labeling. Plant Soil 117, 207–218 (1989).
5. Puente, M. E., Holguin, G., Gleich, B. R. & Bashan, Y. Root-surface colonization of black mangrove seedlings by Azospirillum halophylaeferens and Azospirillum brasilense in seawater. FEMS Microbiol Ecol. 29, 283–292 (1999).
6. Bashan, Y. & Levanony, H. Horizontal and vertical movement of Azospirillum brasilense Cd in the soil and along the rhizosphere of wheat and weeds in controlled and field environments. J Gen Microbiol. 133, 3473–3480 (1987).
25. Meza, B., de-Bashan, L. E. & Bashan, Y. Involvement of indole-3-acetic acid produced by
28. Ryu, C.-M.
27. Farag, M. A., Zhang, H. & Ryu, C. M. Dynamic chemical communication between plants and bacteria through airborne signals:
26. Bailly, A. & Weisskopf, L. The modulating effect of bacterial volatiles on plant growth: current knowledge and future challenges.
20. Gonzalez, L. E. & Bashan, Y. Increased growth of the microalga
19. de-Bashan, L. E., Antoun, H. & Bashan, Y. Cultivation factors and population size control uptake of nitrogen by the microalgae
16. Choix, F. J., de-Bashan, L. E. & Bashan, Y. Enhanced accumulation of starch and total carbohydrates in alginate-immobilized
15. Choix, F. J., Bashan, Y., Mendoza, A. & de-Bashan, L. E. Enhanced activity of ADP glucose pyrophosphorylase and formation of
14. Powell, R. J. & Hill, R. T. Mechanism of algal aggregation by
13. Leyva, L. A., Bashan, Y., Mendoza, A. & de-Bashan, L. E. Accumulation of fatty acids in Chlorella vulgaris under heterotrophic conditions in relation to activity of acetyl-CoA carboxylase, temperature, and co-immobilization with Azospirillum brasilense.
12. Wisniewski-Dyte, E. et al. Azospirillum genomes reveal transition of bacteria from aquatic to terrestrial environments. PLOS Genet. e1002430. doi: 10.1371/journal.pgen.1002430 (2011).
11. Michiels, K., Croes, C. L. & Vanderleyden, J. Two different modes of attachment of Azospirillum brasilense Sp? to wheat roots. J Gen Microbiol. 137, 2241–2246 (1991).
10. de Oliveira Pinheiro, R., Boddey, L. H., James, E. K., Sprent, J.-I. & Boddey, R. M. Adsorption and anchoring of bacterial volatiles to roots of wheat seedlings. Plant Soil 246, 151–166 (2002).
9. Bashan, Y., Levanony, H. & Klein, E. Evidence for a weak active external adsorption of Azospirillum brasilense Cd to wheat roots. J Gen Microbiol. 132, 3069–3073 (1986).
8. Bashan, Y., Levanony, H. & Klein, E. Ammonia causes necrosis in tomato leaves infected with Pseudomonas chlororaphis pv. tabaci. Microbial volatiles promote accumulation of exceptionally high levels of starch in leaves in mono- and co-cultures of Bacillus pumilus and Pseudomonas chlororaphis O6 is a major determinant for eliciting systemic resistance against Erwinia carotovora but not against Pseudomonas syringae pv. tabaci in tobacco. Mol Plant Microbe Interact. 19, 924–930 (2006).
7. Perug, L., de-Bashan, L. E. & Bashan, Y. Assessment of affinity and specificity of Azospirillum for plants. Plant Soil 399, 389–414 (2016).
45. Farag, M. A., Rasheed, D. M. & Kamal, I. M. Volatiles and primary metabolites profiling in two Hibiscus sabdariffa (roselle) cultivars via headspace SPME-GC-MS and chemometrics. Food Res Int. 78, 327–335 (2015).

46. Yang, Y. & Gao, K. Effects of CO2 concentrations on the freshwater microalgae, Chlamydomonas reinhardtii, Chlorella pyrenoidosa and Scenedesmus obliquus (Chlorophyta). J Appl Phycol. 15, 337–389 (2003).

47. Higgins, B. T. & VanderGheynst, J. S. Effects of Escherichia coli on mixotrophic growth of Chlorella minutissima and production of biofuel precursors. PLoS ONE 9, e96807. doi:10.1371/journal.pone.0096807 (2014).

48. Walker, V. et al. Unexpected phytostimulatory behaviour for Escherichia coli and Agrobacterium tumefaciens model strains. Mol. Plant Microbe Int. 26, 495–502 (2013).

49. Farag, M. A., Ryu, C.-M., Sumner, L. W. & Paré, P. W. GC–MS SPME profiling of rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants. Phytochemistry 67, 2262–2268 (2006).

50. Ann, M. N., Cho, Y. E., Ryu, H. J., Koo, H. T. & Park, K. Growth promotion of tobacco plant by 3-hydroxy-2-butanone from Bacillus subtilis. J Pest Sci. 17, 388–393 (2013).

51. Doleschall, F., Recsei, K., Kemeny, Z. & Kovari, K. Comparison of differently coated SPME fibres applied for monitoring volatile substances in vegetable oils. Eur J Lipid Sci Technol. 105, 333–338 (2003).

52. Kai, M. et al. Bacterial volatiles and their action potential. Appl Microbiol Biotechnol. 81, 1001–1012 (2009).

53. Vonschalk, A. & Torzillo, G. Environmental stress physiology in Handbook of Microalgal Culture: Biotechnology and Applied Phycology (ed. Richmond, A.) 57–82 (Blackwell Publishing, Oxford, UK, 2004).

54. Bashan, Y., Trejo, A. & de-Bashan, L. E. Development of two culture media for mass cultivation of Azospirillum spp. and for production of inoculants to enhance plant growth. Biol Fertil Soils 47, 963–969 (2011).

55. Bashan, Y., Lopez, B. R., Huss, V. A. R., Amavizca, E. & de-Bashan, L. E. Chlorella sorokiniana (formerly C. vulgaris) UTEX 2714, a non-thermotolerant microalgal species useful for biotechnological applications and as a reference strain. J Appl Phycol. 28, 113–121 (2016).

56. de-Bashan, L. E., Hernandez, J.-P., Bashan, Y. & Maier, R. M. Bacillus pumilus ES4: Candidate plant growth-promoting bacterium to enhance establishment of plants in mine tailings. Environ Exp Bot. 69, 343–352 (2010).

57. Gonzalez, L. E., Cañizares, R. O. & Baena, S. Efficiency of ammonia and phosphorus removal from a Colombian agroidinal wastewater by the microalgae Chlorella vulgaris and Scenedesmus dimorphus. Bioresource Technol. 60, 259–262 (1997).

58. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. Colorimetric method for determination of sugars and related substances. Anal Chem. 28, 350–356 (1956).

59. Bligh, G. E. & Dyer, J. W. A rapid method f total lipid extraction and purification. Can J Biochem Physiol. 37, 911–917 (1959).

60. Pande, S. V., Parvin, R. K. & Venkitasubramanian, T. A. Microdetermination of lipids and serum total fatty acids. Anal Biochem. 6, 415–423 (1963).

61. Youngman, R. E. Measurement of chlorophyll. Water Research Centre. Tech. Rep. TR82. Medmenham, United Kingdom (1978).

62. Murashige, T. & Skoog, F. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15, 473–497 (1962).

63. Farag, M. A. & Wassjohann, L. A. Volatiles profiling in medicinal licorice roots using steam distillation and solid-phase microextraction (SPME) coupled to chemometrics. J Food Sci. 77, 1179–1184 (2012).

64. Farag, M. A. Comparative mass spectrometry & nuclear magnetic resonance metabolomic approaches for nutraceuticals quality control analysis: A Brief Review. Recent Patents on Biotechnology 8, 17–24 (2014).

65. Schulz, S. & Dickschat, J. S. Bacterial volatiles: the smell of small organisms. Appl Microbiol Biotechnol. 81, 1001–1012 (2009).

66. Ponnusamy, L. Effects of low temperatures (9–33 °C) and pH (3.3–5.7) in the loss of bioactive compounds from powdered Chlorella vulgaris. Phytochemistry 79, 1465–1471 (2003).

67. Hassan, S. B., Gali-Muhtasib, H., Göransson, H. & Larsson, R. Alpha terpineol: a potential anticancer agent which acts through induction of apoptosis. Anticancer Res 29, 1789–1805 (2009).

68. Hassan, S. B., Gali-Muhtasib, H., Göransson, H. & Larsson, R. Alpha terpineol: a potential anticancer agent which acts through suppressing NF-kappaB signalling. Anticancer Res 30, 1911–1919 (2010).

69. Ryan, R. P. & Dow, J. M. Diffusible signals and interspecies communication in bacteria. Microbiology 154, 1845–1858 (2008).

70. Ponnusamy, L. et al. Role of bacteria and bacteria-associated chemical cues that mediate oviposition site preferences by Aedes aegypti. Proc Natl Acad Sci USA 105, 9826–9827 (2008).

71. Winter, J. & Lee, J. Indole as an intercellular signal in microbial communities. FEMS Microbiol Rev. 34, 426–444 (2010).

72. Yu, J. Z. & Wang, R. L. Identification of bacteria and bacteria-associated chemical cues that mediate oviposition site preferences by Aedes aegypti. Proc Natl Acad Sci USA 105, 9826–9827 (2008).

73. Springer, M. G. & Krieg, N. R. Identification of microorganisms by 16S rRNA sequences. Methods Microbiol. 30, 77–106 (2002).

74. Youngman, R. E. Measurement of chlorophyll. Water Research Centre. Tech. Rep. TR82. Medmenham, United Kingdom (1978).

75. Murashige, T. & Skoog, F. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15, 473–497 (1962).

Acknowledgements

We thank Soohyun Lee of the Korean Research Institute of Bioscience and Biotechnology (KRIBB) in Daejeon, South Korea and Kyungseok Park of the Rural Development Administration (RDA) in Suwon, South Korea for ideas and technical support. At CIBNR, we thank Manuel Moreno for technical assistance and Ira Fogel for editorial services. We thank Mike Kubo and Angela Detweiler, NASA Ames Research Center, for assistance with CO2 headspace concentration analysis. At Auburn University, we thank Esther Ngumbi for providing information related to VOCs. This study was supported by Consejo Nacional de Ciencia y Tecnologia of Mexico (CONACYT-Basic Science-2015, contract 251102) and time for writing by The Bashan Foundation, USA. E.A. was mainly supported by a graduate fellowship from CONACYT (321403) and small periodic grants from The Bashan Foundation USA. M.A.F. received financial support from the Alexander von Humboldt Foundation, Germany. This work was supported by the Next-Generation BioGreen 21 Program (SSAC grant PJ009524) funded by the RDA and the KRIBB Research Initiative Program of South Korea. This is a contribution 2017–18 of The Bashan Institute of Science, USA.

Author Contributions

Edgar Amavizca - Designed some experiments, performed most experiments and microalgal analyses; Yoav Bashan - Design all experiments, wrote most of the manuscript, Choong-Min Ryu-advised in analysis of volatiles, Mohamed A. Farag-analyzed most volatiles and participate in writing on that topic, Brad M. Bebout-analyzed CO2 and helped with the final preparation of the manuscript, and Luz E. de-Bashan, managed the entire project, design the experiments, supervised the laboratory work and participate in writing of the manuscript.
Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Amavizca, E. et al. Enhanced performance of the microalga Chlorella sorokiniana remotely induced by the plant growth-promoting bacteria Azospirillum brasilense and Bacillus pumilus. Sci. Rep. 7, 41310; doi: 10.1038/srep41310 (2017).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2017