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# Plant growth promotion due to rhizobacterial volatiles – An effect of $CO_2$?

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ABSTRACT

**_Serratia odorifera_, an antagonistic rhizobacterium, emits a diverse and complex bouquet of volatiles. Three different in vitro experimental culture systems indicated that these volatiles promote the growth of _Arabidopsis thaliana_. $CO_2$ trapping and significant rise of $CO_2$ levels (390–3000 ppm $CO_2$ within 24 h) due to bacterial growth in sealed Petri dishes verified the enhanced effects of rhizobacterial $CO_2$ on _A. thaliana's_ growth. In contrast, open cocultivations abrogated growth promotion, and inhibitory effects come to the fore at ambient $CO_2$ concentrations.**


## 1. Introduction

Plant growth-promoting rhizobacteria (PGPR) were first defined by Kloepper and Schroth [1]. These bacteria primarily colonize the roots, since exudations of up to 40% of the photosynthate from the plant roots establish a preferred ecological niche [2]. The rhizobacteria in return can be beneficial to the plant by acting as biocontrol agent, biofertilizer, or phytostimulant. Various modes of action are known how PGPRs can support plant growth [3], e.g., nitrogen-fixing rhizobia generate a host-specific symbiosis with leguminous plants, other PGPR stimulate the bioavailability of phosphate or iron by secreting phosphatases or siderophores, respectively, or they produce hormones, such as auxin, gibberellins, octadecanoids, or ethylene. Antibiotics, e.g., pyoluteorin, phenazines, pyrrolnitrin, and 2,4-diacetylphloroglucinol are often released by rhizobacteria, which indirectly promote plant growth by inhibiting, among others, phytopathogenic fungi living in the same ecosystem. Recently, it was shown that 2,3-butanediol and acetoin, two volatiles emitted by *Bacillus subtilis* (GB03) and *Bacillus amyloliquefaciens* (IN937a), positively affect growth of *Arabidopsis thaliana* [4,5]. Following up on this observation, the same working group discovered that *B. subtilis* GB03 volatiles regulate the homeostasis of auxin and cell expansion and augment photosynthetic capacity by increasing photosynthetic efficiency and chlorophyll content [6]. Respective studies also indicated increased sugar accumulation as well as suppression of classic glucose responses (hypocotyl elongation and seed germination). While earlier experiments by Ryu et al. [4] demonstrated plant growth-promoting effects due to acetoin and 2,3-butanediol, the later experiments by Zhang et al. [6,7] were performed with the volatile mixture emitted by *B. subtilis* GB03.

Increase of photosynthetic efficiency, chlorophyll content, and sugar accumulation are typical photosynthesis markers that usually rise due to elevated $CO_2$ levels [8]. We hypothesize that in the laboratory experiment (cocultivation in sealed bi-partite Petri dishes) $CO_2$ is emitted during bacterial catabolism and accumulates to ultimately stimulate the growth of *A. thaliana*.

## 2. Materials and methods

### 2.1. Bacterial growth

*Serratia odorifera* 4Rx13 was obtained from the Strain Collection of Antagonistic Microorganisms (SCAM, University of Rostock, Microbiology, Germany) [9]. It was originally isolated from the rhizosphere of *Brassica napus*. Bacteria were grown on nutrient agar II (NA II; peptone from casein 3.5 $gL^{-1}$, peptone from meat 2.5 $gL^{-1}$, peptone from gelatine 2.5 $gL^{-1}$, yeast extract 1.5 $gL^{-1}$, NaCl 5 $gL^{-1}$, agar–agar 15 $gL^{-1}$, pH 7.2) or in nutrient broth II liquid culture (NBII) (NAII without addition of agar).

### 2.2. Plant growth

Seeds of *A. thaliana* (cv. Columbia 0) were surface sterilized (2 min 70% ethanol, 5 min 5% calcium hypochlorite, rinsed four

*Abbreviation:* PGPR, plant growth-promoting rhizobacteria

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times with sterile $A_{dest}$), and placed on Petri dishes containing half-strength Murashige–Skoog medium (MS medium). The seeds were vernalized for 3 days at 4 °C in the absence of light, then placed in a growth chamber (16 h light/8 h darkness, light: 5 Osram L58 W/76 lamps, 84 μmol $m^{-2}s^{-1}$, 20 °C).

### 2.3. Cocultivation

#### 2.3.1. Petri dish

*Arabidopsis* seedlings were transferred after 6 days from a normal Petri dish to one side of a bi-partite Petri dish. After 24 h, 20 μL of an overnight bacterial culture (1–3 × $10^7$ cfu) was spotted onto the other side. If appropriate, Nescofilm® was wrapped around to seal the Petri dishes. Cocultivation was performed for 14 days (20 °C, 84 μmol $m^{-2}s^{-1}$ light, 16 h/8 h light/darkness). Fresh weights of the stems and leaves were determined and images of the plants were recorded (digital camera C-3030 Zoom, Camedia, Olympus, Tokyo, Japan). Standard deviation (S.D.) was calculated as follows:

$$X = \sqrt[2]{\frac{\sum(x-\bar{x})^2}{(n-1)}}$$

#### 2.3.2. 'Mini greenhouse'

Surface sterilized, vernalized *A. thaliana* Col-0 seedlings were grown for 6 days on half-strength MS medium in Petri dishes. Seedlings were transferred to pots containing a mixture of sterilized soil/vermiculite (3 vol/2 vol) (Deutsche Vermiculite Dämmstoffe GmbH, Sprockhövel, Germany) and after 4 weeks placed into the 'mini greenhouse' (25.5 cm × 12 cm × 7 cm).

A preculture of *S. odorifera* ($OD_{600}$: 0.8–1.2) was used to adjust 100 mL NBII to an $OD_{600}$ of 0.005. A control was performed with uninoculated NBII. The modified Erlenmeyer flask was equipped with an in- and outlet. Charcoal-purified, sterile air entered the Erlenmeyer flask through the inlet with a constant airflow of 3 L $min^{-1}$ (Diaphragm Pump, 5010, Gardner Denver, Puchheim, Germany). The volatile enriched air of the bacterial culture directly streamed into the 'mini greenhouse' and left the container through two holes at the opposite site (Fig. 2A). Light conditions: 60 μmol $m^{-2}s^{-1}$, 8 h light/16 h darkness. Every seventh day a new bacterial culture was affixed to the greenhouse inlet. The experiment lasted 32 days and was completed by determining fresh weight and image recording (digital camera C-3030 Zoom, Camedia, Olympus, Tokyo, Japan). Standard deviation (S.D.) was calculated (see above).

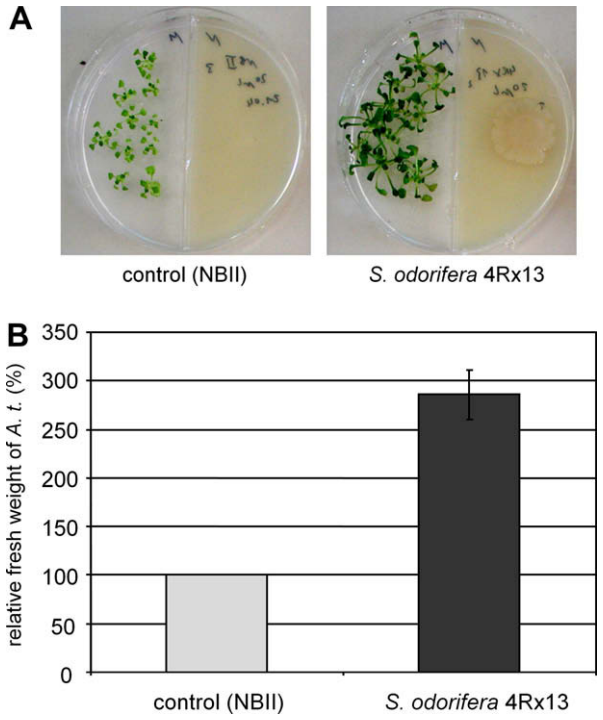


**Fig. 1.** Cocultivation of *Serratia odorifera* and *Arabidopsis thaliana*. (A) *A. thaliana* seedlings were transferred into one compartment of a bi-partite Petri dish, and 20 μL of an overnight culture (1–3 × $10^7$ cfu) of *S. odorifera* was spotted into the other compartment. The Petri dish was sealed with Nescofilm® and cocultivation was performed for 14 days at 20 °C, 84 μmol $m^{-2}s^{-1}$ light, 16 h/8 h light/darkness. (B) Fresh weight of the stems and leaves was determined and images of the plants were recorded. $n = 3$ with 3–5 replicates, S.D. was calculated.

### 2.4. Double glass dish

Bacteria and plants were treated as described for the 'mini greenhouse' experiment. Instead of the 'mini greenhouse' an especially designed double glass dish was applied (Fig. 2B). The glass construct consisted of two glass dishes, one (lower) dish with an inlet for the incoming air and a second (upper) dish that was perforated at the bottom. The latter was tightly sealed to the lower dish. Three plants were planted into soil/vermiculite (3 vol/2 vol) of the upper dish. The bacterial culture was changed every 7 days. The experiment lasted 32 days and was finished by determining fresh weight and by image recording (digital camera C-3030 Zoom). Standard deviation (S.D.) was calculated (see above).

### 2.5. $CO_2$ experiments

Trapping experiments were performed in tri-partite Petri dishes. Plant and bacterial growth was performed as described above. The third compartment was filled with 7 mL 0.1 M $Ba(OH)_2$. After 14 days, the dry weight of $BaCO_3$ was determined by filtering the solution through filter paper (MN 640 m Ø 70 mm; Macherey–Nagel, Düren, Germany) and drying at 50 °C for 4 days.

$CO_2$ determination of 100 mL bacterial culture was performed with a Li-840 $CO_2/H_2O$ gas analyzer (LICOR, Bad Homburg, Germany). Li-840 was connected to the Erlenmeyer flask (Fig. 2) and $CO_2$ levels were analyzed for 55 h using the 840–500 PC communication software.

$CO_2$ levels in Petri dishes were measured using infrared photometry. About 20 μL of culture of *S. odorifera* (OD 1–2 × $10^7$ cfu) was dropped on NB II. The Petri dish was covered with a glass lid equipped with an in- and outlet. To ensure that no air escaped through the lid the Petri dish and the lid were pressed between two metal plates furnished with eight screws. The airflow was adjusted by a mass flow controller (3 L $h^{-1}$). Volatile enriched air was recorded for 30 min in the $CO_2$-detector (Advance Optima, ABB, Automation Production GmbH, Hartmann and Braun Analytical, Frankfurt a.M., Germany). $CO_2$ was determined directly after inoculation and 24 h after inoculation; between these monitoring time points the system was completely closed. Standard deviation (S.D.) was calculated (see above).

## 3. Results and discussion

### 3.1. Growth promotion of A. thaliana cocultivated with S. odorifera

It was shown that volatiles of *B. subtilis* (Gram-positive, Firmicutes) promote the growth of *A. thaliana* [4]. This observation was extended by cocultivation of *S. odorifera*, a Gram-negative γ-proteobacteria, with ten *A. thaliana* plantlets in bi-partite sealed Petri dishes allowing only volatiles to diffuse from one side to the other (Fig. 1A). After 14 days the fresh weight of leaves and stems of *Arabidopsis* was determined (Fig. 1B). Compared to the

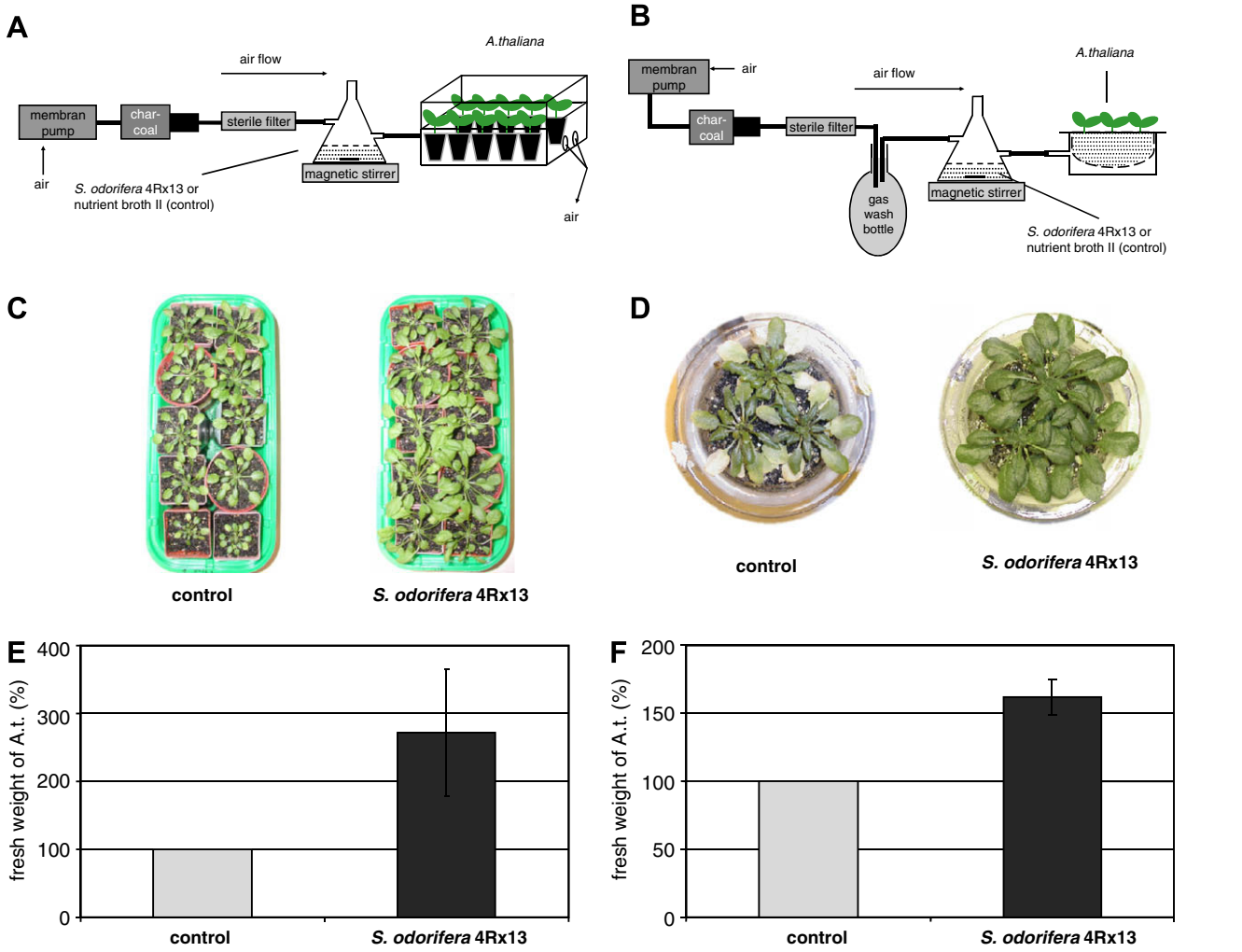


**Fig. 2.** Volatiles of *S. odorifera* directed on *Arabidopsis thaliana*. (A, C, E) *A. thaliana* seedlings were transferred into pots containing a mixture of sterilized soil/vermiculite and placed into the 'mini greenhouse'. The volatiles of *S. odorifera* directly streamed into the 'mini greenhouse'. (B, D, F) Plants were transferred to the double glass dish. The volatiles of *S. odorifera* streamed through the holes of the upper dish into the soil. Light conditions were set at 60 μmol $m^{-2}s^{-1}$, 8 h light/16 h darkness. Every seventh day a new bacterial culture was fixed to the plant growth container. Fresh weight was determined after 32 days and images were taken. S.D. was calculated.

control experiment (growth without cocultivation of bacteria), the fresh weight reached an approximately 3-fold increase. It can be concluded that in addition to a Gram-positive bacteria also a Gram-negative bacteria can support the growth of plants via volatiles.

To demonstrate that the observed growth promotion not only occurs when bacteria grow on agar plates (solid media), we performed two additional experiments. *S. odorifera* was grown in liquid media and the volatiles were channeled either into a small plastic container ('mini greenhouse') containing ten *A. thaliana* plants growing in pots (Fig. 2A) or into a specially designed double glass dish (Fig. 2B). The double glass dish, which was filled with soil and three plants, was perforated to allow the penetration of volatiles from the bottom. In the first experimental setup, the bacterial volatiles streamed directly over the leaves and aboveground parts of the plant, while in the second experiment the volatiles streamed through the soil and alongside of the roots. In both setups, fresh weight of *A. thaliana* was determined and approximately 3-fold or 1.5-fold plant growth promotion was observed, respectively (Fig. 2C and E, D and F). The $CO_2$ concentration leaving the Erlenmeyer flask, which was inoculated with *S. odorifera*, was 365 ± 12 ppm, and without bacteria 320 ± 5 ppm. These experiments further underline the growth-promoting effects of rhizobacterial volatiles and indicate a possible role of $CO_2$. Strong growth promotion occurred when bacterial volatiles were directed to the leaves, less promotion was observed when the volatiles were channeled through the soil.

### 3.2. Bacterial $CO_2$ production promotes plant growth

Since $CO_2$ emitted by the bacteria might contribute to the growth promotion of *A. thaliana* during cocultivation, its effect was tested in tri-partite sealed Petri dishes (Fig. 3). Normal growth of the plants was observed when the second and third compartments were not filled (Fig. 3A) (Fig. 3E; fresh weight = 100%). The growth of *A. thaliana* was reduced 5-fold after the addition of $Ba(OH)_2$ in one of the compartments (Fig. 3B) (Fig. 3E; fresh weight = 25%), most likely because atmospheric $CO_2$ was captured by $Ba(OH)_2$. The cocultivation of *A. thaliana* with *S. odorifera* stimulated its growth 3.5-fold (Fig. 3C) (Fig. 3E; fresh weight = 371%), while addition of $Ba(OH)_2$ into the third compartment caused a growth reduction of *A. thaliana* (Fig. 3D) (Fig. 3E; fresh weight = 126%). The captured $CO_2$, which precipitated as $BaCO_3$, was increased 1.6-fold during bacterial cultivation, and 1.4-fold during bacterial/plant co-cultivation (Fig. 3F). These experiments allowed the conclusion that elevated $CO_2$ levels resulted from bacterial emission, which ultimately explained the plant growth promotion in sealed Petri dish experiments.

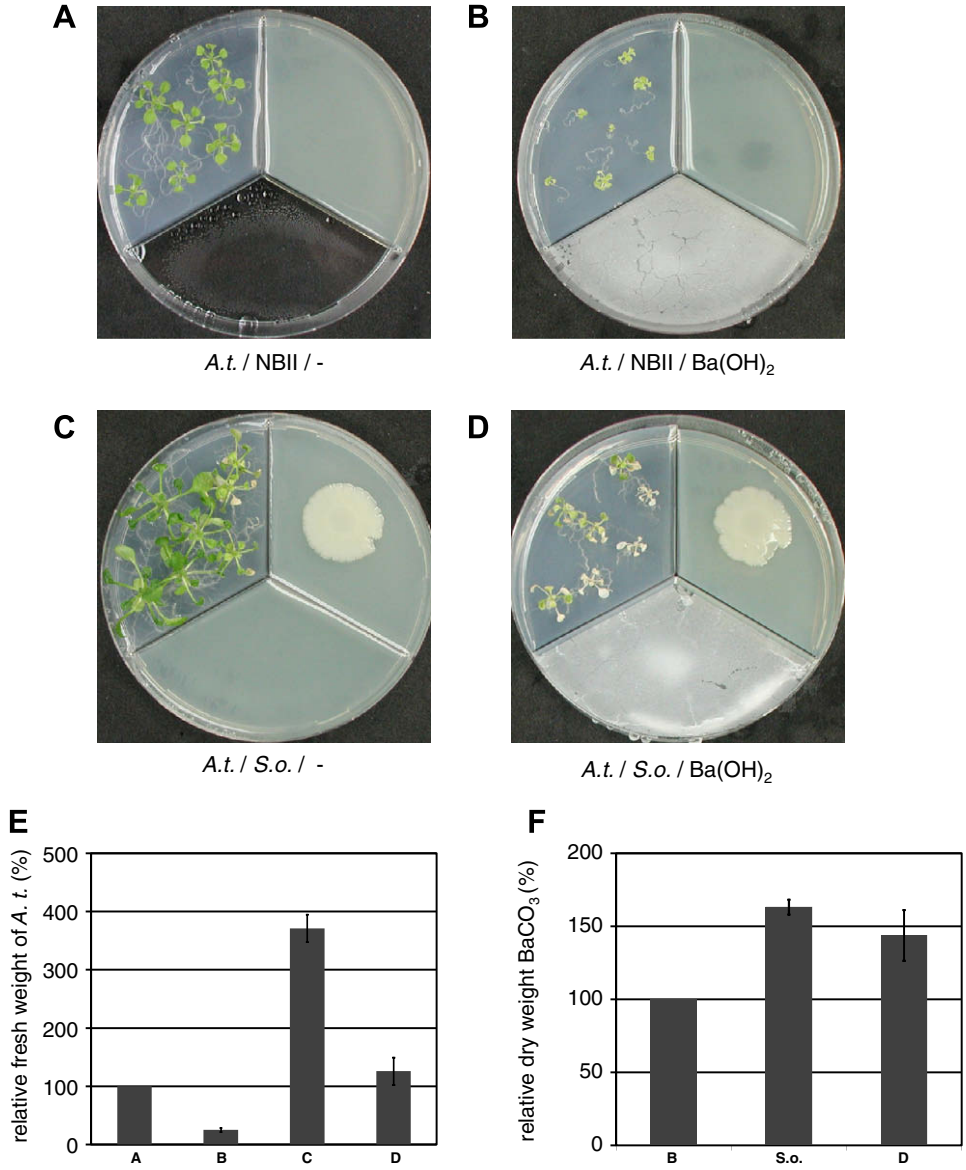


**Fig. 3.** Cocultivation of *S. odorifera* and *Arabidopsis thaliana* in the presence of $Ba(OH)_2$. (A) *A. thaliana* growing in one compartment of a sealed tri-partite Petri dish. (B) *A. thaliana* and 7 mL 0.1 mM $Ba(OH)_2$ were incubated in a sealed tri-partite Petri dish. (C) *A. thaliana* and *S. odorifera* were cocultivated in a sealed tri-partite Petri dish. (D) *A. thaliana, S. odorifera,* and 7 mL 0.1 mM $Ba(OH)_2$ were incubated in a sealed tri-partite Petri dish. Cultivation was performed for 14 days at 20 °C, 84 μmol $m^{-2}s^{-1}$ light, 16 h/8 h light/darkness. (E) Fresh weight of the stems and leaves was determined and images taken. (F) The dry weight of $BaCO_3$ precipitates of experiments B, and D, and S. odorifera (S.o.) were determined. $n = 3$ with five replicates, S.D. was calculated.

Infrared laser photometry allowed a quantitative measurement of $CO_2$ accumulation due to the emission of *S. odorifera*. Bacteria were grown in sealed Petri dishes and $CO_2$ concentrations at the beginning of the experiment was determined to be 390 ppm, which approximately reflects the normal atmospheric concentration. After 24 h of growth, when bacteria reach the end of the log phase in the batch culture, the $CO_2$ concentration in the Petri dish was significantly increased, reaching 3000 ppm. It can be assumed that the approximately 8-fold increased $CO_2$ levels in sealed Petri dishes are due to bacterial metabolism. Therefore during plant–bacteria cocultivation in bi-partite sealed Petri dishes, $CO_2$ has to be considered as a plant growth-promoting volatile, in addition to acetoin and 2,3-butanediol.

The above-mentioned experiments suggest $CO_2$ emission of *S. odorifera* while growing on NBII medium. In 1928 it was demonstrated by Pederson and Breed [10] that *S. marcescens* and *S. indica* catabolize peptone, most likely via the tricarboxylic acid cycle by releasing $CO_2$. Bacilli usually release $CO_2$ via the tricarboxylic acid cycle, however, many bacilli carry out incomplete oxidations when growing on carbohydrates and under anaerobic conditions. Under these conditions the synthesis of the enzymes of the tricarboxylic acid cycle are repressed and metabolites are partially converted to acetate, pyruvate, acetoin, and 2,3-butanediol. During this conversion two pyruvate units are condensed to form acetoacetate and $CO_2$. Subsequently acetoacetate decarboxylates to acetoin, which is further reduced to 2,3-butanediol. As a result, 2 mol $CO_2$ are produced to yield 1 mol acetoin (or 2,3-butanediol as its reduced product) [5]. Therefore, $CO_2$ is simultaneously emitted during acetoin and 2,3-butanediol synthesis.

Another controversial issue has to be addressed. Here it was demonstrated that during cocultivation with *S. odorifera*, the growth of *A. thaliana* was promoted. In the publication by Vespermann et al. [11], the same combination of organisms revealed severe inhibition of *A. thaliana*. The different growth effects were

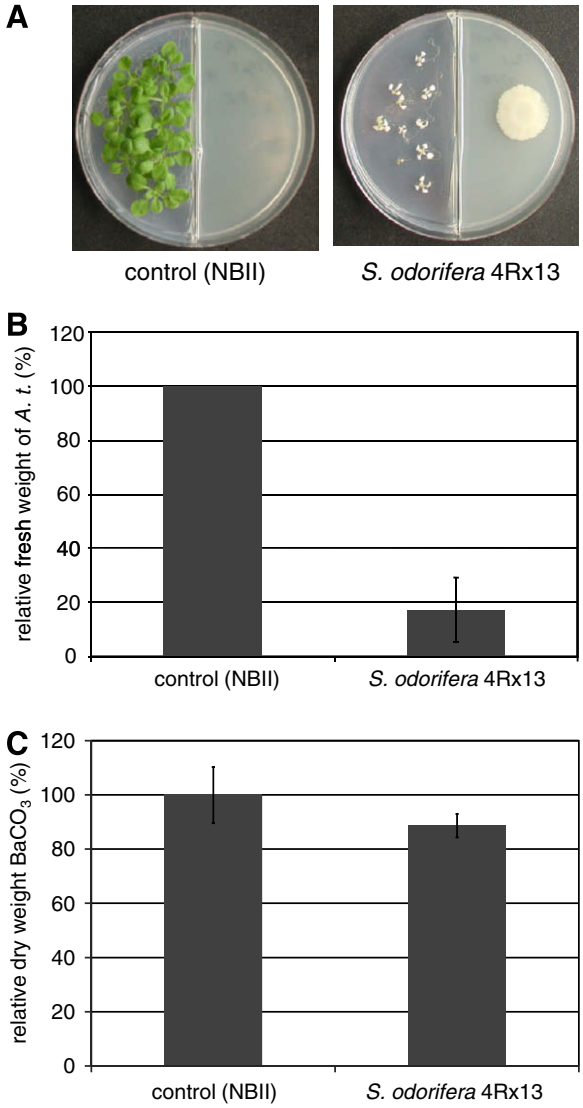


**Fig. 4.** Cocultivation of *Serratia odorifera* and *Arabidopsis thaliana* (open system). (A) Cultivation was performed as described in Fig. 1A without sealing the Petri dish. (B) Fresh weight of the stems and leaves was determined and images were taken. (C) *A. thaliana* and 10 mL 0.1 mM $Ba(OH)_2$ were incubated with or without *S. odorifera* in a bi-partite Petri dish. $n = 2$ with five replicates, S.D. was calculated.

due to sealed or non-sealed Petri dishes. Petri dishes in Figs. 1 and 3 were sealed with parafilm so that volatiles accumulated in the plastic container, which resulted in the dramatic accumulation of $CO_2$. In the non-sealed setup (Fig. 4), plants were severely inhibited (5-fold decrease of fresh weight compared to control) (Fig. 4B). Two facts may explain the inhibitory result. First, $CO_2$ (produced by *S. odorifera*) does not accumulate but equilibrates with the normal atmospheric $CO_2$ concentration by diffusing out of the Petri dish. This is shown by the very similar $CO_2$ precipitations ($BaCO_3$ dry weight) without or with cultivation of *S. odorifera* (Fig. 4C). Secondly, the volatile mixture released from *S. odorifera* was qualitatively identical under the sealed and unsealed conditions (Kai and Piechulla, unpublished). This complex mixture contained dimethyl disulfide, ß-phenylethanol, and several compounds of unknown structure [9]. It cannot be excluded that respective compounds of the volatile mixtures of *S. odorifera* individually or in combination have an effect on the growth of the plants. For example, dimethyl disulfide causes growth inhibition of *A. thaliana* (Kai and Piechulla, unpublished) and ß-phenylethanol was shown to have a negative effect on microorganisms [12]. More volatiles of *S. odorifera* await structural elucidation and it will be a future task to determine their biological potential. The volatile bouquet of *B. subtilis* GB03 also contained other compounds, 3-methyl-1-butanol, 2-methyl-butanol, and 1-methoxy-3-methylbutane among others [13] and their biological functions are not known at present.

Considering all results, it is very likely that the growth promotion of *A. thaliana* cocultivated with *S. odorifera* in the sealed Petri dish (Figs. 1 and 3) was at least partially due to the privileged $CO_2$ accumulation, while in the open system negatively-acting volatiles come to the fore at ambient $CO_2$ concentrations. Often researchers dealing with volatiles tend to capture the volatiles by sealing the experimental setup, however, this does not resemble a natural situation. Surely, both experimental setups, 'mini greenhouse' and 'double glass dish', used here are open systems but a continuous flow of volatiles at rates of 3 L $min^{-1}$ are unlikely to appear in nature. Nevertheless, the results open up a crucial ecological question regarding whether the $CO_2$ emission by soil bacteria influences roots and close aboveground leaf growth and to what extent.

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