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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).

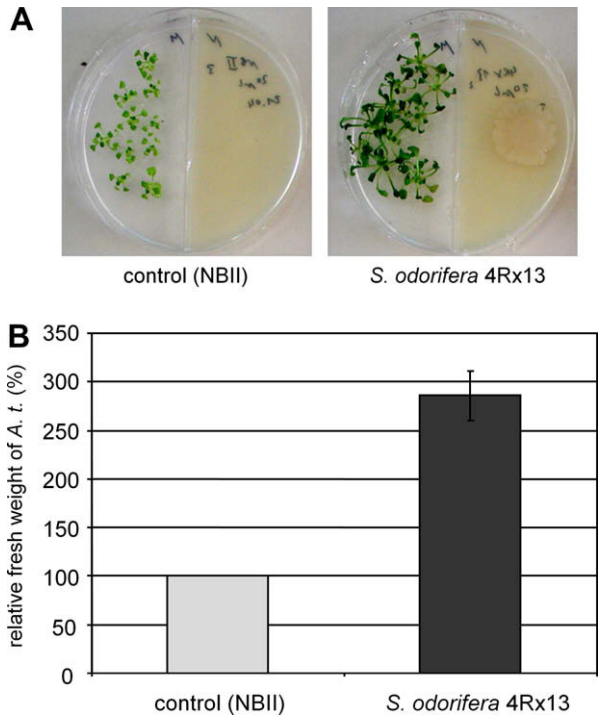

Fig. 1. Cocultivation of *Serratia odorifera* and *Arabidopsis thaliana*. (A) *A. thaliana* seedlings were transferred into one compartment of a bi-partite Petri dish, and 20 μL of an overnight culture (1–3 × 10^7 cfu) of *S. odorifera* was spotted into the other compartment. The Petri dish was sealed 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