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RESEARCH PAPER

Pseudomonas brassicacearum strain Am3 containing 1-aminocyclopropane-1-carboxylate deaminase can show both pathogenic and growth-promoting properties in its interaction with tomato

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Abstract

The role of bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity in the interaction between tomato (*Lycopersicon esculentum*=*Solanum lycopersicum*) and *Pseudomonas brassicacearum* was studied in different strains. The phytopathogenic strain 520-1 possesses ACC deaminase activity, an important trait of plant growth-promoting rhizobacteria (PGPR) that stimulates root growth. The ACC-utilizing PGPR strain Am3 increased *in vitro* root elongation and root biomass of soil-grown tomato cv. Ailsa Craig at low bacterial concentrations (10^6 cells ml^{-1} *in vitro* and 10^6 cells g^{-1} soil) but had negative effects on *in vitro* root elongation at higher bacterial concentrations. A mutant strain of Am3 (designated T8-1) that was engineered to be ACC deaminase deficient failed to promote tomato root growth *in vitro* and in soil. Although strains T8-1 and 520-1 inhibited root growth *in vitro* at higher bacterial concentrations (>10^6 cells ml^{-1}), they did not cause disease symptoms *in vitro* after seed inoculation, or in soil supplemented with bacteria. All the *P. brassicacearum* strains studied caused pith necrosis when stems or fruits were inoculated with a bacterial suspension, as did the causal organism of this disease (*P. corrugata* 176), but the non-pathogenic strain *Pseudomonas* sp. Dp2 did not. Strains Am3 and T8-1 were marked with antibiotic resistance and fluorescence to show that bacteria introduced to the nutrient solution or on seeds *in vitro*, or in soil were capable of colonizing the root surface, but were not detected inside root tissues. Both strains showed similar colonization ability either on root surfaces or in wounded stems. The results suggest that bacterial ACC deaminase of *P. brassicacearum* Am3 can promote growth in tomato by masking the phytopathogenic properties of this bacterium.

Key words: ACC deaminase, colonization, ethylene, GFP, PGPR, phytopathogen, plant–bacteria interactions, *Pseudomonas*, rhizosphere, tomato.

Introduction

A number of plant growth-promoting rhizobacteria (PGPR), belonging to various taxonomic groups including *Pseudomonas*, contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyses ACC, the immediate precursor of the plant hormone ethylene (Honma and Shimomura, 1978; Glick *et al*., 1995; Belimov *et al*., 2001, 2005). These bacteria, occurring on the root surface, degrade ACC to ammonium and α-ketobutyrate for use as carbon and nitrogen sources. Since a dynamic equilibrium of ACC concentration exists between root, rhizosphere, and bacterium, bacterial uptake of rhizospheric ACC stimulates plant ACC efflux,

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decreases root ACC concentration and root ethylene evolution, and can increase root growth (Glick *et al*., 1998). The ability of ACC-utilizing PGPR to ameliorate plant growth inhibition caused by ethylene through a decrease in ACC content (Penrose *et al*., 2001) and ethylene production (Burd *et al*., 1998; Belimov *et al*., 2002; Mayak *et al*., 2004) has been demonstrated. Stimulation of root elongation and biomass production of different plant species by inoculations with PGPR having ACC deaminase activity has been repeatedly documented, particularly when the plants were subjected to stressful growth conditions (Hall *et al*., 1996; Glick *et al*., 1997; Burd *et al*., 1998; Belimov *et al*., 2001, 2005; Van Loon and Glick, 2004; Safronova *et al*., 2006).

Pseudomonas brassicacearum has been described as a typical bacterium inhabiting the rhizoplane of *Arabidopsis thaliana* and *Brassica napus*, and phenotypically related to the phytopathogen *Pseudomonas corrugata* (Achouak *et al*., 2000). Contradictory data about the nature of the interaction between *P. brassicacearum* and plants have been reported. The strain *P. brassicacearum* 520-1 was described as a pathogen for tomato, causing chlorosis, browning, and necrotic lesions when plant wounds were infected (Sikorski *et al*., 2001). Endophytic strains of *P. brassicacearum* have been isolated from potato stem tissue infected with *Erwinia carotovora* (Reiter *et al*., 2003), but pathogenicity or plant growth-promoting activity of these isolates was not reported. On the other hand, several *P. brassicacearum* strains exhibited take-all disease suppression in wheat infected with *Gaeumannomyces graminis* and were suggested as potential biocontrol agents (Ross *et al*., 2000). Furthermore, *P. brassicacearum* strain Am3, which has ACC deaminase activity, promoted root elongation of Indian mustard (*Brassica juncea*) *in vitro* and increased root and shoot biomass of rape (*B. napus*) and pea (*Pisum sativum*) in pot trials (Belimov *et al*., 2001; Safronova *et al*., 2006). However, the presence of ACC deaminase in other *P. brassicacearum* strains and its effect on plant root development has not been studied.

The objective of the current work was to determine the effects of different *P. brassicacearum* strains on tomato, a species for which *P. brassicacearum* was reported to be phytopathogenic (Sikorski *et al*., 2001). The role of ACC deaminase in plant–bacteria interactions was examined by measuring *in vitro* ACC deaminase activity of the strains studied, and by creating a mutant of *P. brassicacearum* strain Am3 with decreased ACC deaminase activity.

Materials and methods

Characteristics of the bacterial strains

A list and description of strains used is given in Table 1. PGPR strains *P. brassicacearum* Am3, *Pseudomonas* sp. Dp2 (Belimov *et al*., 2001), and *Variovorax paradoxus* 5C-2 (Belimov *et al*., 2005) containing ACC deaminase, and *Escherichia coli* strain S17-1::pSUP5011 (Simon *et al*., 1983) were obtained from the ARRIAM Collection (St-Petersburg). Phytopathogenic strains *P. brassicacearum* 520-1 (Sikorski *et al*., 2001) and *P. corrugata* 176 (Bykova *et al*., 1991) were kindly provided by Dr W Wackernagel (University of Oldenburg, Germany) and Dr AM Lazarev (All-Russia Research Institute of Plant Protection, St-Petersburg), respectively. Strain *Escherichia coli* S17-1::pAG408 (Suarez *et al*., 1997) was kindly provided by Dr T Charles (University of Waterloo, Canada).

An ACC deaminase-deficient mutant of *P. brassicacearum* Am3 was obtained using Tn*5* mutagenesis via conjugation with *E. coli* S17-1::pSUP5011. For this purpose, 0.1 ml aliquots of cell suspensions (10^9 cells ml^{-1}) of strain S17-1::pSUP5011 grown on LB agar, supplemented with 50 μg ml^{-1} kanamycin, and strain Am3 grown on DF agar (Belimov *et al*., 2001), supplemented with 0.3 mg ml^{-1} ACC as a nitrogen source (DFA medium), were spotted on LB agar plate and incubated overnight at 28 °C. Then bacteria were resuspended in sterile water, plated on DF agar supplemented with 50 μg ml^{-1} kanamycin and 0.3 mg ml^{-1} $(NH_4)_2SO_4$ as a nitrogen source (DFN medium), and incubated for 24 h at 22 °C. Under these growth conditions recipient strain Am3 formed colonies characteristic of fluorescent pseudomonads, whereas growth of the donor *E. coli* strain was delayed by low temperature. The frequency of Km^r clones of Am3 (calculated as a ratio of the recombinant titre to the recipient) was 10^{-6}, whereas the frequency of Am3 spontaneous Km^r mutants was lower than 10^{-9}. Three hundred Km^r transconjugant clones were picked up on replica plates containing DFA (selection medium) and DFN (control medium) agar, incubated for 48 h at 28 °C, and the transconjugant T8-1 that lacked ability to grow on DFA medium was selected. The 16S rDNA gene of T8-1 was partially sequenced as described earlier (Belimov *et al*., 2005) and the sequence obtained (accession number AY916776) showed 99.8% homology with wild-type Am3, confirming that the isolated mutant T8-1 is a derivative of this strain. To ascertain whether Tn*5-mob* had been inserted into the isolate T8-1, PCR was performed to amplify the *nptII* gene (Chuang *et al*., 1999) using oligonucleotides CTCGACGTTGTCACTGAAGCGGGAAG (*TNF*) and AAAGCACGAGGAAGCGGTCAGCCCAT (*TNR*). Isolate T8-1 produced a short DNA fragment of 500 bp, indicating the presence of the Tn*5-mob* insert in the mutants, whereas the parental strain Am3, which does not harbour the *nptII* gene, showed no result (data not shown).

To monitor tomato root and tissue colonization by bacteria, the strains Am3 and T81 were marked with the gene encoding green fluorescent protein (GFP) of the jellyfish *Aequorea victoria*. For this purpose, the spontaneous mutants Am3R and T8-1R resistant to 20 μg ml^{-1} rifampicin were obtained via incubation of bacteria on Bacto-Pseudomonas F (BPF) agar containing (g l^{-1}): peptone, 10; casein hydrolysate, 10; glycerol, 12.5; K_2HPO_4, 1.5; $MgSO_4$, 1.5; agar, 15. Strains Am3R and T8-1R were conjugated with *E. coli* S17-1 carrying the promoter-probe *gfp*-based mini-transposon suicide delivery system pAG408 with kanamycin (30 μg ml^{-1}) and gentamycin (15 μg ml^{-1}) resistance as described by Suarez *et al*. (1997). Transconjugants named Am3RF and T8-1RF, having high and visible fluorescence intensity under UV light suggesting chromosomal integration downstream of strong promoters of the mini-transposon, were finally selected and used for further experiments.

Utilization of ACC

The ACC deaminase activity of the bacteria was determined by monitoring the amount of α-ketobutyrate (αKB) generated by enzymatic hydrolysis of ACC in cell-free extracts as described previously (Belimov *et al*., 2005).

Table 1. *Strains and plasmids used*

Name	Description/relevant characteristics	Source or reference
Escherichia coli		
S17-1::pSUP5011	Donor of pSUP5011, pBR325:: (Tn*5-mob*), Km^r, Cm^r	Simon *et al*., 1983
S17-1:: pAG408	Donor of pAG408, Km^r, Gm^r	Suarez *et al*., 1997
Pseudomonas brassicacearum		
Am3	Wild type	Belimov *et al*., 2001
Am3R	Spontaneous Rif^r mutant of Am3	This work
Am3RF	GFP-tagged Am3R, Am3R::pAG408, Rif^r, Km^r, Gm^r	This work
T8-1	ACCD-deficient mutant of Am3, Am3::Tn*5-mob*, Km^r, Cm^r	This work
T8-1R	Spontaneous Rif^r mutant of T8-1, Km^r, Cm^r	This work
T8-1RF	GFP-tagged T8-1R, T8-1R::pAG408, Rif^r, Km^r, Cm^r, Gm^r	This work
520-1	Wild type	Sikorski *et al*., 2001
Pseudomonas sp.		
Dp2	Wild type	Belimov *et al*., 2001
Variovorax paradoxus		
5C-2	Wild type	Belimov *et al*., 2005
Pseudomonas corrugata		
176	Wild type	Bykova *et al*., 1991

In batch culture, strains *P. brassicacearum* Am3 and T8-1 were incubated for 22 d at 25 °C without shaking in flasks containing 50 ml of liquid SMY (Belimov *et al*., 2005) medium (l^{-1}): glucose, 1.2 g; KH_2PO_4, 0.4 g; K_2HPO_4, 2 g; $MgSO_4$, 0.2 g; $CaCl_2$, 0.1 g; $FeSO_4$, 5 mg; H_3BO_3, 2 mg; $ZnSO_4$, 5 mg; Na_2MoO_4, 1 mg; $MnSO_4$, 3 mg; $CoSO_4$, 1 mg; $CuSO_4$, 1 mg; $NiSO_4$, 1 mg; yeast extract, 50 mg; pH 6.4. The SMY medium was supplemented with 10 mM ACC or 5 mM $(NH_4)_2SO_4$ such that the amounts of N were equal. Initial bacterial concentration in SMY medium was 10^5 cells ml^{-1}. Bacterial growth was determined daily by monitoring the optical density (OD) at 540 nm. The inoculated SMY medium without supplements was used as a blank to each strain.

Root elongation assay

The plant root elongation-promoting activity of the bacteria was determined using the modified root elongation assay of Belimov *et al*. (2001). Bacteria were grown for 48 h at 28 °C on BPF agar supplemented with antibiotics where necessary. Cells were collected and resuspended to 10^6, 10^7, and 10^8 cells ml^{-1} in sterile nutrient solution (μM): 500, $MgSO_4$; 500, K_2HPO_4; 200, KH_2PO_4; 500, $CaCl_2$; 20, KNO_3; 5, NaFeEDTA; 0.1, $MnSO_4$; 0.1, $ZnSO_4$; 0.1, H_3BO_3; 0.01, $CoCl_2$; 0.01, $CuSO_4$; 0.01, Na_2MoO_4; 0.01, $NiCl_2$; 0.01, KJ. Five millilitres of the bacterial suspensions or sterile nutrient solution (uninoculated control) were added to filter paper (Whatman #1) in Petri dishes (diameter 90 mm). For treatments with chemical inhibitors of ethylene biosynthesis or action, the nutrient solution was supplemented with 0.1 μM aminoethoxyvinylglycine (AVG), 1 μM Ag_2SO_4, or 2 μM $CoCl_2$ (final concentration). Tomato [*Lycopersicon esculentum* (=*Solanum lycopersicum*) Mill. cv. Ailsa Craig] seeds were surface-sterilized with 5% sodium hypochlorite solution for 15 min, washed carefully with sterile water and placed on wetted filter paper. The treated seeds were tested for surface sterility in a set of preliminary experiments via incubation on BPF agar for 5 d at 28 °C. After incubation of closed Petri dishes for 8 d at 25 °C in the dark, root length of seedlings was measured and root samples were taken for enumeration of bacteria and for microscopy as described below. The assay was repeated at least twice with four dishes (15 seeds per dish) for each treatment.

Agar culture

Tomato cv. Ailsa Craig root colonization by the GFP-tagged transconjugants *P. brassicacearum* Am3RF and T8-1RF was assayed using agar culture. Surface-sterilized seeds were germinated on filter paper wetted with bacterial suspensions (10^7 cells ml^{-1}) for 3 d as described above. Then germinated seeds having a root length of about 5 mm were transferred to Petri dishes (diameter 150 mm) containing 100 ml of the sterile nutrient solution (see above) solidified with 0.7% agarose. Four seeds per each Petri dish (three dishes for each treatment) were placed 2 cm from each other in a line located 3.5 cm from the box edge. The Petri dishes were installed at 45° to the horizontal to allow the roots to grow under the agarose layer and were incubated for 10 d in a greenhouse with a day/night cycle of 16 h/8 h at 25/16 °C and a photon flux density of 400 μmol quanta m^{-2} s^{-1}. After incubation, colonization of roots by the bacteria was monitored microscopically and root samples were taken for enumeration of bacteria as described below.

Pot experiments

These were carried out in the ARRIAM greenhouse (St-Petersburg) under natural illumination and temperature in June–August 2005 (experiments 1 and 2) and in July 2006 (experiment 3). Surface-sterilized tomato seeds were transferred to enamel pots containing 4 kg of unsterilized peat:sod-podzolic soil:dolomite (4:5:0.1, v/v/v) mixture having the following characteristics (mg kg^{-1}): total C, 58 000; total N, 6550; available P, 130; available K, 380; pH_{KCl}, 6.5. The mixture was fertilized with (mg kg^{-1}): $Ca(NO_3)_2$, 250; KH_2PO_4, 125; KCl, 65; $MgSO_4$, 50; $ZnSO_4$, 1.5; $MnSO_4$, 1.5; H_3BO_3, 5. In the course of the experiments the pots were watered daily to maintain 70% of the water holding capacity.

In experiment 1 with cv. Ailsa Craig, the soil mixture was irrigated up to 80% of water holding capacity with tap water, or with bacterial suspensions of *P. brassicacearum* strains Am3RF, T8-1RF, and 520-1 (inoculation resulted in a final concentration of 10^6 cells g^{-1} soil) before planting. Roots of seedlings grown in inoculated and uninoculated soil were sampled 15 d after planting (DAP) for determination of bacterial root colonization (three replicas per determination with two to four seedlings each) and seven seedlings remained in each pot. Three seedlings per pot were taken for a second determination of bacterial root colonization (three replicas per determination with four seedlings each) 30 DAP. The remaining plants (16 plants per treatment) were harvested 38 DAP and dried at 40 °C until constant mass.

In experiments 2 (cv. Ailsa Craig) and 3 (cv. Ailsa Craig and cv. Moneymaker) plants grown in pots containing soil mixture irrigated

with tap water were wound-infected by injecting 5 µl of bacterial suspensions of Am3RF, T8-1RF, and 520-1 (10^7 cells ml^{-1} in sterile 0.85% NaCl solution) into the stem 28 and 35 DAP (experiment 2) and 17 DAP (experiment 3). In the same manner, injections with strains Dp2, 5C-2, and 176 were performed in experiment 3 only. Control plants were injected with sterile 0.85% NaCl solution. A syringe needle of 0.5 mm diameter was passed completely through the stem between the cotyledons and the first leaf and bacterial suspension injected into the stem as the needle was withdrawn. Plant shoots were harvested 26 d (six plants per treatment) and 54 d (nine plants per treatment) (experiment 2) or 37 d (eight plants per treatment) (experiment 3) after injection, weighed, and inspected for internal infection symptoms in the stems. Stem samples of uninfected control plants, and wound-infected plants having discoloration and necrotic lesions were aseptically taken with a sterile surgical blade for bacterial enumeration. Stem cross-sections were photographed using a digital camera (model EOS10D, Canon, Japan).

Tomato fruit infection bioassay

Unripe (green) tomato fruits of cv. Urozhainy having no visual disease symptoms or disorders were collected at the ARRIAM experimental field in August 2006. Fruits were surface-sterilized with 1% sodium hypochlorite solution for 5 min and washed carefully with sterile water. Then 10 µl of 0.85% NaCl solution (control) or bacterial suspensions of Am3, T8-1, 520-1, Dp2, 5C-2, and 176 (10^7 cells ml^{-1}) were injected into the central pith area in six fruits per treatment. After 10 d of incubation in the dark at room temperature, fruit cross-sections were inspected and photographed as described above.

Enumeration of bacteria associated with roots or localized in infected tissues

Root tips (10 mm) of all seedlings from each Petri dish of filter paper culture were cut, pooled, and washed by plunging into sterile water 10 times to eliminate bacterial cells not attached to the roots. Root segments located 3–7 cm from the seeds of each Petri dish of agar culture were carefully removed from the medium and combined without any subsequent washing procedure. Roots from plants grown in soil were thoroughly shaken to remove adhering soil particles. Stem tissues were used for analysis immediately after sampling. The plant samples were homogenized in sterile tap water with a sterile mortar and pestle, the homogenates were serially diluted in 10-fold steps, and 50 µl aliquots were plated in two replicates on BPF agar supplemented with the required amounts of antibiotics where necessary (details in Tables 3, 4). In experiments with filter paper and agar cultures, no antibiotics were added to estimate the total amount of bacteria, to examine stability of transconjugants, and to check contamination of samples with extraneous microorganisms. To analyse the root samples taken from soil, the medium was additionally supplemented with 40 µg ml^{-1} nystatin to prevent growth of fungi. Colony-forming units (CFU) were counted after incubation of plates for 4 d at 25 °C. Presence of GFP marker in the isolated colonies was verified under UV light.

Microscopy

The presence and distribution of GFP-tagged bacteria on roots taken from filter paper and agar cultures was monitored using a laser scanning confocal microscope (LEICA SP2A0BS; Leica, Germany). In filter paper culture, root segments located 3 cm from the root tip were visualized directly. In agar culture, root segments located 3–7 cm from the seeds were carefully taken along with the agarose layer and immediately placed between microscope slides and cover slips. Three roots per treatment were scanned in each of two independent experiments. Cross-sections of the infected tomato stem tissues (10 plants per treatment) and the roots of plants grown in the inoculated soil were examined using a light microscope Axiolab equipped with filter set #01 (Carl Zeiss, Germany). Microphotographs of typical colonization patterns of bacteria on roots and in the infected stem tissues were taken.

Statistical analysis

The data were processed by variance and correlation analysis using the software STATISTICA version 5.5 (StatSoft, Inc., USA). SE and LSD stand for standard error and Fisher's least significant difference test, respectively.

Results

Utilization of ACC

ACC deaminase activity of the mutant *P. brassicacearum* T8-1 was 37 times less than that of the wild-type Am3 (Table 1). During the first 15 d of incubation in batch culture, when ACC was the sole source of nitrogen, the T8-1 mutant also showed a low rate of ACC utilization (Fig. 1). Then the growth rate of T8-1 increased, probably as the selection pressure exerted by high ACC concentrations induced reverse mutations. The growth curves of T8-1 and Am3 in the presence of $(NH_4)_2SO_4$ were identical, suggesting that there were no significant metabolic losses in T8-1 caused by transformation.

Strain *P. brassicacearum* 520-1 showed the highest ACC deaminase activity of all the strains studied, with *V. paradoxus* 5C-2 the lowest (Table 2). Rifampicin-resistant mutants Am3R and T8-1R had an ACC deaminase activity similar to that of wild-type strains, whereas the activity of GFP-tagged derivatives of these strains decreased slightly, probably due to a metabolic load caused by insertion and/or expression of pAG408.

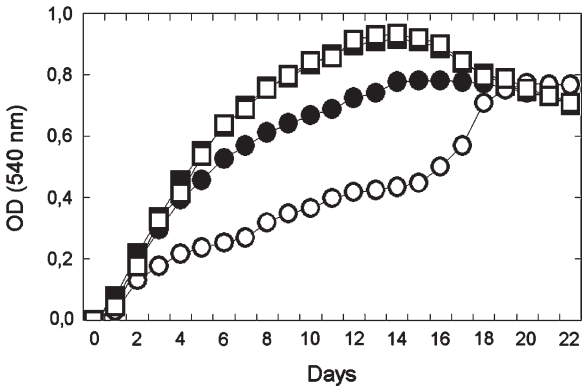

Fig. 1. Growth of *P. brassicacearum* strains Am3 (closed squares and circles) and T8-1 (open squares and circles) on a liquid SMY medium supplemented with 5 mM $(NH_4)_2SO_4$ (closed and open squares) or 10 mM ACC (closed and open circles). OD stands for optical density of bacterial suspensions at 540 nm. In all instances the standard error of the means is smaller than the symbol size. The data are of one of two representative experiments.

Table 2. *ACC deaminase activity of the strains studied*

The data are given as means ±SE of four experiments. Values followed by different letters are significantly different (P <0.05; Fisher's LSD test).

Strain	ACC deaminase activity (μM αKB mg^{-1} h^{-1})
P. brassicacearum Am3	8.1±0.8 a
P. brassicacearum Am3R	7.7±0.4 a
P. brassicacearum Am3RF	6.5±0.4 ad
P. brassicacearum T8-1	0.22±0.03 b
P. brassicacearum T8-1R	0.16±0.03 b
P. brassicacearum T8-1RF	0.14±0.02 b
P. brassicacearum 520-1	10.2±1.0 c
Pseudomonas sp. Dp2	8.6±0.7 ac
V. paradoxus 5C-2	5.2±0.5 d
P. corrugata 176	0

Filter paper culture

All *P. brassicacearum* strains caused stepwise changes in tomato root elongation depending on the concentration of bacteria in the nutrient solution (Fig. 2). *Pseudomonas brassicacearum* Am3 stimulated (by 8%), had no effect, and inhibited (by 25%) root elongation in the presence of 10^6, 10^7, and 10^8 cells ml^{-1}, respectively. The mutant T8-1 had no effect on roots at 10^6 cells ml^{-1} and significantly inhibited root elongation at higher bacterial concentrations. Significant (P <0.05) differences between treatments with wild-type Am3 and the mutant T8-1 occurred in the presence of 10^7 and 10^8 cells ml^{-1}. *Pseudomonas brassicacearum* 520-1 significantly inhibited root elongation at all bacterial concentrations used. Visual observations revealed no visible disease symptoms on the roots inoculated with *P. brassicacearum* strains, except a brownish root colour in the presence of Am3 and T8-1 at 10^8 cells ml^{-1} and in the presence of 520-1 at 10^7 and especially at 10^8 cells ml^{-1}. Contrary to these observations, strains *Pseudomonas* sp. Dp2 and *V. paradoxus* 5C-2, applied as positive controls, significantly stimulated tomato root elongation irrespective of bacterial concentration.

Treatments with chemical inhibitors of ethylene biosynthesis (AVG and Co^{2+}) and action (Ag^{+}) also stimulated root elongation to a similar extent as strains Dp2 and 5C-2. Addition of Co^{2+} to the solution supplemented with bacteria (10^7 cells ml^{-1}) partially alleviated root length inhibition by strains T8-1 and 520-1, increased the stimulating effect of 5C-2, and tended to increase root length of seedlings in the presence of strains Am3 and Dp2.

There was no significant difference between the number of *P. brassicacearum* Am3 and T8-1 on tomato roots (Table 3). Similar numbers of bacteria on roots detected on BPF medium, with and without necessary antibiotics, confirmed a high stability of Tn*5-mob* and pAG408 inserted into the tagged strains, and suggested that genetic modifications had no effect on colonization ability of bacteria. No contamination of roots with extraneous microorganisms was detected. The attempt to visualize the bacteria on roots using laser scanning confocal microscopy was not successful because the root tissues had very high auto-fluorescence.

Agar culture

Study with a microscope showed that distribution of the introduced bacteria on roots was relatively random. Single cells and small microcolonies presented along the root segments and dividing bacterial cells (pairs of cells) were often observed, suggesting that multiplication of the bacteria occurred and the bacteria were in an active physiological state (Fig. 3A, B). Few larger microcolonies were also observed on the root surface where the bacteria were localized in a mucilage layer, but no bacteria were observed inside root tissues (Fig. 3C). No visible differences were found between *P. brassicacearum* Am3RF and its mutant T8-1RF in their root colonization patterns. Study with a microscope and visual observations revealed no morphological changes or disease symptoms such as necrotic spots, browning, lesions, and other root tissue damage caused by inoculation with either Am3RF or T8-1RF. Enumeration of bacteria using the root homogenate serial dilution method showed that the root colonization capability of *P. brassicacearum* Am3RF and its mutant T8-1RF was similar and the GFP-tagged cells comprised all the introduced bacteria (Table 2). No contamination of roots with extraneous microorganisms was detected.

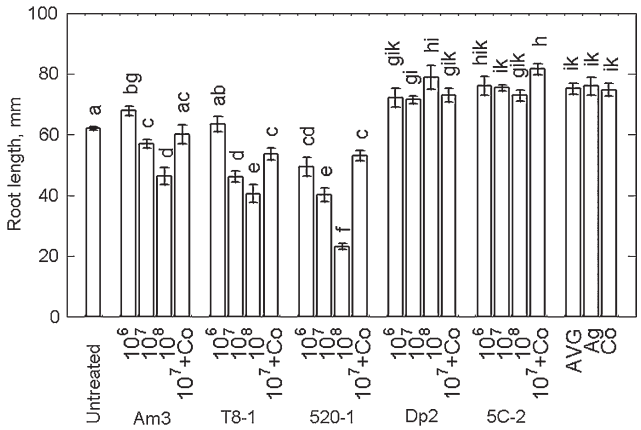

Fig. 2. Effect of bacteria and chemical ethylene inhibitors on tomato cv. Ailsa Craig root elongation. Treatments: Am3, *P. brassicacearum* strain Am3; T8-1, *P. brassicacearum* strain T8-1; 520-1, *P. brassicacearum* strain 520-1; Dp2, *Pseudomonas* sp. strain Dp2; 5C-2, *V. paradoxus* strain 5C-2; AVG, 0.1 μM AVG; Ag, 1 μM Ag_2SO_4; Co, 2 μM $CoCl_2$. Concentration of bacteria in nutrient solution is indicated at the bottom of columns and was 10^6, 10^7, and 10^8 cells ml^{-1}, respectively. Vertical bars represent the standard error. The data are means of at least two experiments for each treatment. Values followed by different letters are significantly different (P >0.05; Fisher's LSD test).

Table 3. *Colonization of tomato cv. Ailsa Craig roots by* P. brassicacearum *strains Am3 and T8-1*

Concentrations of antibiotics added to BPF medium were 20 μg ml^{-1} rifampicin (Rif), 30 μg ml^{-1} kanamycin (Km), and/or 15 μg ml^{-1} gentamycin (Gm). The data for filter paper and agar cultures are given as means ±SE of two experiments, and the data for soil culture are means ±SE of one experiment, with three replicas each. nd stands for not determined. Values followed by the same letter within each experiment type are not significantly different ($P > 0.05$; Fisher's LSD test).

Experiment type and the medium used for determination	Number of cells on roots (CFU×10^3 mg^{-1} FW)			
	Am3	Am3RF	T8-1	T8-1RF
Filter paper culture[a]				
BPF without antibiotics	14.7±1.1 a	nd	15.1±3.0 a	nd
BPF with Rif	nd	10.2±2.0 a	nd	nd
BPF with Km	nd	nd	14.9±2.8 a	nd
BPF with Km and Rif	nd	nd	nd	11.2±2.7 a
BPF with Km, Rif, and Gm	nd	15.1±7.6 a	nd	9.6±1.2 a
Agar culture				
BPF without antibiotics	nd	9.0±4.1 b	nd	8.2±4.3 b
BPF with Km, Rif, and Gm	nd	7.9±3.0 b	nd	5.5±2.8 b
Soil culture[b]				
BPF with Km, Rif, and Gm (15 DAP)	nd	0.5±0.1 c	nd	0.7±0.2 c
BPF with Km, Rif, and Gm (30 DAP)	nd	0.4±0.1 c	nd	0.4±0.1 c

[a] Concentration of bacteria in the nutrient solution was 10^7 cells ml^{-1}.
[b] Bacteria were introduced into soil before planting as described in Materials and methods (experiment 1) and survival of the bacteria on tomato roots was determined 15 and 30 d after planting (DAP).

Pot experiments

Tomato plants grown in soil supplemented with *P. brassicacearum* Am3RF (experiment 1) had increased root biomass as compared with those plants grown in untreated soil and in soil inoculated with strains T8-1RF or 520-1 (Fig. 4). Inoculation with T8-1RF slightly decreased (−11%) root biomass relative to control plants. Shoot growth was not affected by inoculations with T8-1RF or 520-1, whereas inoculation with Am3RF slightly increased (+14%) shoot biomass relative to control and a significant difference was evident as compared with T8-1RF. Visual observations revealed no disease symptoms on roots and shoots caused by any of the strains used. The introduced strains Am3RF and T8-1RF were detected at levels of several hundreds of cells per milligram root FW during the course of the experiment (Table 3). Visualization of GFP-tagged bacteria on roots using light microscopy revealed few cells per field of vision; however, no representative pictures were taken because of the relatively low number of cells on the roots and the high fluorescence level of root tissues and some soil particles (data not shown).

In experiment 2, all plants injected with suspensions of *P. brassicacearum* strains had necrotic brown lesions inside the stems localized in pith tissue above the injection point (Table 4; Fig. 3D). Longitudinal stem sections showed that lesion size increased during the course of the experiment and was maximal on plants wound-infected with strain 520-1 (Table 4). Injection with 520-1 decreased shoot biomass by 17%, whereas the effect of injections with Am3RF and T8-1RF on shoot weight was not significant. Leaves of the infected plants showed no chlorosis or other disease symptoms. The introduced strains Am3RF and T8-1RF were detected in necrotic brown lesions of stems by light microscopy (Fig. 3E, F). The bacteria formed numerous microcolonies located on plant cell walls. No bacteria were observed outside the lesions (data not shown), suggesting that bacterial proliferation and infection were restricted by the plant to the lesion zone. The number of each of the introduced strains inhabiting stem lesions was similar at both 26 d and 54 d after wound-infection and the number of strain 520-1 was about twice more than strains Am3RF and T8-1RF (Table 4).

In experiment 3, the number of infected plants having necrotic lesions inside the stems was maximal after infection with *P. corrugata* 176, whereas the negative control strain *Pseudomonas* sp. Dp2 induced no lesions, and only three plants with lesions were observed after infection with *V. paradoxus* 5C-2 (Table 4). Lesion dimensions were significantly less than in experiment 2, and no significant genotypic differences were observed between tomato cultivars or between lesion-inducing strains. Shoot biomass was not affected by bacterial inoculation. Bacterial numbers in the stem lesions were similar for all the pseudomonad strains, whereas the number of 5C-2 was about 20 times less (Table 4). No bacteria were found in the stem when strain Dp2 was injected.

Tomato fruit infection bioassay

All fruits infected with *P. brassicacearun* Am3, T8-1, and 520-1 and *P. corrugata* 176 had brown lesions in the

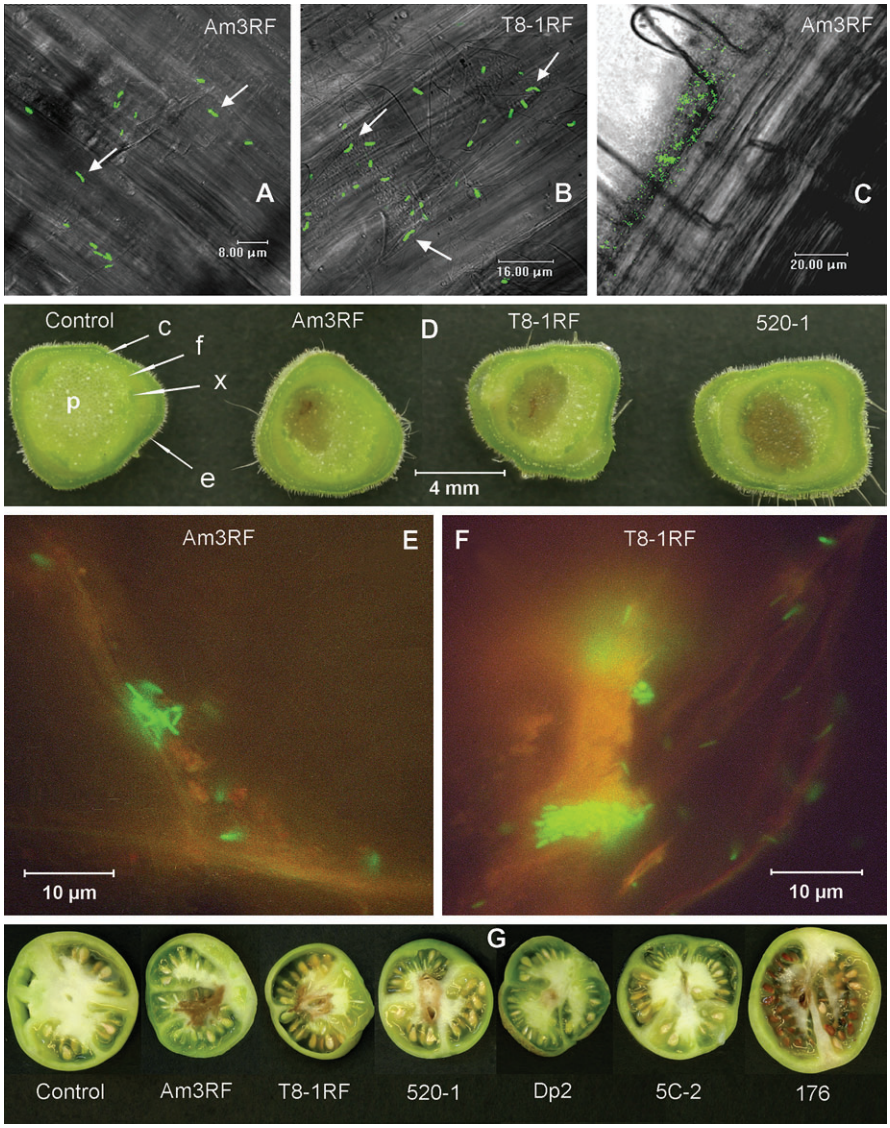

Fig. 3. Confocal laser scanning microscopy images of primary tomato cv. Ailsa Craig root surface colonized by *P. brassicacearum* strains Am3RF (A) and T8-1RF (B) when seeds were treated with bacteria and transferred to agar culture (see Materials and methods). Arrows point to the dividing bacterial cells. (C) Confocal laser scanning microscopy image of primary root longitudinal section shows localization of Am3RF cells on the root surface and in the mucilage layer. (D) Pictures of tomato stem cross-sections, from left to right, of uninfected control plants (injected with 0.85% NaCl only) and the plants wound-infected with 0.85% NaCl suspensions of strains Am3RF, T8-1RF, and 520-1 (26 d after infection). c, Cortex; e, epidermis; f, fibre; p, pith; x, xylem. (E) and (F) Fluorescence light microscopy images of typical colonization patterns of the strains Am3RF and T8-1RF, respectively, in the stem brown lesions of the wound-infected tomato plants (54 d after infection). (G) Pictures of tomato cv. Urozhainy fruit cross-sections, from left to right, of uninfected control fruits (injected with 0.85% NaCl only) and the fruits wound-infected with 0.85% NaCl suspensions of strains Am3RF, T8-1RF, 520-1, Dp2, 5C-2, and 176 (10 d after infection).

central pith area, whereas no lesions were observed in control (0.85% NaCl sterile solution) fruit. Strains Dp2 and 5C-2 induced no lesions with the exception that only two fruits of each treatment had small, slightly brownish lesions (cross-sections of these fruits are shown in Fig. 3G). Visual changes in the colour of the gel area (from transparent to brownish) and seeds (from yellow-white to brown) were detected in the fruits infected with strain 176 only. Cross-sections of representative fruits are presented in Fig. 3G.

Discussion

Pseudomonas brassicacearum strain 520-1, a bacterium described previously as phytopathogenic for tomato (Sikorski *et al*., 2001), possesses ACC deaminase activity, an important mechanism of plant growth promotion by beneficial root-associated bacteria (Glick *et al*., 1998; Van Loon and Glick, 2004). This observation is in agreement with searches of the NCBI sequence database, where putative ACC deaminase genes were found in complete

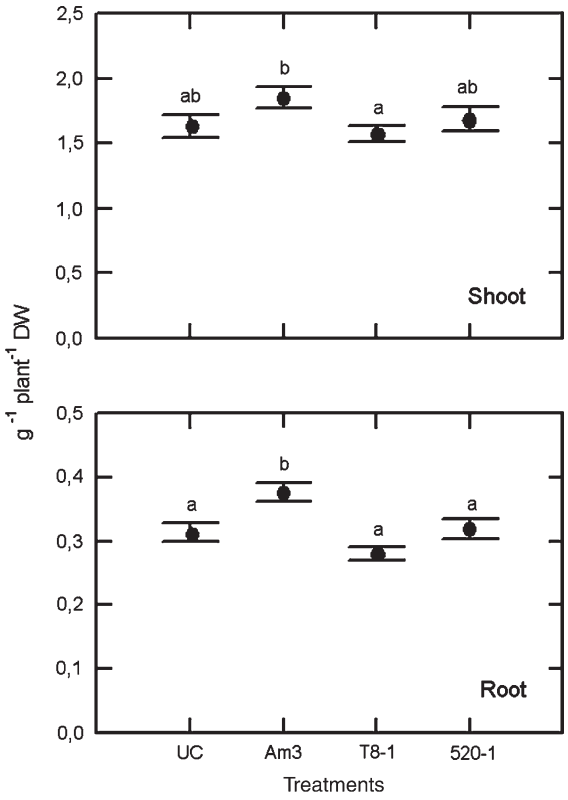

Fig. 4. Shoot and root biomass of tomato cv. Ailsa Craig grown in soil supplemented with *P. brassicacearum* strains. Treatments: UC, untreated control; Am3, *P. brassicacearum* strain Am3RF; T8-1, *P. brassicacearum* strain T8-1RF; 520-1, *P. brassicacearum* strain 520-1. Vertical bars represent the standard error. The data are means of one experiment with 16 determinations for each treatment. Values followed by different letters are significantly different (P >0.05; Fisher's LSD test).

genome sequences of several phytopathogens such as *Pseudomonas syringae* pv. *tomato* DC3000 (NP793449), *P. syringae* pv. *syringae* B728a (ZP00124451), and *E. carotovora* subsp. *atroseptica* SCRI1043 (YP049633), although the ability of these strains to degrade ACC was not determined. Recently, Blaha *et al.* (2006) detected the ACC deaminase gene in a number of phytopathogenic *Agrobacterium tumefaciens* strains, in *Burkholderia gladioli* LMG2216, *B. caryophylli* LMG2155, and *B. cepacia* LMG1222, and showed that strain LMG1222 possessed ACC deaminase activity *in vitro*. Furthermore, it is shown here that an ACC deaminase-containing bacterium (*P. brassicacearum* Am3), characterized as a PGPR for pea, rape, and Indian mustard (Belimov *et al.*, 2001; Safronova *et al.*, 2006), is pathogenic for tomato.

The effects of *P. brassicacearum* strain Am3 (and particularly its ACC deaminase-deficient T8-1 mutant) resembled those of phytopathogenic strain 520-1 on *in vitro* root elongation in the presence of high bacterial concentrations (10^7 and 10^8 cells ml^{-1}) (Fig. 2). However, at low cell concentrations (10^6 cells ml^{-1}), strain Am3 stimulated root elongation in filter paper culture (Fig. 2) and increased biomass of plants grown in inoculated soil (Fig. 4). By contrast, *P. brassicacearum* strains 520-1 and T8-1 had no detectable effect on biomass of plants grown in inoculated soil. *Pseudomonas brassicacearum* Am3 (and T8-1) successfully colonized the root surface of both *in vitro* and in soil-grown plants (Table 3), in agreement with previous reports describing this species as a common rhizosphere bacterium (Achouak *et al.*, 2004). However, it was not able to infect plant tissues when bacterial cells were present around the root surface of nutrient solution- or soil-grown plants.

Disease symptoms (necrotic lesions) caused by *P. brassicacearum* were only evident when bacteria were injected into wounded plant tissues (stem or fruit). The area of damaged stem tissues was limited, and infiltrated bacteria were found in this area only (Table 4). Localization of bacteria in the pith might be associated with reduced resistance of this tissue to bacterial toxicity. Necrosis of tomato stem pith is a typical disease symptom induced by the phytopathogen *P. corrugata* (Sutra *et al.*, 1997), a species closely related to *P. brassicacearum* (Achouak *et al.*, 2000). Although bacteria were introduced to the plant stem using a similar technique to Sikorski *et al.* (2001), the response of tomato cv. Ailsa Craig and cv. Moneymaker in the present experiments was much less pronounced than the chlorosis, vascular browning, and necrotic lesions on leaflets reported in cv. Moneymaker (Sikorski *et al.*, 2001). In particular, very small stem pith lesions induced by *P. brassicacearum* and *P. corrugata* were observed in experiment 3 on both cultivars. At present, much of the biology of tomato disease caused by *P. corrugata* is not well understood, but disease severity appears to be worse when temperature is low and humidity is high (Momol and Pernezny, 2006). Climatic data for St-Petersburg (http://www.pogoda.ru.net) showed that conditions (mean temperature and monthly precipitation) for disease development were more conducive in experiment 2 (17.8 °C; 85 mm month^{-1}) than experiment 3 (19.1 °C; 16 mm month^{-1}). Therefore, it is suggested that phytopathogenicity of *P. brassicacearum* depends on plant genotype and environmental conditions, and occurs particularly when bacteria invade (penetrate) wounded plant tissues, a property characteristic of 'opportunistic' pathogenesis.

It is possible that the inhibition of root elongation *in vitro* and necrotic lesions observed in wound-infected tomato stems and fruits caused by *P. brassicacearum* strains Am3 and 520-1 were due to production of bacterial toxins. Various phytopathogenic *Pseudomonas* species produce a wide spectrum of phytotoxic compounds (Bender *et al.*, 1999), and the phytotoxin coronatine from *P. syringae* inhibited root growth of *Arabidopsis thaliana* (Feys *et al.*, 1994) and wheat (Sakai, 1980). Toxins

Table 4. *Response of tomato cultivars to stem wound infection with the bacterial strains studied*

The medium for bacterial isolation was supplemented with (μg ml^{-1}): no antibiotics (*); 20, rifampicin and 30, kanamycin (†); 20, rifampicin, 30, kanamycin and 15, gentamycin (‡). The data are given as means ±SE. nd and nt stand for not determined and not detected, respectively. Values followed by different letters within each parameter and experiment are significantly different (P <0.05; Fisher's LSD test).

Parameters and treatments	Experiment 2		Experiment 3	
	cv. Ailsa Craig		cv. Ailsa Craig (37 DAI)	cv. Moneymaker (37 DAI)
	26 DAI	54 DAI		
Number of plants having lesions in stem[a]				
Control (0.85% NaCl)	0	0	0	0
P. brassicacearum Am3RF	6	9	5	3
P. brassicacearum T8-1RF	6	9	5	5
P. brassicacearum 520-1	6	9	4	3
Pseudomonas sp. Dp2	nd	nd	0	0
V. paradoxus 5C-2	nd	nd	2	1
P. corrugata 176	nd	nd	7	4
Length of lesion zone in longitudinal sections of stem (mm)				
Control (0.85% NaCl)	nt	nt	nt	nt
P. brassicacearum Am3RF	28±2 a	36±3 b	2.6±0.9 a	1.0±0.0 a
P. brassicacearum T8-1RF	30±3 ab	37±3 b	1.6±0.6 a	1.8±0.4 a
P. brassicacearum 520-1	35±2 ab	46±4 c	1.3±0.3 a	2.7±0.9 a
Pseudomonas sp. Dp2	nd	nd	nt	nt
V. paradoxus 5C-2	nd	nd	1.5±0.5 a	1.5 a
P. corrugata 176	nd	nd	1.7±0.4 a	1.5±0.3 a
Shoot FW (g^{-1} plant)				
Control (0.85% NaCl)	nd	99±8 a	32±1 a	33±5 a
P. brassicacearum Am3RF	nd	88±9 a	32±3 a	33±4 a
P. brassicacearum T8-1RF	nd	87±7 a	32±2 a	32±5 a
P. brassicacearum 520-1	nd	82±7 a	31±2 a	31±2 a
Pseudomonas sp. Dp2	nd	nd	31±2 a	34±2 a
V. paradoxus 5C-2	nd	nd	32±3 a	33±5 a
P. corrugata 176	nd	nd	32±2 a	33±4 a
Number of bacteria in the stem lesions (104 CFU mg^{-1} FW)				
Control (0.85% NaCl)*	nt	nt	nt	nt
P. brassicacearum Am3RF‡	4.0±0.2 a	4.3±0.8 a	2.7±0.8 ab	2.6±0.4 b
P. brassicacearum T8-1RF‡	4.2±0.1 a	4.9±0.5 a	3.8±0.5 a	2.8±0.5 ab
P. brassicacearum 520-1*	7.9±0.7 b	9.5±1.5 b	1.3±0.1 c	1.9±0.2 b
Pseudomonas sp. Dp2*	nd	nd	nt	nt
V. paradoxus 5C-2†	nd	nd	0.03±0.02 d	0.07±0.01 d
P. corrugata 176*	nd	nd	1.8±0.7 bc	0.9±0.3 c

[a] Number of infected plants per treatment examined in experiment 2 was six and nine at the 26th and 54th day after infection (DAI), respectively; eight plants per treatment were examined in experiment 3.

produced by the phytopathogenic bacteria, including pseudomonads, increase ACC accumulation and ethylene evolution in infected plants (Pegg and Cronshaw, 1976; Dutta and Biggs, 1991; Kenyon and Turner, 1992). The ethylene-insensitive tomato mutant Never ripe (Nr) showed significant reduction in disease symptoms in response to different pathogens including *P. syringae*, suggesting that stress ethylene evolution can promote disease development (Lund *et al.*, 1998). Analogously, the reduced ethylene biosynthesis in tomato plants transformed with bacterial ACC deaminase from *Enterobacter cloacae* UW4 decreased disease symptoms of *Verticillum* wilt (Robison *et al.*, 2001). In agreement with these observations that decreasing plant ethylene sensitivity or biosynthesis can limit disease development, it is shown here that ACC deaminase activity of the phytopathogenic bacterium *P. brassicacearum* Am3 counteracted its phytotoxic effects. This hypothesis relies on previous observations that rhizosphere inoculation with ACC deaminase-containing bacteria decreases root ACC levels and ethylene evolution (Burd *et al.*, 1998; Penrose *et al.*, 2001; Belimov *et al.*, 2002; Mayak *et al.*, 2004). Indeed, the ACC deaminase-deficient mutant T8-1 decreased root elongation and biomass production in comparison with the wild-type Am3. Furthermore, the chemical inhibitor of ethylene biosynthesis Co^{2+} partially alleviated *in vitro* growth inhibition caused by the T8-1 mutant, but not by wild-type Am3. In this context, it should be noted also that *P. brassicacearum* Am3 does not produce ethylene and auxins (Belimov *et al.*, 2001), although many pseudomonads produce ethylene or auxins that may promote infection (Weingart and Volksch, 1997) or inhibit root growth (Persello-Cartieaux *et al.*, 2001), respectively.

Stimulation of tomato root elongation at a low cell concentration (10^6 cells ml^{-1}) and an increase in root biomass observed in soil culture suggest that, under

certain growth conditions, the effect of ACC deaminase may overcome the toxic effects of *P. brassicacearum* Am3 on plants. By contrast, the effects of other PGPR (*Pseudomonas* sp. Dp2 and *V. paradoxus* 5C-2) were independent of bacterial concentration in the nutrient solution (Fig. 2). *Pseudomonas brassicacearum* Am3 was initially isolated as a PGPR that stimulated root elongation of Indian mustard and rape in the presence of a high cell concentration (5×10^7 cells ml^{-1}) and increased growth of the inoculated rape and pea plants in pot experiments (Belimov *et al*., 2001; Safronova *et al*., 2006). Here, experiments with tomato cultivar Ailsa Craig showed that a particular bacterial strain might exert either beneficial or deleterious effects on plant growth depending on plant species, concentration of bacteria, and growth conditions. This observation may be of crucial importance for investigations aimed at isolation, characterization, and further application of PGPR as biopreparations in agriculture that presupposes a large-scale release of bacteria to the environment.

Colonization of tomato roots and wound-infected stems by *P. brassicacearum* Am3 and its mutant T8-1 was similar, suggesting that (i) the difference between the effects of Am3 and T8-1 on plants was not related to the colonization ability of the strains, and (ii) lowering the ACC deaminase activity did not affect colonization and distribution of the bacteria associated with the plant. The latter observation suggests that the utilization of ACC as a nutrient substance gives the bacteria no advantages in colonization of rhizosphere or plant tissues. By contrast, it is likely that *P. brassicacearum* may use ACC deaminase as a tool for masking (swindling) their intention for infection via decreasing the stress induced by toxins and impeding plant recognition of an 'opportunistic' pathogen, since ethylene is a key signal intermediate in the expression of induced systemic resistance in plants (Van Loon *et al*., 1998). Interestingly, *E. cloacae* (recently reclassified as *P. putida* by Hontzeas *et al*., 2005) strain UW4, but not its ACC deaminase minus mutant UW4/AcdS$^-$, caused down-regulation of stress-related genes encoding glycine-rich RNA-binding protein and ras-GTPase-activating protein in canola roots (Hontzeas *et al*., 2004).

In conclusion, the interactions between plants and *Pseudomonas* are very variable, since this genus has plant growth-promoting, saprophytic, deleterious, and phytopathogenic species and strains, and in many cases it is difficult to discriminate between beneficial and injurious individuals (Preston, 2004). The present data reinforce this view, as a single strain (*P. brassicacearum* Am3) can have growth-promoting, neutral, or phytopathogenic effects on growth of a single plant cultivar according to the dose and environmental conditions. The outcome of the *P. brassicacearum*–tomato interaction is influenced by bacterial ACC deaminase activity, as decreasing this activity modified the root growth response of tomatoes to inoculation. Whether the coincidence of bacterial properties such as ACC degradation and phytopathogenicity is a casual circumstance or interdependent, and the role that ACC deaminase plays in pathogenesis require further study.

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