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Modulation of tomato root architecture and root hair traits by *Pseudomonas brassicacearum* and *Variovorax paradoxus* containing 1-aminocyclopropane-1-carboxylate deaminase

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Abstract

By decreasing root 1-aminocyclopropane-1-carboxylate (ACC) content and plant ethylene production, the microbial enzyme ACC deaminase is a widespread beneficial trait of plant growth-promoting rhizobacteria (PGPR), ameliorating ethylene-mediated root growth inhibition. However, relatively little is known about whether bacterial ACC deaminase modulates root architecture and root hair traits. Thus the dwarf tomato (*Solanum lycopersicum*) cultivar Micro-Tom was inoculated *in vitro* with *Pseudomonas brassicacearum* Am3, its ACC deaminase deficient mutant T8-1, a known PGPR strain *Variovorax paradoxus* 5C-2 or chemically treated with agents that promoted or inhibited ethylene production or sensitivity (Ag^+, Co^{2+}, and ACC). ACC treatment reduced both root elongation and the number of lateral roots, while ethylene inhibitors (Ag^+, Co^{2+}) and *V. paradoxus* 5C-2 promoted primary root elongation, but differentially affected lateral root length and number. Ag^+ stimulated lateral root development, while Co^{2+} and *V. paradoxus* 5C-2 did not. Inoculation with *P. brassicacearum* Am3 and T8-1 inhibited elongation of the primary and lateral roots at a high inoculum concentration (10^6 cells cm^3). All bacterial strains significantly increased the length and number of root hairs, with these effects more pronounced in *P. brassicacearum* Am3 than in the mutant T8-1. Treatment with Ag^+ inhibited root hair formation and elongation, while Co^{2+} had the opposite effects. ACC treatment had no effect on root hair elongation but increased root hair density. While root growth inhibition caused by *P. brassicacearum* Am3 was independent of ACC deaminase, the promotion of root hair elongation and density by this strain was augmented by ACC deaminase activity. Thus ACC deaminase can modulate the morphological impacts of bacteria on root hair response by affecting plant ethylene content.

Keywords: ACC deaminase, cobalt, ethylene, plant growth-promoting rhizobacteria, *Pseudomonas brassicacearum*, rhizosphere, silver, tomato, *Variovorax paradoxus*.

Introduction

Plant growth-promoting rhizobacteria (PGPR) influence root architecture and root hair formation (summarized in Table 1), generally increasing one or more parameters related to root and/or root hair length and number. Nevertheless, many studies often only measure a few parameters, and the mechanisms causing these effects may

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Abbreviations: ACC - 1-aminocyclopropane-1-carboxylate; GFP - green fluorescent protein; LSD - least significant difference; PGPR - plant growth-promoting rhizobacteria; WT - wild type.
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Table 1. Examples of rhizobacterial effects on plant root architecture and root hairs: + means positive effect; - means negative effect; = means no effect, and nd means not determined.

Bacterial strain	Host plant	Primary root length	Total lateral root length	Lateral root number	Mean lateral root length	Root hair length	Root hair density	Proposed mechanism of action	Reference
Azospirillum brasilense Cd	maize, wheat	+	+	nd	nd	+	+	plant growth promoting substances	Okon and Kapulnik 1986
Azospirillum brasilense Cd	tomato	+	nd	nd	nd	nd	+	unknown	Hadas and Okon 1987
Azospirillum brasilense Sp245	*Arabidopsis*	+	nd	nd	nd	+	nd	unknown	Dubrovsky *et al.* 1994
Azospirillum brasilense Sp245	*Arabidopsis*	-	nd	+	nd	nd	+	production of auxins	Spaepen *et al.* 2014
Azospirillum brasilense Sp7	soybean	+	+	+	+	+	+	unknown	Molla *et al.* 2001
Azospirillum brasilense FT326	tomato	=	nd	nd	nd	+	+	increase in plant IAA and ethylene	Ribaudo *et al.* 2006
Bacillus subtilis AP-3, PRBS-1	soybean	=	nd	+	nd	nd	+	production of IAA	Araújo *et al.* 2005
Mesorhizobium loti MAFF 303099	*Arabidopsis*	=	=	+	-	+	nd	ACC deaminase	Contesto *et al.* 2008
Ochrobactrum sp. NBRISH6	maize	+	nd	nd	nd		+	unknown	Mishra *et al.* 2020
Phyllobacterium brassicacearum STM196	*Arabidopsis*	=	+	=	nd	+	nd	ACC deaminase	Contesto *et al.* 2008
Phyllobacterium brassicacearum STM196	*Arabidopsis*	nd	nd	nd	nd	+	nd	modulation of plant ethylene pathway	Galland *et al.* 2012
Pseudomonas fluorescens WCS417	*Arabidopsis*	-	+	+	+	+	+	production of auxins	Zamioudis *et al.* 2013
Pseudomonas putida UW4	*Arabidopsis*	-	=	=	nd	+	nd	ACC deaminase	Contesto *et al.* 2008
Rhizobium leguminosarum bv. *viciae* 128C53K	*Arabidopsis*	-	=	=	nd	+	nd	ACC deaminase	Contesto *et al.* 2008
Sphingomonas sp. Cra20	*Arabidopsis*	nd	nd	+	nd	+	+	production of volatile organic compounds	Luo *et al.* 2019
Variovorax paradoxus 5C-2	maize	+	+	nd	nd	+	nd	ACC deaminase	Jin *et al.* 2021

be uncertain within divergent plant-microbe interactions (Table 1). PGPR of various taxonomic groups contain 1-aminocyclopropane-1-carboxylate (ACC) deaminase (reviewed in Dodd *et al.* 2010, Nascimento *et al.* 2014) and ameliorate plant growth inhibition caused by ethylene by decreasing root content of the ethylene precursor ACC (Penrose *et al.* 2001, Belimov *et al.* 2009) and thus ethylene production (Burd *et al.* 1998, Belimov *et al.* 2002, Mayak *et al.* 2004, Jin *et al.* 2021). Ethylene plays important role in root development and root hair formation (Tanimoto *et al.* 1995, Bibikova and Gilroy 2002, Vandenbussche *et al.* 2012, Zhang *et al.* 2016, Vissenberg *et al.* 2020). It was shown that bacterial ACC deaminase is involved in mediating root and root hair development and root morphology. For example, *Pseudomonas putida* UW4 increased primary root elongation of canola, whereas its ACC deaminase minus mutant lost this ability (Li *et al.* 2000), and both wild-type and the same ACC deaminase minus mutant inhibited primary root length of *Arabidopsis* similarly (Contesto *et al.* 2008). While the ACC-deaminase minus mutants of multiple PGPR strains promoted root hair elongation of *Arabidopsis* (Contesto *et al.* 2008), the ACC-utilizing *Variovorax paradoxus* 5C-2 promoted root hair formation and root density of maize (Jin *et al.* 2021). However, root hair formation is regulated by complex metabolic networks and strongly depends on environmental factors (Bibikova and Gilroy 2002, Vissenberg *et al.* 2020), making it difficult to generalize how PGPR might affect root architecture, especially since they can produce or metabolise multiple plant hormones that regulate root responses (Dodd *et al.* 2010, Vacheron *et al.* 2013).

The ACC deaminase-containing *Pseudomonas brassicacearum* Am3 was initially characterized as a PGPR for pea, canola, and Indian mustard, for which it increased root elongation and/or biomass (Belimov *et al.* 2001, Safronova *et al.* 2006). Later, *P. brassicacearum* Am3 was found to exert positive effects on primary root elongation of seedlings *in vitro* and on root biomass of tomato cultivar Ailsa-Craig cultivated in soil supplemented with this strain (Belimov *et al.* 2007). However, root growth inhibition at high bacterial cell content *in vitro* and lesions in the inoculated stems and fruits of tomato were also observed, suggesting this strain can be deleterious or even

pathogenic for this plant species (Belimov *et al.* 2007). Moreover, the tomato phytopathogen *P. brassicacearum* 520-1 (Sikorski *et al.* 2001) also possessed very high ACC deaminase activity (Belimov *et al.* 2007) while ACC deaminase of *P. brassicacearum* Am3 promoted primary root growth of tomato (cv. Ailsa-Craig), to some extent masking its deleterious properties (Belimov *et al.* 2007). As pseudomonads colonise various parts of the root system, including the root hairs (Beauchamp and Kloepper 2003, Gamalero *et al.* 2005), variation in colonisation pattern may alter different root morphological variables.

Other deleterious and phytopathogenic bacteria also affect root architecture and root hairs. Inoculating *Petunia hybrida* seedlings with *Ralstonia solanacearum* strains inhibited lateral root elongation, caused swelling of the root tips and increased root hair length and number (Zolobowska and Van Gijsegem 2006). *Pseudomonas syringae* pv. *maculicola* 795 or *P. syringae* pv. *tomato* DC3000 stimulated root hair elongation and initiation of wild-type *Arabidopsis* but not of the N8844 (At5g03280, *ein2*) mutant affected in ethylene perception, suggesting that ethylene signaling is involved in this phenomenon (Pecenkova *et al.* 2017). However, the presence of ACC deaminase in these bacteria was not studied and such limited information makes it difficult to understand the role of bacterial ACC deaminase in plant-bacteria interactions.

We compared the effects of two contrasting ACC-utilizing rhizobacteria *V. paradoxus* 5C-2 (a known PGPR) and *P. brassicacearum* Am3 (which has both plant growth-promoting and deleterious traits) on root and root hair formation in tomato seedlings. To explore the role of ACC deaminase in this interaction, we utilised a transposon mutant (*P. brassicacearum* T8-1) with low ACC deaminase activity (Belimov *et al.* 2007) and chemical modulators of plant hormone ethylene. Particular attention was focused on understanding how bacteria may use a PGPR trait such as ACC deaminase activity to alter root growth by modulating plant ethylene status. We hypothesised that deleterious bacterial traits inhibiting primary and lateral root elongation may promote compensatory root hair responses.

Materials and methods

Bacteria and plants: Strain *Pseudomonas brassicacearum* Am3 resistant to 20 μg cm^{-3} rifampicin, its ACC deaminase deficient mutant T8-1 also resistant to 40 μg cm^{-3} kanamycin (Belimov *et al.* 2007) and strain *Variovorax paradoxus* 5C-2 resistant to 40 μg cm^{-3} kanamycin and 20 μg cm^{-3} rifampicin (Spaepen *et al.* 2014), were obtained from the Russian Collection of Agricultural Microorganisms (RCAM, Saint-Petersburg, http://www.arriam.ru/kollekciya-kul-tur1/). Strains Am3 and T8-1 were tagged previously with a green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* (Belimov *et al.* 2007) using the strain *Escherichia coli* S17-1::pAG408 kindly provided by Dr. T. Charles (University of Waterloo, Waterloo, Canada). Seeds of tomato (*Solanum lycopersicum* Mill.) cultivar Micro-Tom were obtained from *Moles Seeds* (Colchester, Essex, UK), and utilised as their dwarf stature allowed growth in a relatively small environment (Petri dishes).

Agar culture: Petri dishes (d = 150 mm) were filled with 100 cm^3 of the sterile nutrient solution solidified with 0.7 % plant cell culture tested agar (*Sigma-Aldrich*, St. Louis, USA) and containing [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 $NiCl_2$, and 0.01 KJ. The nutrient solution was supplemented either with 1 μM Ag_2SO_4 (antagonist of ethylene perception), 2 μM $CoCl_2$ (inhibitor of ethylene biosynthesis) or 10 μM ACC, selected based on preliminary dose-response curves with each reagent, as used in our previous work (Belimov *et al.* 2007). The bacteria were grown for 48 h at 28 °C on *Bacto-Pseudomonas F* (BPF) agar supplemented with antibiotics. Cells were collected, resuspended to 10^8 cells per cm^3 in sterile tap water and added to the nutrient solution just before solidification in a final concentration of 10^5 or 10^6 cells per cm^3.

Tomato seeds were surface-sterilized with 5 % sodium hypochlorite solution for 15 min, washed carefully with sterile water and placed on sterile moistened filter paper (*Whatman #1*) in Petri dishes (d = 90 mm). 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. Four germinated seeds per each Petri dish (d = 150 mm) with nutrient agar were placed 2 cm from each other in a line located 3.5 cm from the box edges. The Petri dishes were installed at 45 degrees to the horizontal to allow the roots to grow under the agar layer and incubated for 10 d in a greenhouse at a 16-h photoperiod, day/night temperatures of 16/25 °C, and a photon flux density of 400 μmol(quanta) m^{-2} s^{-1}. All plants (all treatments) of each experiment were grown simultaneously in the same greenhouse near to each other, to ensure similar irradiance and temperature for all treatments. The closed dishes were sealed ¾ with *PARAFILM* tape and the upper ¼ was sealed with micropore surgical tape to minimise impacts on plant gas exchange. During incubation, dish positions were swapped daily and the greenhouse was ventilated making the atmosphere uniform. After incubation, the Petri dishes were scanned and the obtained images were used to determine the lateral root number and the primary and lateral root length. The assay was repeated three times with three dishes for each treatment, each containing four seedlings, resulting in 12 replicates.

Microscopy: The primary root segments located 13 to 16 cm from the root tip (where root hairs were fully developed) and lateral root segments located 0.5 to 1.5 cm from the primary root were used for microscopic observations. Images of intact root hairs located on the primary root were taken using a light microscope (*Axio Scope.A1*, *Carl Zeiss*, Oberkochen, Germany). These images were printed (20 to 36 images per treatment) and root hair length and number on the primary root were measured and expressed as mean values for each image. The presence and distribution of GFP-tagged bacteria on roots and root hairs taken from agar cultures were

monitored using a laser scanning confocal microscope (*LEICA SP2A0BS*, *Leica*, Wetzlar, Germany). Root segments were carefully taken along with the agar layer, immediately placed between microscope slides and cover slips and microphotographs of various colonization patterns of bacteria on roots and root hairs were taken.

Enumeration of bacteria on roots: After microscopy observation, the primary root segments located 3 to 7 cm from the seeds of each Petri dish were carefully removed from the medium and homogenized in sterile tap water with sterile mortar and pestle as previously described (Belimov *et al.* 2007). The homogenates were serially diluted in 10-fold steps and 50 mm^3 aliquots were plated in two replicates on BPF agar supplemented with required amounts of antibiotics. No antibiotics were added to check the contamination of samples with extraneous microorganisms. Colony forming units (CFU) were counted after incubating plates at 25 °C for 4 d.

Statistical analysis: Statistical analysis of the data was performed using the software *STATISTICA v. 10* (*TIBCO Software*, Palo-Alto, CA, USA). MANOVA analysis with Fisher's LSD test and Student's t-test were used to evaluate differences between means. SE and LSD stand for standard error and Fisher's least significant difference test, respectively.

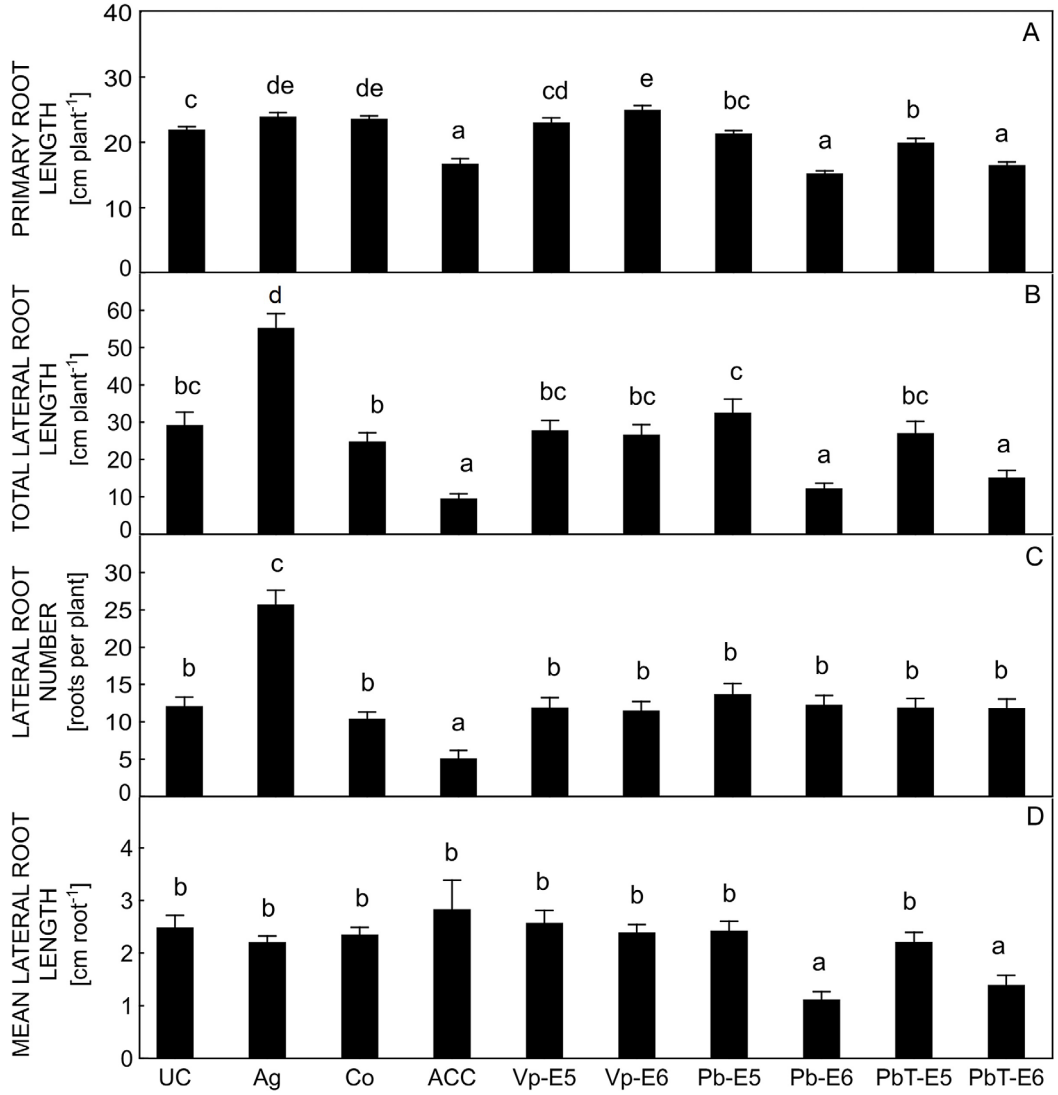

Fig. 1. Effect of chemical ethylene modulators and rhizobacteria on tomato root architecture. Treatments: UC - uninoculated control; Ag - 1 µM Ag_2SO_4; Co - 2 µM $CoCl_2$; ACC - 10 µM ACC; Vp-E5 - *V. paradoxus* 5C-2 (10^5 cells cm^{-3}); Vp-E6 - *V. paradoxus* 5C-2 10^6 cells cm^{-3}); Pb-E5 - *P. brassicacearum* Am3 (10^5 cells cm^{-3}); Pb-E6 - *P. brassicacearum* Am3 (10^6 cells cm^{-3}); PbT-E5 - *P. brassicacearum* T8-1 (10^5 cells cm^{-3}); PbT-E6 - *P. brassicacearum* T8-1 (10^6 cells cm^{-3}). Means ± SEs of three experiments. Different letters indicate significant differences between treatments (Fisher's LSD test, $P < 0.05$, n varied from 20 to 36 depending on parameter and treatment).

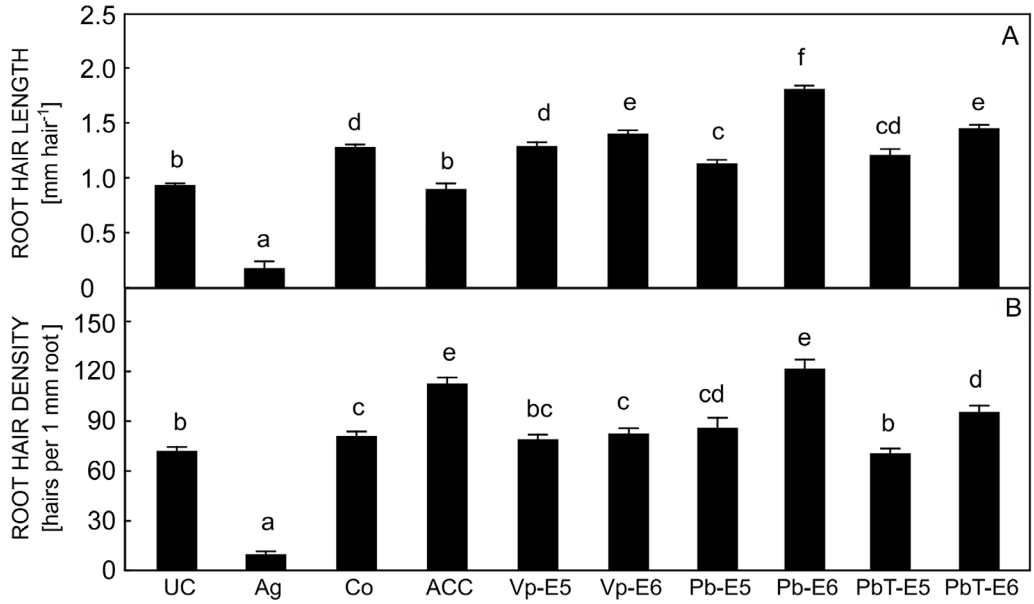

Fig. 2. Effect of chemical ethylene modulators and rhizobacteria on tomato root hair traits. Treatments: UC - uninoculated control; Ag - 1 µM Ag_2SO_4; Co - 2 µM $CoCl_2$; ACC - 10 µM ACC; Vp-E5 - *V. paradoxus* 5C-2 (10^5 cells cm^{-3}); Vp-E6 - *V. paradoxus* 5C-2 (10^6 cells cm^{-3}); Pb-E5 - *P. brassicacearum* Am3 (10^5 cells cm^{-3}); Pb-E6 - *P. brassicacearum* Am3 (10^6 cells cm^{-3}); PbT-E5 - *P. brassicacearum* T8-1 (10^5 cells cm^{-3}); PbT-E6 - *P. brassicacearum* T8-1 (10^6 cells cm^{-3}). Means ± SEs of three experiments. Different letters indicate significant differences between treatments (Fisher's LSD test, $P < 0.05$, n varied from 20 to 36 depending on parameter and treatment).

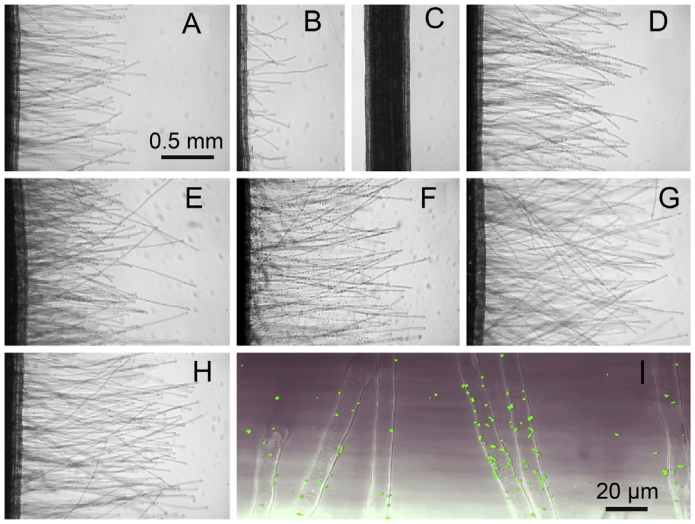

Fig. 3. Images showing typical effects of chemical ethylene modulators and rhizobacteria on tomato root hairs. Treatments: *A* - uninoculated control; *B* and *C* - 1 µM Ag_2SO_4; *D* - 2 µM $CoCl_2$; *E* - 10 µM ACC; *F* - *V. paradoxus* 5C-2 (10^6 cells cm^{-3}); *G* - *P. brassicacearum* Am3 (10^6 cells cm^{-3}); *H* - *P. brassicacearum* T8-1 (10^6 cells cm^{-3}); *I* - green cells of GFP-tagged *P. brassicacearum* Am3 on root hairs. Two roots (*B* and *C*) given for Ag_2SO_4 treatment demonstrate presence and absence of root hairs.

Results

The introduction of the bacteria to the agar did not result in visual disease or damage symptoms on the roots (data not shown). Adding 10^5 cells cm^{-3} of rhizobacteria had no effects on the primary root length (Fig. 1*A*), total lateral root length (Fig. 1*B*), the number of lateral roots (Fig. 1*C*) and the mean lateral root length (Fig. 1*D*), except that the ACC deaminase deficient *P. brassicacearum* T8-1 decreased primary root length by 10 % (Fig. 1*A*). Adding 10^6 cells cm^{-3} of *V. paradoxus* 5C-2 stimulated primary root elongation by 14 %, while the same concentrations of *P. brassicacearum* Am3 and T8-1 were inhibitory (Fig. 1*A*). While *V. paradoxus* 5C-2 had no effect on elongation of lateral roots, *P. brassicacearum* Am3 and T8-1 approximately halved their elongation (Fig. 1*B*,*D*). Lateral root number was not affected by any bacterial treatment, or by adding Co^{2+} ions (Fig. 1*C*). Treatment with Ag^+ ions increased the primary root length (Fig. 1*A*) and total lateral root length (Fig. 1*B*) by increasing the number of lateral roots (Fig. 1*C*). Treatment with Co^{2+} ions also increased the primary root length (Fig. 1*A*), but did not affect the

Table 2. Number of the introduced bacteria on tomato roots at the end of experiments. Means ± SEs of three experiments with 2 replicas each. Different letters indicate significant differences between treatments (Student *t*-test, $P < 0.01$, $n = 6$).

Treatments	Bacteria number [10^4 CFU mg^{-1} (root f.m.)]
V. paradoxus 5C-2 (10^5 cells cm^{-3})	13.5 ± 3.3 c
V. paradoxus 5C-2 (10^6 cells cm^{-3})	29.1 ± 7.1 d
P. brassicacearum Am3 (10^5 cells cm^{-3})	1.1 ± 0.2 a
P. brassicacearum Am3 (10^6 cells cm^{-3})	5.1 ± 0.7 b
P. brassicacearum T8-1 (10^5 cells cm^{-3})	1.1 ± 0.2 a
P. brassicacearum T8-1 (10^6 cells cm^{-3})	6.7 ± 1.7 b

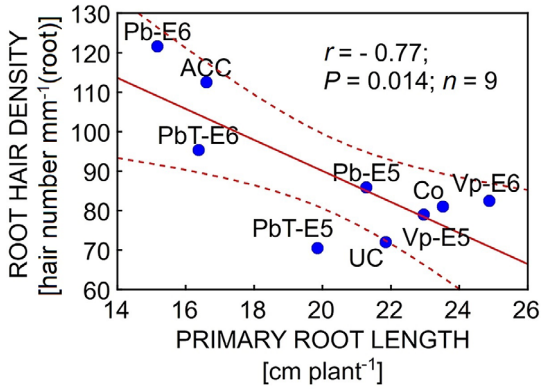

Fig. 4. Scatterplot showing correlation between the primary root length and root hair density on primary roots (Ag treatment was omitted). Mean values are shown by *blue symbols*. Treatments: UC - uninoculated control; Co - 2 µM $CoCl_2$; ACC - 10 µM ACC; Vp-E5 - *V. paradoxus* 5C-2 (10^5 cells cm^{-3}); Vp-E6 - *V. paradoxus* 5C-2 (10^6 cells cm^{-3}); Pb-E5 - *P. brassicacearum* Am3 (10^5 cells cm^{-3}); Pb-E6 - *P. brassicacearum* Am3 (10^6 cells cm^{-3}); PbT-E5 - *P. brassicacearum* T8-1 (10^5 cells cm^{-3}); PbT-E6 - *P. brassicacearum* T8-1 (10^6 cells cm^{-3}). *Red line* shows linear regression. *Red dotted lines* show confidence level ($P = 0.05$) of the regression.

other parameters measured. Contrary to the ethylene inhibitors, treating the plants with ACC decreased primary root length (Fig. 1*A*), total lateral root length (Fig. 1*B*) and lateral root number (Fig. 1*C*). Thus, high concentrations of *P. brassicacearum* inhibited primary and lateral root elongation independent of its ACC deaminase activity, but had no effect on lateral root initiation.

All bacterial strains stimulated root hair elongation at 10^5 cells cm^{-3} (by 22 - 39 %) and even more so (by 50 - 95 %) at 10^6 cells cm^{-3} (Fig. 2*A*). At 10^5 cells cm^{-3}, both wild-type (WT) *P. brassicacearum* Am3 and the ACC deaminase mutant T8-1 had similar effects on root hair elongation, but a higher concentration of *P. brassicacearum* Am3 increased root hair elongation 25 % more than the T8-1 mutant. While ACC treatment had no significant effect on root hair elongation, treatment with Co^{2+} ions increased root hair elongation by 38 %, and treatment with Ag^+ ions decreased root hair elongation by five times (Fig. 2*A*). Although inhibiting ethylene perception (Ag^+ ions) and ACC synthesis (Co^{2+} ions) had opposing effects on root hair elongation, all bacterial strains stimulated root hair elongation, with a diminished response of *P. brassicacearum* T8-1 at high bacterial concentrations owing to its low ACC deaminase activity (Fig. 2*A*).

All three bacterial strains increased the number of root hairs after inoculation with 10^6 cells cm^{-3}, whereas *P. brassicacearum* Am3 increased the number of root hairs also in the presence of 10^5 cells cm^{-3} (Fig. 2*B*). At both bacterial concentrations, the number of root hairs of plants inoculated with *P. brassicacearum* Am3 exceeded those inoculated with T8-1 mutant. Treatment with Ag^+ ions dramatically inhibited root hair number, while Co^{2+} ions and ACC had the opposite effect (Fig. 2*B*), with root hair density of ACC-treated plants 43 % greater than Co^{2+}-treated plants. Typical root hairs of plants treated with ethylene modulating compounds (Fig. 3*B-D*), ACC (Fig. 3*E*), or inoculated with rhizobacteria at 10^6 cells cm^{-3} (Fig. 3*F-H*) are presented. Ag^+ treatment dramatically inhibited (Fig. 3*B*) or sometimes completely prevented (Fig. 3*C*) root hair formation, whereas ACC stimulated elongation of only some single hairs (Fig. 3*E*). While the ACC deaminase activity of *P. brassicacearum* had no effect on root hair length at 10^5 cells cm^{-3}, otherwise the higher ACC deaminase activity of *P. brassicacearum* Am3 enhanced root hair length and number compared to *P. brassicacearum* T8-1.

Inoculating the roots with a higher bacterial concentration (10^6 cells cm^{-3}) increased the bacterial number on tomato roots several-fold (Table 2). The number of *V. paradoxus* 5C-2 was about 5 - 10 times higher than *P. brassicacearum* Am3 and T8-1, but no difference was found between the latter two strains. Confocal microscopy observations revealed the GFP-tagged bacteria *P. brassicacearum* Am3 and T8-1 on root surfaces as single cells and small clumps of cells. The bacteria mostly actively colonized the surface of the primary root and root hairs located on the primary root, as illustrated for *P. brassicacearum* Am3 (Fig. 3*I*). Some bacteria were also located in the agar at some distance from the plants. However, no bacterial cells of *P. brassicacearum* Am3 and T8-1 were observed inside the roots or root hairs. Thus, bacteria colonised the root surface.

Discussion

Bacterial ACC deaminase is a well-known trait of PGPR that promotes root elongation (Glick *et al.* 1994, Belimov *et al.* 2007), but its occurrence in strains such as *P. brassicacearum* suggests that caution must be applied when isolating novel ACC-utilizing organisms to develop microbial inocula. Although low concentrations of ACC-utilizing *P. brassicacearum* Am3 promoted elongation of tomato (cv. Ailsa-Craig) primary roots grown on filter paper, higher concentrations inhibited root growth, as did the recognised ACC-utilizing phytopathogen *P. brassicacearum* 520-1 (Belimov *et al.* 2007). Comparing root growth responses to inoculation with WT *P. brassicacearum* Am3 and its ACC deaminase deficient mutant T8-1 demonstrated that ACC deaminase activity did

not alter tomato root architecture (Fig. 1), as in *Arabidopsis* roots inoculated with a range of WT bacteria and their respective ACC deaminase mutants (Contesto *et al.* 2008). Whereas these mutants consistently promoted *Arabidopsis* root hair elongation to a greater extent than their respective WTs (Contesto *et al.* 2008), here *P. brassicacearum* T8-1 inhibited root hair elongation and density compared to its WT, which promoted root hair elongation and density compared to uninoculated controls. Thus inhibition of primary and lateral root elongation by *P. brassicacearum* was to some extent compensated by promotion of root hair length and density, with ACC deaminase activity augmenting these root hair traits. Bacterial mediation of root surface area available for colonisation possibly is of adaptive significance, highlighting the complexity of plant interactions with ACC-utilizing bacteria, which may depend on plant genotype.

Whereas a typical tall indeterminate tomato cultivar (cv. Ailsa Craig) was previously used to determine the *P. brassicacearum* Am3 effect on tomato primary root elongation (Belimov *et al.* 2007), here a dwarf semi-determinate tomato (cv. Micro-Tom) allowed roots and root hairs to develop fully. Its dwarf stature allows genetic studies as many fruiting plants can be grown within controlled environment facilities of limited space (Meissner *et al.* 1997), but its multiple mutations (the self-pruning and dwarf genes and another that decreases internode length independently of active gibberellin content - Marti *et al.* 2006) might affect its plant-microbe interactions. Its brassinosteroid deficiency may actually enhance the detection of phenotypes mediated by bacterial ACC deaminase. Synthetic brassinosteroid treatment attenuated salt-induced ethylene synthesis of lettuce (Serna *et al.* 2015) while a brassinosteroid-deficient pea mutant produced high ethylene content (Ross and Reid 1986), although it is not known whether such tomato mutants behave similarly. The ethylene overproducing *Arabidopsis eto1-1* mutant produced a greater vegetative response (leaf area and shoot fresh mass) than WT *Arabidopsis* following inoculation with the ACC-utilizing PGPR *V. paradoxus* 5C-2 (Chen *et al.* 2013). Furthermore, different tomato genotypes can show contrasting physiological responses to inoculation with ACC-utilising PGPR (Calvo-Polanco *et al.* 2016). Irrespective of these potential genotype × microbe interactions, Micro-Tom showed typical root responses to ethylene inhibitor Ag^+ ions (that antagonize ethylene perception) stimulating primary root elongation (Fig. 1*A*) and the emergence of lateral roots (Fig. 1*C*), thus doubling total lateral root length (Fig. 1*B*). In contrast, Co^{2+} treatment (that inhibits ethylene biosynthesis) increased primary root length (Fig. 1*A*) but had no effect on total lateral root length while ACC treatment decreased both these variables (Fig. 1*A*,*B*). Similarly, Ag^+ and Co^{2+} ions stimulated primary root elongation of cv. Ailsa Craig (Belimov *et al.* 2007), indicating that ethylene inhibits root elongation of both tall and dwarf tomato cultivars. Ag^+ ions have multiple effects on plant growth and physiological and biochemical characteristics, varying from positive to negative depending on plant species and concentrations (Siddiqi and Husen 2021). Particularly, treating tomato seedlings with 100 - 300 µM $AgNO_3$ induced oxidative stress and increased expression of ethylene-inducing xylanase (Noori *et al.* 2019). Similarly to Ag^+ ions, Co^{2+} ions could be beneficial (essential micronutrient) or toxic (heavy metal in contaminated soils) for plants (Akeel and Jahan 2020, Banerjee and Roychoudhury 2021). Tomato seedling growth inhibition caused by 200 µM $CoSO_4$ was accompanied by increased peroxidase activity (Gopal *et al.* 2003). It can be assumed that the effect of these metals on tomato plants in our experiments could be associated not only with the modulation of ethylene but also with other metabolic processes.

Strain *V. paradoxus* 5C-2 promoted primary root elongation of both tall (Belimov *et al.* 2007) and dwarf (Fig. 1) tomato cultivars. Involvement of bacterial ACC deaminase in promoting primary root elongation of canola seedlings was first documented using the ACC deaminase deficient mutant of the PGPR *Pseudomonas putida* UW4 (Glick *et al.* 1994), and also by comparing tomato (cv. Ailsa Craig) seedlings inoculated with *P. brassicacearum* Am3 and its T8-1 mutant (Belimov *et al.* 2007). In contrast, other ACC-utilizing PGPR had no (*Phyllobacterium brassicacearum* STM196, *Rhizobium leguminosarum* bv. *viciae* 128C53K, *Mesorhizobium loti* MAFF303099) or an inhibitory (*P. putida* UW4) effect on *Arabidopsis* root elongation *in vitro*, with variation in ACC deaminase activity not affecting responses to these PGPR (Contesto *et al.* 2008). That different plant species vary in their root responses to a single PGPR (*P. putida* UW4) suggests commercial opportunities to develop bespoke microbial inocula for specific crops, even if microbial ACC deaminase is almost universally regarded as a growth-promoting trait (Nascimento *et al.* 2018), albeit with modest effects in certain environmental conditions (Contesto *et al.* 2008).

Varying the microbial load on the root surface can profoundly influence plant responses to PGPR. Whereas the positive effect of *V. paradoxus* 5C-2 on tomato (Ailsa Craig) primary root elongation was concentration independent (Belimov *et al.* 2007) across 3 orders of magnitude (10^6 - 10^8 cells cm^{-3} applied), here the highest bacteria concentration (10^6 cells cm^{-3}) highly promoted Micro-Tom primary root elongation, possibly because more bacteria on the roots (Table 2) increased ACC consumption. In contrast, high bacterial loads exacerbated the negative effects of *P. brassicacearum* Am3 and T8-1 on root length, when tomato Ailsa Craig seedlings were grown on filter paper (Belimov *et al.* 2007). Various *Pseudomonas* species produce phytotoxins inhibiting root growth (Bender *et al.* 1999, Adeniji *et al.* 2020) causing ACC and ethylene accumulation in infected plants (Pegg and Cronshaw 1976, Kenyon and Turner 1992), and this mechanism likely applied in our experiments, since *P. brassicacearum* Am3 was deleterious for tomato. Although *P. brassicacearum* Am3 did not produce ethylene (Belimov *et al.* 2001), it might produce some compounds that mimic ethylene, inducing its biosynthesis or increasing plant sensitivity to ethylene. Inoculating stems and fruits of tomato with *P. brassicacearum* Am3 produced disease symptoms such as lesions (Belimov *et al.* 2007), but these

symptoms were not found on tomato seedlings grown on agar *in vitro* as the bacteria did not invade the roots. Interestingly, ACC deaminase modulates root growth inhibition, as the ACC deaminase deficient T8-1 mutant, but not the WT Am3, significantly inhibited primary root elongation at a low (10^5 cells cm^{-3}) bacterial load (Fig. 1*A*). Similarly, higher concentrations of the ACC deaminase deficient T8-1 inhibited primary root growth to a greater extent than Am3 (Belimov *et al.* 2007), indicating that bacterial ACC deaminase can partially alleviate the effects of pseudomonad toxins. We propose that two opposite processes occur in inoculated plants: deleterious bacterial properties cause root ACC and ethylene accumulation while ACC deaminase allows bacteria to utilize more ACC as a nutrient, thereby modulating plant ethylene status.

While the concentration of *P. brassicacearum* applied determined the impacts of ACC deaminase activity on primary root elongation (Fig. 1*A*), its effects on lateral root length and number were independent of ACC deaminase activity. *P. brassicacearum* Am3 and T8-1 both inhibited elongation of lateral roots (Fig. 1*B*) when applied at 10^6 cells cm^{-3}, but not their initiation (Fig. 1*C*). An ACC deaminase deficient mutant of *P. putida* UW4 stimulated *Arabidopsis* lateral root number in contrast to its WT, while both strains promoted total lateral root length (Contesto *et al.* 2008). Lateral roots appear later in development, and fewer *P. brassicacearum* Am3 and T8-1 cells were observed on lateral roots than on the primary root, probably explaining the absence of bacterial effects in our experiments.

Nevertheless, the roots and root hairs show divergent responses to ethylene, and possibly also ACC-utilizing bacteria. Many ACC-utilizing bacteria promoted *Arabidopsis* root hair length, even in the ethylene-insensitive *ein2-1* mutant, with a greater response to inoculation with ACC deaminase deficient mutants than WT bacteria (Contesto *et al.* 2008). This agrees with reports that root hair formation in *Arabidopsis* requires ethylene or ACC treatment (Tanimoto *et al.* 1995, He *et al.* 2005), and that blocking ethylene perception with Ag^+ ions significantly inhibited root hair length and number (Fig. 2). However, Co^{2+} treatment along with ACC increased root hair length and number, suggesting that ethylene finely regulates root hair formation, depending on root ACC biosynthesis (Angulo *et al.* 2021). Although Ag^+ inhibits ethylene perception and Co^{2+} inhibits ethylene biosynthesis, little is known about their effects on root hair formation (Romera and Alcántara 2004). Their opposing effects on root hair phenotypes could be explained by the relative importance of ethylene biosynthesis and tissue sensitivity to ethylene. Root hair formation (especially) seems to require ethylene-sensitive tissue, as Ag^+ substantially decreased root hair density. Root hair growth depended on ethylene biosynthesis, possibly by stimulating auxin signaling and potentially modulating auxin transport (Zhang *et al.* 2016). Alternatively, the opposing effects of Co^{2+} and Ag^+ on root hair formation might result from complex effects of Co^{2+} acting as a micronutrient (at low concentrations) and a toxic heavy metal (at elevated concentrations). Although Co inhibits the conversion of ACC to ethylene causing accumulation of ACC (Yu and Yang 1979), micromolar Co^{2+} concentrations could be toxic for various plant species (Liu *et al.* 2000, Waters *et al.* 2007, Lwalaba *et al.* 2017) including tomato (Gopal *et al.* 2003), leading to stress ethylene biosynthesis. As an essential micronutrient, Co^{2+} can be easily translocated into shoots and included in plant enzymes and other compounds (Akeel and Jahan 2020, Banerjee and Roychoudhury 2021), thereby both modulating plant metabolism and decreasing its concentrations in the rhizosphere and roots. Thus the effects of Co^{2+} might vary with time in our experiments. Initially, it acted as an ethylene inhibitor by stimulating primary root elongation but simultaneously causing ACC to accumulate in root tissues. Subsequent Co^{2+} uptake and translocation likely enhanced its phytotoxicity thereby stimulating root hair formation (much later in root development) by an ethylene-dependent mechanism. More detailed measurements of the temporal dynamics of root Co^{2+} accumulation and ethylene emission are needed to test this hypothesis. Nevertheless, caution should be applied in using Co^{2+} as an ethylene inhibitor to study root hairs.

Strain *V. paradoxus* 5C-2 decreased rhizosphere ACC content of potato (Belimov *et al.* 2015) and xylem sap ACC content of pea (Belimov *et al.* 2009), suggesting lower root ACC content. Inoculation with *V. paradoxus* 5C-2 increased root hair length at both bacterial concentrations and root hair number at 10^6 cells cm^{-3}, similar to the effects of Co^{2+} treatment (Fig. 2). *P. brassicacearum* Am3 and its ACC deaminase deficient mutant T8-1 also increased root hair length and number, especially at 10^6 cells cm^{-3} (Fig. 2). Nevertheless, the higher concentration of T8-1 had less effect, even though *P. brassicacearum* Am3 and T8-1 colonised the root hairs similarly (estimated visually by confocal microscopy), as in stem lesions and on roots of cv. Ailsa-Craig inoculated with *P. brassicacearum* Am3 and T8-1 (Belimov *et al.* 2007). Thus, ACC deaminase of *P. brassicacearum* seems involved in root hair responses to inoculation. Similarly, the ACC-utilizing PGPR *Phyllobacterium brassicacearum* STM196 linearly enhanced root hair elongation of *Arabidopsis* as inoculum concentration increased without affecting ethylene production (Zhang *et al.* 2016), suggesting bacterial mediation of ethylene signaling pathways. Other PGPR such as *A. brasilense* (Egorenkova *et al.* 2000), *Bacillus subtilis* (Yi *et al.* 2018, Hernández *et al.* 2020) and *Paenibacillus polymyxa* (Egorenkova *et al.* 2013) also actively colonized tomato root hairs. However, phytopathogenic bacteria like *Pseudomonas syringae* can induce defence reactions in *Arabidopsis* root hairs hindering colonization (Rodriguez-Furlán *et al.* 2016), while other strains of the same organism stimulated *Arabidopsis* root hair growth (Pecenkova *et al.* 2017), conditional on functional ethylene signaling within the host. Thus, both the studied strains can stimulate root hair growth, with bacterial ACC deaminase inhibiting this response in *Arabidopsis* (Contesto *et al.* 2008) but promoting tomato root hair response to *P. brassicacearum* (Fig. 2).

Alternatively, PGPR may stimulate root hair formation by producing auxins (Araújo *et al.* 2005, Spaepen *et al.* 2014). Inoculating *Arabidopsis* plants with the auxin-

producing *Pseudomonas fluorescens* WCS417 inhibited primary root growth, but stimulated the formation of lateral roots and root hairs, with these root hair responses requiring functional auxin signalling (Zamioudis *et al.* 2013). *V. paradoxus* 5C-2 (Belimov *et al.* 2005), but not *P. brassicacearum* Am3 (Belimov *et al.* 2001) produced auxins. *In vitro* ACC deaminase activity of the latter strain was 25 % higher (Hontzeas *et al.* 2005), while the number of *V. paradoxus* 5C-2 on the roots was about 10 times more than *P. brassicacearum* Am3 and T8-1 (Table 2). Nevertheless, the qualitatively similar responses of root hair length and density to *V. paradoxus* 5C-2 and *P. brassicacearum* Am3 inoculation suggests that microbial ACC deaminase activity may be more influential in regulating root hair traits than auxin production, at least in tomato. A possibility for feedback regulation of ethylene biosynthesis as a response to ACC deaminase activity should also be considered (Vandenbussche *et al.* 2012).

Two mechanisms were suggested to increase specific root hair number in maize and wheat following *A. brasilense* inoculation: *1*) stimulation of differentiation of cells into root hairs independently of root length effects and *2*) shortening of the root by reducing cell elongation (Okon and Kapulnik 1986). Across all treatments (excluding the Ag^+ treatment as there were so few root hairs), root hair density was significantly (r = -0.77; P = 0.014; n = 9) inversely correlated with primary root length (Fig. 4), thus faster growing roots had lower root hair density due to greater cell expansion. This correlation remains significant even when including the Ag^+ treatment (r = -0.68; P = 0.032; n = 10). The first mechanism is proposed to apply to both *V. paradoxus* 5C-2 (which increased both the primary root length and root hair number by 14 %) and *P. brassicacearum* Am3 (which decreased the primary root length by 31 % but increased root hair number by 68 %). Greater root hair density following *P. brassicacearum* Am3, than T8-1, inoculation suggests ACC deaminase involvement in this process independently of microbial effects on primary root elongation.

Although bacterial ACC deaminase of *P. brassicacearum* Am3 did not affect overall root colonization (Table 2) and probably also root hair colonization patterns, its impacts on root hair length and density might alter overall rhizobacterial colonization of the root system, and also affect chemical and physical processes in the soil. Root hairs strongly contribute to forming the rhizosheath (soil adhesion to the root) along with root exudation, with root hairless mutants forming a limited rhizosheath (Burak *et al.* 2021). Rhizosheath development was positively correlated with root hair length in both WT and ABA-deficient tomato genotypes grown in drying soil, and with root hair density in well-watered plants (Karanja *et al.* 2021). Enhanced rhizosheath development provides a niche for bacterial proliferation that stimulates chemical and biological processes (Mahmood *et al.* 2014), and also minimizes the risk of soil detachment in response to simulated precipitation (De Baets *et al.* 2020). Inoculating rice with the PGPR *Enterobacter aerogenes* G3 and its knockout ACC deaminase$^-$ mutant demonstrated an important role of ACC deaminase in rhizosheath formation (Zhang *et al.* 2020). Beyond its deleterious effects, by behaving as a saprophyte or PGPR (Belimov *et al.* 2001, Safronova *et al.* 2006), *P. brassicacearum* may also positively affect rhizosheath formation, indicating the complexity of rhizosphere processes mediated by microorganisms.

Conclusions

Different ACC-utilising bacteria had contrasting effects on primary and lateral root elongation of tomato seedlings, as *V. paradoxus* 5C-2 stimulated primary roots without affecting lateral roots, while *P. brassicacearum* Am3 inhibited primary and lateral root elongation without altering lateral root number. An ACC deaminase deficient mutant of the latter strain more strongly inhibited primary root elongation than the WT, at relatively low cell concentrations (10^5 cells cm^{-3}) in the medium. Both *V. paradoxus* 5C-2 and *P. brassicacearum* Am3 increased root hair formation and elongation, with greater effects of *P. brassicacearum* Am3 on root hairs than its ACC deaminase deficient mutant T8-1. At high cell concentrations, the promotion of root hair density was possibly due to stimulating the differentiation of cells into root hairs. Thus ACC deaminase can modulate the morphological impacts of bacteria on root hair response by affecting plant ethylene content and mask the deleterious properties of bacteria. We hypothesize that bacterial traits inhibiting primary and lateral root elongation may promote compensatory root hair responses. Further studies of the role of ACC deaminase in the studied strains in mediating root architecture and root hair traits in Micro-Tom could utilize a range of ethylene-related mutants (Carvalho *et al.* 2011, Fracetto *et al.* 2013) that have been introgressed into this cultivar.

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