

RESEARCH PAPER

# Multiple impacts of the plant growth-promoting rhizobacterium *Variovorax paradoxus* 5C-2 on nutrient and ABA relations of *Pisum sativum*

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Received 3 August 2012; Revised 24 September 2012; Accepted 28 September 2012

## Abstract

Resolving the physiological mechanisms by which rhizobacteria enhance plant growth is difficult, since many such bacteria contain multiple plant growth-promoting properties. To understand further how the 1-aminocyclopropane-1-carboxylate (ACC) deaminase (ACCD)-containing rhizobacterium *Variovorax paradoxus* 5C-2 affects plant growth, the flows and partitioning of mineral nutrients and abscisic acid (ABA) and ABA metabolism were studied in pea (*Pisum sativum*) plants following rhizosphere bacterial inoculation. Although root architecture was not affected, inoculation increased root and shoot biomass, and stomatal conductance, by 20, 15, and 24%, respectively, and increased N, P, K, Ca, and Mg uptake by 16, 81, 50, 46, and 58%, respectively. P deposition in inoculated plant roots was 4.9 times higher than that in uninoculated controls. Rhizobacterial inoculation increased root to shoot xylem flows and shoot to root phloem flows of K by 1.8- and 2.1-fold, respectively. In control plants, major sinks for K deposition were the roots and upper shoot (43% and 49% of total uptake, respectively), while rhizobacterial inoculation increased K distribution to the lower shoot at the expense of other compartments (xylem, phloem, and upper shoot). Despite being unable to metabolize ABA *in vitro*, *V. paradoxus* 5C-2 decreased root ABA concentrations and accumulation by 40–60%. Although inoculation decreased xylem ABA flows, phloem ABA flows increased. Whether bacterial ACCD attenuates root to shoot ABA signalling requires further investigation, since ABA is critical to maintain growth of droughted plants, and ACCD-containing organisms have been advocated as a means of minimizing growth inhibition of plants in drying soil.

**Key words:** Abscisic acid, ACC deaminase, hormone flow modelling, nutrient uptake, pea, plant–microbe interaction, rhizobacteria, *Variovorax paradoxus*.

## Introduction

Plant growth-promoting rhizobacteria (PGPR) colonize the rhizosphere and can enhance plant growth via a set of biocontrol mechanisms (Lugtenberg and Kamilova, 2009) or by increasing plant nutrient uptake via multiple mechanisms including

biological nitrogen fixation, siderophore production, and phosphate solubilization (Dey *et al.*, 2004; Lugtenberg and Kamilova, 2009). Additionally, many rhizobacteria can alter plant hormone status by producing auxins and cytokinins (Costacurta and Vanderleyden, 1995; Dodd *et al.*, 2010) or by decreasing plant ethylene levels via the bacterial enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (ACCd) which hydrolyses the immediate ethylene precursor ACC into ammonia and  $\alpha$ -ketobutyrate (Honma and Shimomura, 1978; Glick *et al.*, 1998) for bacterial use.

Various ACCd-containing rhizobacteria were repeatedly shown to promote plant growth, particularly when plants were subjected to environmental stresses likely to stimulate stress-induced ethylene production (Glick *et al.*, 2007; Belimov *et al.*, 2009). Although bacterial auxin production by some ACCd-containing rhizobacteria (Glick *et al.*, 1998, 2007) may stimulate root growth, the creation of bacterial mutants with severely diminished ACCd activity abolished their root growth-promoting effect (Glick *et al.*, 1994; Belimov *et al.*, 2007, 2009). ACCd-containing bacteria decreased root ACC concentrations and ethylene production (Penrose *et al.*, 2001), whole plant ethylene production (Mayak *et al.*, 2004), and the xylem ACC concentration of plants exposed to drying soil (Belimov *et al.*, 2009). Since ethylene often acts as a growth inhibitor (Pierik *et al.*, 2006), it seems likely that decreased ACC levels *in planta* lowered ethylene production, thereby increasing shoot growth and yield particularly under soil water deficit (Arshad *et al.*, 2008; Belimov *et al.*, 2009).

However, rhizobacterial impacts on *in planta* concentrations of one phytohormone may have feedback effects on the concentration of other hormones. Applying 0.5 mM of the ethylene-releasing chemical ethephon (2-chloroethylphosphonic acid) to the roots of hydroponically or sand-grown plants increased endogenous ethylene production and stimulated abscisic acid (ABA) biosynthesis (Hansen and Grossmann, 2000; F. Jiang, unpublished results). Conceivably, decreased ethylene production of plants inoculated with ACCd-containing rhizobacteria (Mayak *et al.*, 2004) may cause feedback reductions of plant ABA levels. In contrast, inoculation of pea (*Pisum sativum*) with *Variovorax paradoxus* 5C-2 apparently increased xylem ABA concentration of plants in drying soil, probably due to the greater soil drying of larger plants (Belimov *et al.*, 2009). Another possibility, as yet untested for *V. paradoxus* 5C-2, is that it produces ABA, as do other rhizosphere bacteria (Cohen *et al.*, 2008; Sgroj *et al.*, 2009). However, rhizosphere inoculation of maize plants with *V. paradoxus* 5C-2 did not affect xylem ABA concentration over a wide range of soil water availability (Dodd *et al.*, 2009a). Limited evidence of systemic rhizobacterial effects on phytohormone relations (Dodd *et al.*, 2010) suggests that detailed empirical hormone flow models (*sensu* Jeschke *et al.*, 1995; Jiang *et al.*, 2007) may be necessary to resolve subtle differences.

There has also been much recent interest in using PGPR inoculants to decrease the application of chemical fertilizers (Adesemoye *et al.*, 2009), either by stimulating root growth (thereby increasing root foraging for nutrients) or by directly stimulating plant nutrient uptake. Some ACCd-containing rhizobacteria increased shoot and grain nutrient concentrations in

specific plant–microbe interactions: pea and *Pseudomonas brassicacearum* Am3, *P. marginalis* Dp1, or *Rhodococcus* sp. Fp2 (Safronova *et al.*, 2006); peanut (*Arachis hypogea*) and various *Pseudomonas* spp. isolates (Dey *et al.*, 2004); and wheat (*Triticum aestivum*) and *Azospirillum brasilense* Sp245 (Creus *et al.*, 2004). Following inoculation of pea with the ACCd-containing rhizobacterium *V. paradoxus* 5C-2, increased seed nitrogen concentration of plants grown in drying soil (Belimov *et al.*, 2009) may have been due to enhanced nodulation, since ethylene typically inhibits nodulation (Guinel and Geil, 2002). The multiplicity of mechanisms by which a single bacterium can affect plant nutrient status suggests that more sophisticated methods of determining plant nutrient budgets are required.

Apart from these fundamental physiological impacts of PGPR on plant nutrient and hormone budgets, they have been much used to stimulate early plant growth and/or stand establishment, even in legume species which form nitrogen-fixing symbiosis with nodule bacteria of the Rhizobiaceae family (e.g. Dey *et al.*, 2004; Ahmad *et al.*, 2011). Since nodulation occurs comparatively late in legume ontogeny (commonly around flowering), there may be considerable agronomic benefits of applying PGPR such as *V. paradoxus* 5C-2 to improve early vegetative growth. Moreover, these ‘free-living’ PGPR may also stimulate legume nodulation (Dey *et al.*, 2004; Belimov *et al.*, 2009) by decreasing root ethylene production and/or other mechanisms.

Previous work has demonstrated that *V. paradoxus* 5C-2 promoted pea vegetative growth and seed yield, especially of plants grown in drying soil, by attenuating a drought-induced increase in xylem sap ACC concentration in non-nodulated plants, and by preventing a drought-induced decrease in seed nitrogen content of nodulated plants by stimulating nodulation (Belimov *et al.*, 2009). Since *V. paradoxus* 5C-2 apparently stimulated xylem ABA concentration in pea (Belimov *et al.*, 2009) but had no effect in maize (Dodd *et al.*, 2009a), further work to understand these contrasting results, using hormonal flow models, seemed necessary. Since empirical flow models (Jeschke *et al.*, 1995; Jiang *et al.*, 2007) have never been used to evaluate PGPR impacts on plant nutrition and/or hormone homeostasis, the objectives of this study were to develop models to determine whether the ACCd-containing rhizobacterium *V. paradoxus* 5C-2 perturbed ABA metabolism and flows, and/or nutrient uptake, fluxes, and distribution in pea. A secondary objective was to determine whether this organism produced other phytohormones [e.g. ABA, gibberellin (GA), and indole-3-acetic acid (IAA)] in batch culture.

## Materials and methods

### *Bacterial culture, phytohormone production, and ABA degradation*

The PGPR strain *V. paradoxus* 5C-2 containing ACCd was obtained from the Russian Collection of Agricultural Microorganisms (Saint Petersburg) and maintained on Bacto-Pseudomonas F (BPF) agar medium as previously described (Belimov *et al.*, 2005). Bacteria were grown on agar BPF medium for 3 d at 28 °C, and cells were suspended to a final concentration of  $10^8$  cells ml<sup>-1</sup> in nutrient solution ( $\mu$ M): KNO<sub>3</sub>, 2800; Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O, 1600; MgSO<sub>4</sub>·7H<sub>2</sub>O, 1000; NH<sub>4</sub>NO<sub>3</sub>, 2000; NaH<sub>2</sub>PO<sub>4</sub>, 60; and microelements NaFeEDTA, 40; H<sub>3</sub>BO<sub>3</sub>, 10; ZnSO<sub>4</sub>, 2; MnSO<sub>4</sub>·4H<sub>2</sub>O, 2; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.5; Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O, 0.2; H<sub>2</sub>MoO<sub>4</sub>, 0.08. Bacterial suspensions were added to the plants as described below.

*V. paradoxus* 5C-2 was cultivated in liquid BPF medium or in a modified minimal salt, minimal N (MSMN) medium (Belimov *et al.*, 2005) containing (mg l<sup>-1</sup>): mannitol, 5000; glucose, 5000; yeast extract, 500; NH<sub>4</sub>NO<sub>3</sub>, 100; KNO<sub>3</sub>, 50; KH<sub>2</sub>PO<sub>4</sub>, 400; K<sub>2</sub>HPO<sub>4</sub>, 1600; MgSO<sub>4</sub>·7H<sub>2</sub>O, 200; CaCl<sub>2</sub>, 10; NaCl, 10; FeSO<sub>4</sub>, 5; biotin, 0.01; pyridoxal-HCl, 0.02; pH 6.4. In some cultures, the MSMN medium was supplemented with either 100 mM NaCl to induce ABA biosynthesis (Cohen *et al.*, 2008) or 500 µg ml<sup>-1</sup> tryptophan as a precursor of auxin biosynthesis (Cohen *et al.*, 2003). Batch cultures were incubated for 7 d at 25 °C, centrifuged at 13 000 rpm for 5 min, and sterilized using 0.2 µm filters (Corning, Germany). Supernatants were stored at -80 °C until used for phytohormone analysis.

For auxin determination, the supernatants and uninoculated media were acidified to pH 3.0 with 0.4 N hydrochloric acid and extracted with equal volumes of ethyl acetate. The organic phase containing auxins was evaporated to dryness under vacuum at 35 °C and suspended in 0.5 ml of 20% acetonitrile (ACN). The obtained extracts were filtered through 0.22 µm nylon centrifuge tube filters Spin-X (Corning, USA) and fractionated by C<sub>18</sub> reverse-phase ultra-performance liquid chromatography (UPLC) (Waters ACQUITY UPLC BEH Shield RP18 1.7 µm, 2.1×50 mm column) with 5 µl sample injections. The UPLC system Waters ACQUITY H-Class (Waters, USA) with fluorescent detector (λ<sub>ex</sub>=280 nm, λ<sub>em</sub>=350 nm) was used to detect auxins. Solvent conditions included a flow rate of 0.3 ml min<sup>-1</sup> with a 5 min linear gradient from 1% ACN–0.1% acetic acid to 18% ACN–0.1% acetic acid, followed by a 3 min isocratic elution and 3 min column washing with 80% ACN–0.1% acetic acid. IAA, indole-3-carboxylic acid (ICA), and indole-3-lactic acid (ILA) supplied by Sigma-Aldrich were used as standards. The content of L-tryptophan was determined directly by 5 µl injections of initial media using the same conditions as for auxin analysis.

The supernatants and uninoculated media were analysed for ABA concentration via radioimmunoassay using the antibody MAC252 (Quarrie *et al.*, 1988). For GA analysis, 1 ml aliquots were diluted with 100 ml of 80% methanol–water containing 2 ng of each <sup>3</sup>H-labelled internal standard (Professor Lewis Mander, Australian National University, Canberra, Australia) and the samples were then purified (Griffiths *et al.*, 2006) and analysed by gas chromatography–mass spectrometry (GC-MS) (Rieu *et al.*, 2008) as previously described.

To test whether *V. paradoxus* 5C-2 could utilize ABA as some other rhizobacteria can (A.A. Belimov and I.C. Dodd, unpublished observations), the MSMN medium (without mannitol, glucose, and yeast extract) was supplemented with 1 mg ml<sup>-1</sup> ABA as a sole carbon source. Bacteria were cultivated for 20 d at 25 °C with shaking at 200 rpm. Bacterial growth was monitored daily via measurement of the optical density of batch cultures at 540 nm against uninoculated medium used as a blank. At the end of the experiment, the ABA concentration in supernatants was determined as described above.

#### Plant culture and measurements

Pea (*P. sativum* L. cv. Alderman) seeds (Moles Seeds, UK) were selected for homogeneity of seed weight, surface-sterilized with 6% NaClO for 15 min, rinsed carefully with sterile water, and germinated in vermiculite (LBS Horticulture, UK) at room temperature for 6 d. Afterwards, seedlings were washed with tap water to remove vermiculite from the roots and then transplanted into 1 litre pots (110 mm diameter, 130 mm height) containing washed sand (Leighton Buzzard 16/30, Sibelco, UK). Plants of similar size and developmental stage were separated into two groups. One group of plants was watered daily with the nutrient solution (see above) while the other was additionally supplied with a suspension of *V. paradoxus* 5C-2 (10<sup>8</sup> cells ml<sup>-1</sup>) every 3 d or 4 d, starting from the fifth day after transplanting. Plants were cultivated in a greenhouse with natural light and the temperature varying between 12 °C (night) and 25 °C (day).

Leaf stomatal resistance was measured 14 d after transplanting (10 d after inoculation of *V. paradoxus* 5C-2) with a transient-time porometer (Model AP4, Delta-T Devices, UK) between 10:00 h and 11:00 h. Following these measurements, the roots were carefully removed from the pots and adhering sand carefully washed away with tap water. The

roots were spread on trays with water for scanning, and the length, diameter, and surface area of all roots were determined with WinRHIZO (Regent Instruments Inc., Canada).

Xylem sap was collected from the main vein of the pea leaves during the study period by placing the pots into a pressure chamber, sealing the shoot into the chamber using a silicone-based dental impression compound (a-gum vinyl polysiloxane impression materials, Dentsply DeTrey GmbH, Germany), and pressurizing the pots until xylem sap exuded from leaf incisions (Jeschke and Pate, 1991). Phloem exudates were obtained by placing excised pea shoots in vials containing 1.5 ml of 5 mM Na<sub>2</sub>EDTA, which were maintained in a humid atmosphere. By chelating Ca<sup>2+</sup>, EDTA prevents the formation of callose, which would otherwise seal the sieve tubes after wounding, and thus stimulates phloem exudation (Wolf *et al.*, 1990). Sap samples were stored at -25 °C prior to analysis.

At the beginning and end of the study (10 d and 16 d after transplanting), plants were separated into lower leaves and internodes (nodes 1–4 numbering from the base of the plant), upper leaves and internodes, and roots. All plant parts were weighed, and placed in liquid nitrogen prior to freeze-drying. Dry tissues were weighed and then finely ground. Ions in different organs and xylem sap were analysed using an ICP spectrometer (JY Plus, Division d'Instruments S.A., France). Total N was analysed by use of a CHN analyser (Elementar, Germany). Cotyledons (which were very small and shrunken at the time of the first harvest) were discarded.

#### Bacterial root colonization

At the end of the experiment (22 d after germination), roots were removed from the pot and adhering sand particles gently removed. Three random samples of inoculated fresh pea root tissue were weighed and homogenized in sterile tap water with a sterile mortar and pestle. Homogenates were serially diluted in 10-fold steps and 50 µl aliquots were plated in two replicates on BPF agar supplemented with 30 µg ml<sup>-1</sup> kanamycin and 20 µg ml<sup>-1</sup> rifampicin, to which *V. paradoxus* 5C-2 naturally shows resistance, and 40 µg ml<sup>-1</sup> nystatin to prevent fungal growth. The characteristic colonies of *V. paradoxus* 5C-2 were counted after incubation at 28 °C for 4 d. The average number of colony-forming units (CFU) of inoculated pea roots was 1.9 × 10<sup>6</sup> g<sup>-1</sup> fresh weight (FW), while no colonies were recovered from uninoculated plants.

#### ABA analysis of plant samples

Freeze-dried tissue samples were homogenized and extracted in 80% aqueous methanol solution. Extracts were passed through Sep Pak C<sup>18</sup>-cartridges. Methanol was removed under reduced pressure and the aqueous residue was taken up in phosphate buffer mixed with 0.1% Tween-20 and 0.1% gelatin, and then subjected to an immunological ABA assay (enzyme-linked immunosorbent assay; as described in Yang *et al.*, 2001). For xylem sap and phloem exudates, no further purification was necessary. The percentage recovery of ABA was >90% and all sample extract dilution curves paralleled the standard curves, indicating the absence of non-specific inhibitors in the extracts.

#### Modelling plant internal flows

Based on the assumption that (i) calcium is transported in the xylem only and (ii) mass flow occurs in the xylem, net xylem potassium flow (moles per plant) from root to shoot (J<sub>k,x</sub>) was calculated from the ratio of potassium to calcium (K/Ca)<sub>x</sub> in xylem sap and the increment of calcium in the shoot, ΔCa (Armstrong and Kirkby, 1979):

$$J_{k,x} = (K/Ca)_x \times \Delta Ca \quad (1)$$

Net potassium flow in the phloem (J<sub>k,p</sub>) was calculated from the difference between the potassium increment ΔK in each organ and the net xylem import to that organ, J<sub>k,x</sub>:

$$J_{k,p} = \Delta K - J_{k,x} \quad (2)$$

The content of each element in the organs in moles per plant and increments in moles per plant over the study period were then calculated from the concentrations and the dry weights; see Table 2.

Estimation of ABA flows was based on the assumption that mass flow occurs in the xylem and phloem, hence solutes are translocated according to their relative concentrations. Potassium flows were the basis for the calculation of ABA flows.

According to this assumption, net xylem flows of ABA ( $J_{ABA,x}$ ) from root to shoot are given by the flows of potassium ( $J_{K,x}$ ) and the ratio of ABA to K in xylem sap  $[ABA/K]_x$ :

$$J_{ABA,x} = J_{K,x} \times [ABA/K]_x \quad (3)$$

The phloem flow of ABA ( $J_{ABA,p}$ ) was estimated on the basis of the obtained K flows, as the product of the phloem K flow ( $J_{K,p}$ ) and the ratio of ABA to K in phloem exudates  $[ABA/K]_p$ :

$$J_{ABA,p} = J_{K,p} \times [ABA/K]_p \quad (4)$$

The differences between the estimated net flows of ABA moving in or out of an organ and its increment ( $\Delta ABA$ ) in that organ yielded the net metabolic changes of ABA ( $J_{ABA,met}$ ) either by degradation or by synthesis of ABA:

$$J_{ABA,met} = \Delta ABA - J_{ABA,x} - J_{ABA,p} \quad (5)$$

(with an influx into, or an efflux from, an organ being a positive or negative flow, respectively). Net degradation must have occurred if the resulting metabolic changes were negative, whereas, if they were positive, net synthesis was indicated.

The flow modelling approach presented herein has been used previously (Jeschke *et al.*, 1995; Jiang *et al.*, 2007) and depends on increments of nutrient and ABA contents between first and second harvest, the standard errors of which are presented (Table 2).

## Results

### *V. paradoxus* 5C-2 produced auxins, but not ABA and GAs, in selected media

*Variovorax paradoxus* 5C-2 produced IAA and ILA most actively after adding L-tryptophan to MSMN medium, whereas the maximum ICA concentration was detected in supernatants of bacteria grown in BPF medium, which also contained a relatively high concentration of L-tryptophan (Table 1). In contrast, neither biologically active GAs nor their precursors could be detected (limits were 30, 70, and 80  $\mu\text{g ml}^{-1}$  for  $GA_1$ ,  $GA_3$ , and  $GA_4$ , respectively) by GC-MS.

Using a radioimmunoassay, ABA was not detected (the limit was 0.2 ng ABA  $\text{ml}^{-1}$ ) in all culture media, suggesting that

*V. paradoxus* 5C-2 was not able to produce this hormone in these media, unlike another organism (*Achromobacter xylosoxidans*) that was tested. No bacterial growth was observed on MSMN medium supplemented with 1 mg ABA  $\text{ml}^{-1}$  as a sole carbon source, and the ABA concentration in the medium at the end of the experiment did not change (data not shown). Thus *V. paradoxus* 5C-2 was not able to metabolize ABA under the conditions tested.

### *V. paradoxus* 5C-2 stimulated growth, stomatal conductance, and nutrient uptake

*Variovorax paradoxus* 5C-2 significantly ( $P < 0.05$ ) increased pea root and shoot biomass (Table 2) by 20% and 15%, respectively. However, bacterial inoculation did not significantly affect total root length and surface area (data not shown) or root length distribution according to diameter (Fig. 1). In Leaf 3 (counting from the base of the plant), rhizobacterial inoculation significantly ( $P < 0.05$ ) decreased stomatal resistance (from  $3.66 \pm 0.25 \text{ s cm}^{-1}$  in control plants to  $2.67 \pm 0.29 \text{ s cm}^{-1}$  in inoculated plants,  $n=4$ ), while no significant effect was detected in Leaf 4 (data not shown).

For all measured elements, nutrient contents of all parts of inoculated pea plants were generally slightly higher at the first harvest (6 d after inoculation), and were more pronounced after a further 6 d. Thus inoculation substantially increased root nutrient increments over 6 d by 41% for N, 4.9-fold for P, 36% for K, 70% for Ca, and 89% for Mg (Table 2). In comparison, inoculation effects on nutrient increments in the lower part of the shoot were much stronger: 4.4-fold for K, 2.6-fold for Ca, and 8.2-fold for Mg. Following inoculation, the nitrogen content of the lower part of the pea shoot increased between the two harvests, but it decreased in control plants. Less P was mobilized from the lower part of the inoculated pea plants in comparison with the control plants. The effects of rhizobacterial inoculation on nutrient increments in the upper part of the shoot were much weaker than in other organs (Table 2).

Rhizobacterial effects on nutrient budgets were investigated by constructing flow models of phosphorus (Fig. 2A) and potassium (Fig. 2B) from the data of Tables 2 and 3. In control plants, the upper part of the shoot was the major P sink. A substantial amount of P was mobilized from the lower shoot. Consequently, P re-translocation in the phloem (52% of xylem flow) exceeded P deposition in the root and hence led to a recirculation of P towards the upper shoot (Fig. 2A).

Rhizobacterial inoculation increased total P uptake by 81%, and most of this was used for upper shoot growth.

**Table 1.** Production of phytohormones by *V. paradoxus* 5C-2 in batch culture.

Medium	L-Tryptophan in growth media ( $\mu\text{g ml}^{-1}$ )	Phytohormone production ( $\text{ng ml}^{-1}$ )				
		IAA	ICA	ILA	ABA	$GA_{1,3,4}$
MSMN	$0.025 \pm 0.002$	$0.9 \pm 0.04$	$0.23 \pm 0.01$	$2.6 \pm 0.1$	ND	ND
MSMN + L-tryptophan	$500 \pm 10$	$75 \pm 6$	ND	$156 \pm 10$	ND	ND
BPF	$67 \pm 5$	$19 \pm 2$	$121 \pm 11$	$2.6 \pm 0.1$	ND	ND

Data are shown as means  $\pm$ SE;  $n=2$ . ND, not detected.

**Table 2.** Biomass (mg dry weight plant<sup>-1</sup>), total nitrogen, phosphorus, potassium, calcium, and magnesium contents (μmol plant<sup>-1</sup>) and ABA contents (pmol plant<sup>-1</sup>) and their increments in pea plants (control and inoculated by *V. paradoxus* 5C-2) at the beginning and end of the study period 10 d and 16 d after transplanting, ~6 and 12 d after inoculation.

		Internodes 1–4 and leaves		Internodes 5–7 and leaves		Roots		Control	<i>V. paradoxus</i> 5C-2
		Control	<i>V. paradoxus</i> 5C-2	Control	<i>V. paradoxus</i> 5C-2	Control	<i>V. paradoxus</i> 5C-2		
Biomass	Harvest 1	109±3.23	123±5.27*	25.3±3.40	36.1±1.18*	84.5±3.03	87.2±4.31		
	Harvest 2	121±3.90	139±4.43*	143±7.65	165±5.95*	143±5.47	172±5.53*		
	Increment	12±1.82	16±1.41	118±3.78	129±3.79	58.5±3.06	85±3.25**		
Total N	Harvest 1	517±15	520±23	147±20	189±6	425±15	421±21		
	Harvest 2	508±16	542±17	702±37	747±27	636±24	719±23*		
	Increment	-9±3.51	22±9.47	556±20.1	558±17.5	211±10.9	298±11.1**		
	Uptake of N by roots							758±87.9	878±30.3
P	Harvest 1	15.3±0.45	15.5±0.65	8.23±1.11	10.1±0.33	12.3±0.44	13.6±0.67		
	Harvest 2	10.3±0.33	15.1±0.45*	24.1±1.27	26.2±0.95	13.6±0.52	20.0±0.64*		
	Increment	-5±0.17	-0.4±0.4**	15.9±0.16	16.1±0.52	1.3±0.22	6.4±0.32**		
	Uptake of P by roots							12.2±2.85	22.1±0.91*
K	Harvest 1	65.1±1.94	72.4±2.98	16.8±2.22	22.8±0.74*	71.0±2.55	103±5.08*		
	Harvest 2	85.8±2.82	165±5.00*	143±7.84	166±5.90*	182±6.97	254±8.14*		
	Increment	21±0.88	93±3.18**	126±5.45	143±3.94*	111±4.42	151±4.49**		
	Uptake of K by roots							258±18.0	387±9.67**
Ca	Harvest 1	22.9±0.68	23.1±1.01	2.47±0.33	3.38±0.11*	8.96±0.32	9.59±0.47		
	Harvest 2	31.4±1.00	45.1±1.41*	31.9±1.70	35.1±1.26	16.7±0.64	22.7±0.73*		
	Increment	8.5±0.39	22±0.64**	29.5±1.34	31.7±0.95	7.7±0.33	13.1±0.39**		
	Uptake of Ca by roots							45.7±3.37	66.8±1.74
Mg	Harvest 1	12.4±0.37	12.1±0.51	2.23±0.30	2.81±0.09	3.66±0.13	3.59±0.18		
	Harvest 2	13.3±0.43	19.5±0.59*	16.2±0.87	17.4±0.62	8.36±0.32	12.5±0.40*		
	Increment	0.9±0.12	7.4±0.27**	13.9±0.54	14.5±0.42	4.7±0.19	8.9±0.26**		
	Uptake of Mg by roots							19.5±1.69	30.8±0.82**
ABA	Harvest 1	39.4±1.20	60.4±2.78*	12.0±1.65	19.4±0.66*	29.6±1.06	28.4±1.40		
	Harvest 2	33.3±1.05	31.8±1.04	53.2±2.84	56.1±2.02	59.1±2.26	41.7±1.34*		
	Increment	-6±0.42	-29±1.61**	41±1.56	37±1.54*	29±1.27	13±0.49**		

Data are shown as means ±SE; n=11–12.

Asterisks indicate significant (\**P* < 0.05) and highly significant (\*\**P* < 0.01) differences between inoculated and control plants.

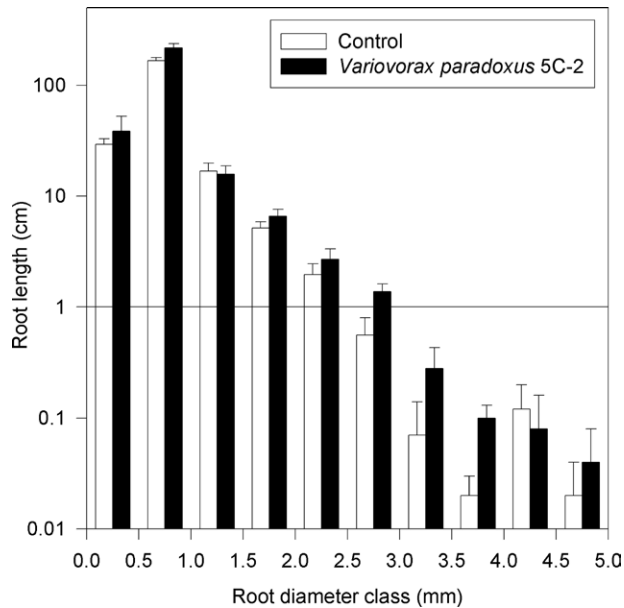
Total P deposition in the roots increased 4.9-fold, while P re-translocation in phloem was relatively lower (44% of xylem flow).

In control plants, major sinks for K were the roots and upper shoot, where 43% and 49% of root K uptake were deposited over 6 d. Only 8% of the K was deposited in the lower shoot. Xylem K transport into the lower and upper shoot exceeded K deposition and resulted in phloem K re-translocation (40% of the xylem flow).

Rhizobacterial inoculation increased K uptake substantially (by 1.5 times), and this increased xylem K flow to the shoot (by 1.8-fold). Since xylem K flow was clearly higher than K accumulation, K flow in the phloem approximately doubled and led to a higher K re-translocation (46% of xylem flow). K was distributed homogeneously, with 39, 24, and 37% in the roots, lower parts, and upper parts of the shoot, respectively.

*V. paradoxus* 5C-2 decreased root ABA concentration despite increased phloem ABA flow

ABA flows within the plants (Fig. 2C) were calculated using the data from Tables 2 and 3. Rhizobacterial inoculation tended to decrease xylem ABA concentration (Table 3) and ABA concentrations in all shoot parts, while root ABA concentrations decreased significantly by 41% (Table 2). After inoculation, xylem ABA flows from the lower to the upper part of the shoots decreased by 21%. Estimated ABA degradation in the upper shoot decreased by 94%. However, ABA deposition in the upper shoot was similar to that of control plants, consistent with increased phloem ABA flow (80% higher than the control) from the upper to the lower part of the shoots. The lower part of the shoots released much more ABA into the phloem, which also contributed to a higher ABA flow (83% higher than in the control) from shoot to roots. The ratio of ABA shoot to root phloem flows to ABA



**Fig. 1.** Relationship between root length and diameter for peas grown in sand that were uninoculated (hollow bars) or inoculated with *V. paradoxus* 5C-2 (filled bars). Data are means  $\pm$ SE;  $n=4$ .

root to shoot xylem flows was also increased 76%. Root ABA biosynthesis and accumulation were decreased by 46% and 55%, respectively.

## Discussion

Adding the ACCd-containing rhizobacterium *V. paradoxus* 5C-2 to the substrate of well-watered, well-fertilized pea plants increased root and shoot growth by 20% and 15%, respectively (Table 2) as previously described (Belimov *et al.*, 2009), independently of any effect on root nodulation. Since bacterial mutants having low ACCd activity (including a transposome mutant of *V. paradoxus* 5C-2) did not stimulate plant growth (Glick *et al.*, 1994; Belimov *et al.*, 2007, 2009), the growth promotion observed here was most probably due to decreased plant production of the growth-inhibitory phytohormone ethylene.

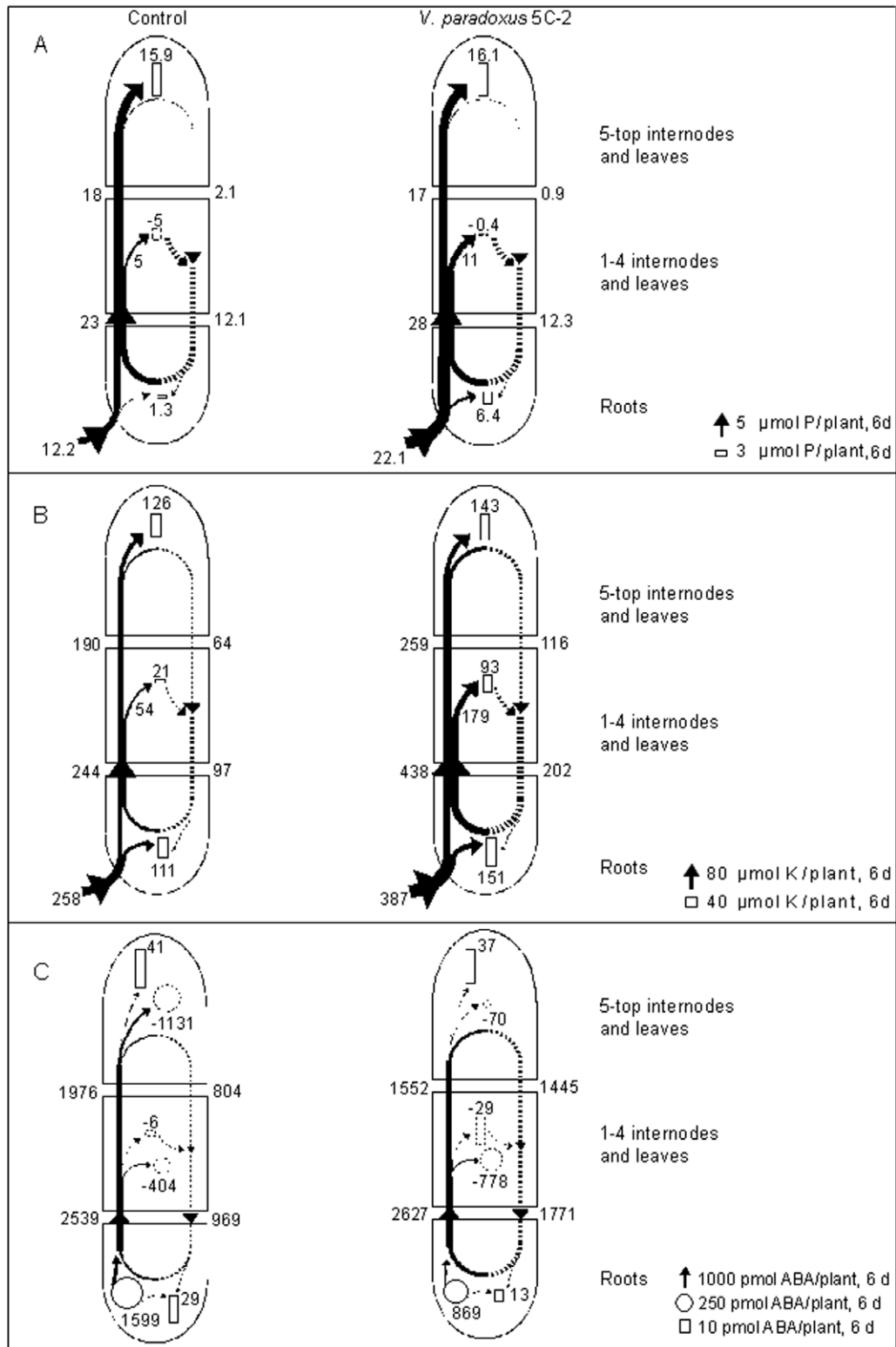
However, many rhizobacteria can also produce multiple plant hormones when cultured *in vitro* (e.g. Sgroj *et al.*, 2009), thus these were assayed in *V. paradoxus* 5C-2 culture filtrate (Table 1). Although *V. paradoxus* 5C-2 apparently did not produce GA *in vitro*, in contrast to some other rhizobacteria such as *Bacillus pumilis* and *B. licheniformis* (Gutierrez-Manero *et al.*, 2001), previous measurements (with a colorimetric method based on Salkovsky's reagent) indicated that *V. paradoxus* 5C-2 produced putative auxins or other indoles (Belimov *et al.*, 2005). While this technique can be non-specific for auxins (Ehman, 1977), UPLC confirmed the production of several auxins by this strain (Table 1), indicating that *V. paradoxus* 5C-2 might synthesize auxins from L-tryptophan, a common root exudate (Kamilova *et al.*, 2006). Irrespective of the mechanism(s) by which bacteria synthesize auxin(s), their effects *in planta* will be concentration dependent, with 10 nM IAA inhibiting pea root growth (Eliasson

*et al.*, 1989) yet stimulating bean (*Vicia faba*) root growth (El-Antably and Larsen, 1974) in hydroponics. Since bacterial mutants with decreased auxin production failed to stimulate root growth (Patten and Glick, 2002), further work with *V. paradoxus* 5C-2 should down-regulate its auxin production and assay effects on root growth.

Enhanced root growth following *V. paradoxus* 5C-2 inoculation probably improved nutrient uptake (Fig. 2A, 2B). These nutritional effects seem partially specific to *V. paradoxus* 5C-2, as other ACCd-containing rhizobacteria (*P. brassicacearum* Am3, *P. marginalis* Dp1, or *Rhodococcus* sp. Fp2) had positive effects on pea foliar N, Ca, S, and Fe concentrations, but not on P and K concentrations (Safronova *et al.*, 2006). Rhizobacterial stimulation of nutrient uptake (81, 50, 46, and 58% for total P, K, Ca, and Mg, respectively) was proportionally greater for many nutrients than the enhancement of root growth (20%). In contrast, the similar enhancement of nitrogen uptake (16%) and root growth (20%) suggests a cause-effect relationship. Increases in nutrient uptake larger than the increased root growth suggest that alternative mechanisms (e.g. ion transporters or channels) may be stimulated by rhizobacterial inoculation.

While some ACCd-containing rhizobacteria (but not *V. paradoxus* 5C-2; A.A. Belimov, unpublished data) can solubilize phosphate (Dey *et al.*, 2004), this is unlikely to benefit plants in the present experiments, to which P was supplied as the soluble  $H_2PO_4^-$  ion. Although altered root morphology of inoculated plants may enhance phosphorus uptake, ACCd-containing rhizobacteria did not affect lateral root development or root architecture in *Arabidopsis thaliana* (Contesto *et al.*, 2008) and *Cucumis sativus* (Gamalero *et al.*, 2008), and *P. sativum* here (Fig. 1). Root hair abundance and length are also positively correlated with increased uptake of relatively immobile elements such as P (Bates and Lynch, 2001; Gahoonia and Nielsen, 2003), yet *in vitro* application of bacterial mutants with decreased ACCd activity resulted in plants with longer root hairs (Contesto *et al.*, 2008; A.A. Belimov and I.C. Dodd unpublished results) than those inoculated with wild-type ACCd-containing rhizobacteria. Nevertheless, *V. paradoxus* 5C-2 stimulated root hair elongation of tomato *in vitro* (Belimov, 2012), which may enhance P uptake of soil-grown plants.

Since *V. paradoxus* 5C-2 decreased xylem ACC concentrations in pea (Belimov *et al.*, 2009), shoot ethylene production should diminish, which may alter the concentrations of other phytohormones *in planta*. Although plant ABA status moderates ethylene production (Sharp *et al.*, 2000; Dodd *et al.*, 2009b), the converse effect is equivocal. However, *V. paradoxus* 5C-2 inoculation decreased ABA biosynthesis and deposition in pea roots (Fig. 2C), more so than in comparable experiments with maize (Dodd *et al.*, 2009a). Unlike maize, where the root hypodermis (=exodermis) strongly inhibits exudation of solutes and plant hormones to the rhizosphere (Hose *et al.*, 2001), legumes never form exodermal Casparian bands (Enstone and Peterson, 1992), and it has been argued that rhizobacterial inoculation will have greater effects on plant hormonal relations in legumes than cereals (Belimov *et al.*, 2009; Dodd *et al.*, 2009a). However, further experiments with a range of leguminous and non-leguminous species are required to determine the generality of this hypothesis. The mechanism(s) by which *V. paradoxus* 5C-2 decreased



**Fig. 2.** Empirical models of the uptake, transport, and utilization of total phosphorus (A) and potassium (B), or the metabolism, transport, and deposition of ABA (C) in whole pea plants over a 6 d study period. Arrow widths [net flows in xylem sap (black) or phloem (dotted)] and rectangle heights (deposition in each organ) are drawn in proportion to flow rates and the magnitude of deposition, respectively. Circled areas in (C) are drawn in proportion to the rates of metabolism.

root ABA concentration is not known, as it was not capable of metabolizing ABA *in vitro* when grown on ABA as a sole carbon source.

Decreased ABA levels following inoculation with ACCd-containing rhizobacteria may regulate plant growth. Although ABA application inhibited root growth of hydroponically

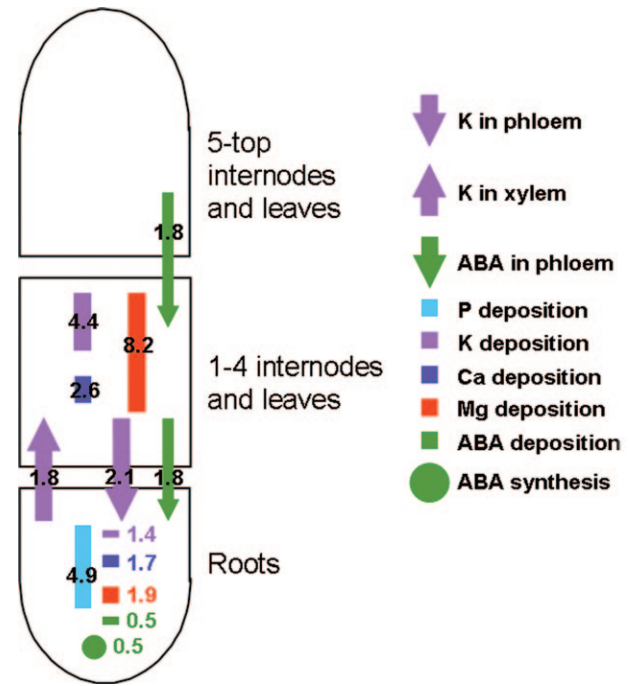
**Table 3.** The concentrations and ratios of Ca, K, P, and ABA in xylem sap in different parts of the shoot, and the ratio of ABA to potassium (K) in phloem exudates obtained with the EDTA technique in pea plants. Further details are as described in Table 2.

	Control	<i>V. paradoxus</i> 5C-2
Xylem sap concentrations in internodes 1–4		
Abscisic acid (ABA) (nM)	15.5 ± 1.76	12.6 ± 1.79
Calcium (Ca) (mM)	0.25 ± 0.03	0.23 ± 0.02
Potassium (K) (mM)	1.35 ± 0.33	2.05 ± 0.33
Phosphorus (P) (mM)	0.14 ± 0.02	0.14 ± 0.05
Xylem sap concentrations in internodes 5–7		
Abscisic acid (ABA) (nM)	15.3 ± 1.67	9.20 ± 2.31
Calcium (Ca) (mM)	0.26 ± 0.05	0.36 ± 0.03
Potassium (K) (mM)	1.84 ± 0.26	2.49 ± 0.24
Phosphorus (P) (mM)	0.15 ± 0.02	0.14 ± 0.01
Xylem sap ratios (entire shoot)		
K/Ca	6.45 ± 0.72	8.17 ± 0.74
P/Ca	0.60 ± 0.07	0.52 ± 0.12
ABA/K	10.4 ± 1.39	6.0 ± 1.37*
ABA/K in phloem exudates		
Internodes 1–4	10.0 ± 3.2	8.8 ± 2.7
Internodes 5–7	12.6 ± 4.0	12.5 ± 2.6

Data are shown as means ± SE;  $n=4-8$ . An asterisk indicates a significant difference at the  $P < 0.05$  level.

grown peas (Tietz, 1973), the ABA-deficient wilted pea mutant showed decreased root growth and allocation of biomass to the roots compared with wild-type plants (I.C. Dodd, unpublished data), suggesting instead that normal ABA levels are required to maintain root growth. Similarly, rhizobacterial alterations in shoot ABA homeostasis (Fig. 2C) may influence shoot growth. However, the ABA-deficient wilted pea mutant (with shoot ABA concentrations 50% lower than those of wild-type peas) had a similar relative foliage expansion rate to wild-type peas when grown in sand at two different external N concentrations (Dodd, 2003). Following rhizobacterial inoculation, the spatial distribution of changes in ABA status suggests that ABA did not mediate leaf growth since there were only limited impacts in the upper shoot where leaves were expanding (Fig. 2C). Nevertheless, greater ABA degradation in the lower nodes and leaves (93% higher than control plants) probably accounted for the increased stomatal conductance of more mature leaves.

While interpreting the effects of ACCd-containing rhizobacteria has logically focused on attenuating the growth-inhibitory effects of ethylene, other mechanisms such as improved nutrient uptake (Fig. 2A, 2B) and CO<sub>2</sub> fixation (because of increased stomatal conductance) may also be important. Empirical modelling of nutrient flows revealed specific changes in plant nutrient relations induced by rhizobacterial inoculation (Fig. 3) that were not apparent from conventional measurements of tissue concentrations. However, rhizobacterial inoculation decreased root ABA concentrations (Table 2), which may mitigate plant adaptation to water deficits, suggesting that the strategy of applying



**Fig. 3.** Fold changes in xylem sap or phloem flows (arrows), deposition (rectangles), and metabolism (circles) of ABA and plant macronutrients induced by inoculation with *V. paradoxus* 5C-2.

ACCd-containing PGPR to overcome the effects of soil drying (Dodd, 2009) requires re-evaluation.

## Acknowledgements

Financial support was provided to FJ by the NSFC (31121003), the State Key Basic Research and Development Plan (2007CB106802), and the Scientific Research Foundation of Beijing Normal University (2009SAT-3). FJ's visit to Lancaster was part supported by an RCUK China Bridge project (to WJD); ICD and AAB thank DEFRA (WU0121), the Royal Society, and MSE RF (GK 16.512.11.2162) for financial support. Rothamsted Research receives strategic funding from the Biotechnology and Biological Sciences Research Council (BBSRC).

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