**RESEARCH PAPER**

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

Fan Jiang¹, Lin Chen², Andrey A. Belimov³, Alexander I. Shaposhnikov³, Fan Gong⁴, Xu Meng¹, Wolfram Hartung⁵, Dieter W. Jeschke⁵, William J. Davies² and Ian C. Dodd²,⁎

¹ Beijing Key Laboratory of Gene Resource and Molecular Development, College of Life Sciences, Beijing Normal University, Beijing, 100875, China
² The Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, UK
³ All-Russia Research Institute for Agricultural Microbiology, Podbelskogo Sh. 3, Pushkin-8, 196608, Saint Petersburg, Russian Federation
⁴ Rothamsted Research, Harpenden, West Common, Hertfordshire AL5 2JQ, UK
⁵ Julius von Sachs Institut für Biowissenschaften der Universität, Lehrstuhl Botanik I, Julius von Sachs Platz 2, D-97082 Würzburg, Germany

* To whom correspondence should be addressed. E-mail: i.dodd@lancaster.ac.uk

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**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 and 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, sideroaphore 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 α-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 ethphen (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; Sgroy 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 hypogaea) and various Pseudomonas spp. isolates (Dey et al., 2004); and wheat (Triticum aestivum) and Azospirillumbrasiliense Sp245 (Crou e 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 10^6 cells ml^-1 in nutrient solution (μM): KNO₃, 2800; Ca(NO₃)₂·4H₂O, 1600; MgSO₄·7H₂O, 1000; NH₄NO₃, 200; NaH₂PO₄, 60; and microelements NaFeEDTA, 40; H₂BO₃, 10; ZnSO₄·2H₂O, 10; MnSO₄·H₂O, 2; CuSO₄·5H₂O, 0.5; Co(NO₃)₂·6H₂O, 0.2; H₂MoO₄·0.08. Bacterial suspensions were added to the plants as described below.
V. paradoxus SC-2 was cultivated in liquid BPF medium or in a modified minimal salt, minimal N (MSMN) medium (Belimov et al., 2005) containing (mg l⁻¹): mannitol, 5000; glucose, 5000; yeast extract, 500; NH₄NO₃, 100; KNO₃, 50; KH₂PO₄, 400; K₂HPO₄, 1600; MgSO₄·7H₂O, 200; CaCl₂, 10; NaCl, 10; FeSO₄·5H₂O; 0.01; pyridoxal-HCl, 0.02; pH 6.4. In some cultures, the MSMN medium was supplemented with either 100 µM NaCl to induce ABA biosynthesis (Cohen et al., 2008) or 500 µg ml⁻¹ tryptophan as a precursor of auxin biosynthesis (Cohen et al., 2003). Batch cultures were incubated for 7 d at 25 °C, centrifuged at 12 000 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 auxin was evaporated to dryness under vacuum at 35 °C with equal volumes of ethyl acetate. The organic phase containing auxin was analyzed by gas chromatography–mass spectrometry (GC-MS) (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.

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 3 min, rinsed carefully with sterile water (Corning, USA) and fractionated by C₁₈ 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 (λex=280 nm, λem=350 nm) was used to detect auxins. Solvent conditions included a flow rate of 0.3 ml min⁻¹ 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 I-tryptophan was determined directly by 5 µl injections of initial media using the same conditions as for auxin analysis.

To test whether V. paradoxus SC-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⁻¹ 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.

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 (Jk,p) was calculated from the ratio of potassium to calcium (K/Ca), in xylem sap and the increment of calcium in the shoot, ΔCa (Armstrong and Kirkby, 1979):
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 \((J_{\text{ABA},x})\) from root to shoot are given by the flows of potassium \((J_{K,x})\) and the ratio of ABA to K in xylem sap \([\text{ABA}/K]_x\):

\[ J_{\text{ABA},x} = J_{K,x} \times [\text{ABA}/K]_x \]  \hspace{1cm} (3)

The phloem flow of ABA \((J_{\text{ABA},p})\) 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 \([\text{ABA}/K]_p\):

\[ J_{\text{ABA},p} = J_{K,p} \times [\text{ABA}/K]_p \]  \hspace{1cm} (4)

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

\[ \Delta \text{ABA} = J_{\text{ABA},x} - J_{\text{ABA},p} \]  \hspace{1cm} (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 pg 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 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 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±0.25 s cm\(^{-1}\) in control plants to 2.67±0.29 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.

<table>
<thead>
<tr>
<th>Medium</th>
<th>L-Tryptophan in growth media (µg ml(^{-1}))</th>
<th>Phytohormone production (ng ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IAA</td>
</tr>
<tr>
<td>MSMN</td>
<td>0.025±0.002</td>
<td>0.9±0.04</td>
</tr>
<tr>
<td>MSMN + L-tryptophan</td>
<td>500±10</td>
<td>75±6</td>
</tr>
<tr>
<td>BPF</td>
<td>67±5</td>
<td>19±2</td>
</tr>
</tbody>
</table>

Data are shown as means ±SE; \(n=2\).

ND, not detected.
Table 2. Biomass (mg dry weight plant⁻¹), total nitrogen, phosphorus, potassium, calcium, and magnesium contents (μmol plant⁻¹) and ABA contents (μmol plant⁻¹) 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.

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Internodes 1–4 and leaves</th>
<th>Internodes 5–7 and leaves</th>
<th>Roots</th>
<th>Control</th>
<th>V. paradoxus 5C-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harvest 1</td>
<td>109±3.23</td>
<td>123±5.27</td>
<td>25.3±3.40</td>
<td>36.1±1.18*</td>
<td>84.5±3.03</td>
</tr>
<tr>
<td>Harvest 2</td>
<td>121±4.90</td>
<td>199±4.43</td>
<td>143±7.65</td>
<td>165±5.95*</td>
<td>143±5.47</td>
</tr>
<tr>
<td>Total N</td>
<td></td>
<td></td>
<td>147±20</td>
<td>189±6</td>
<td>425±15</td>
</tr>
<tr>
<td>Harvest 1</td>
<td>517±15</td>
<td>520±23</td>
<td>702±37</td>
<td>747±27</td>
<td>636±24</td>
</tr>
<tr>
<td>Increment</td>
<td>-9±3.51</td>
<td>22±9.47</td>
<td>556±20.1</td>
<td>558±17.5</td>
<td>211±10.9</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td>12.4±0.46</td>
<td>12.1±0.51</td>
<td>10.3±0.33</td>
</tr>
<tr>
<td>Harvest 1</td>
<td>15.3±0.45</td>
<td>15.5±0.65</td>
<td>8.23±1.11</td>
<td>10.1±0.33</td>
<td>12.3±0.44</td>
</tr>
<tr>
<td>Harvest 2</td>
<td>10.3±0.33</td>
<td>15.1±0.45*</td>
<td>24.1±1.27</td>
<td>26.2±0.95</td>
<td>13.6±0.52</td>
</tr>
<tr>
<td>Increment</td>
<td>-5±0.17</td>
<td>-0.4±0.4**</td>
<td>15.9±0.16</td>
<td>16.1±0.52</td>
<td>1.3±0.22</td>
</tr>
<tr>
<td>K</td>
<td></td>
<td></td>
<td>65.1±1.94</td>
<td>72.4±2.98</td>
<td>16.8±2.22</td>
</tr>
<tr>
<td>Harvest 1</td>
<td>65.1±1.94</td>
<td>72.4±2.98</td>
<td>16.8±2.22</td>
<td>22.8±0.74*</td>
<td>71.0±2.55</td>
</tr>
<tr>
<td>Harvest 2</td>
<td>85.8±2.82</td>
<td>165±5.00*</td>
<td>143±7.84</td>
<td>166±5.90*</td>
<td>182±6.97</td>
</tr>
<tr>
<td>Increment</td>
<td>21±0.88</td>
<td>93±3.18**</td>
<td>126±5.45</td>
<td>143±3.94*</td>
<td>111±4.42</td>
</tr>
<tr>
<td>Ca</td>
<td></td>
<td></td>
<td>22.9±0.68</td>
<td>23.1±1.01</td>
<td>2.47±0.33</td>
</tr>
<tr>
<td>Harvest 1</td>
<td>22.9±0.68</td>
<td>23.1±1.01</td>
<td>2.47±0.33</td>
<td>3.38±0.11*</td>
<td>8.96±0.32</td>
</tr>
<tr>
<td>Harvest 2</td>
<td>31.4±1.00</td>
<td>45.1±1.41*</td>
<td>31.9±1.70</td>
<td>35.1±1.26</td>
<td>16.7±0.64</td>
</tr>
<tr>
<td>Increment</td>
<td>8.5±0.39</td>
<td>22±0.64**</td>
<td>29.5±1.34</td>
<td>31.7±0.95</td>
<td>7.7±0.33</td>
</tr>
<tr>
<td>Mg</td>
<td></td>
<td></td>
<td>12.4±0.37</td>
<td>12.1±0.51</td>
<td>2.23±0.30</td>
</tr>
<tr>
<td>Harvest 1</td>
<td>12.4±0.37</td>
<td>12.1±0.51</td>
<td>2.23±0.30</td>
<td>2.81±0.09</td>
<td>3.66±0.13</td>
</tr>
<tr>
<td>Harvest 2</td>
<td>13.3±0.43</td>
<td>19.5±0.59*</td>
<td>16.2±0.87</td>
<td>17.4±0.62</td>
<td>8.36±0.32</td>
</tr>
<tr>
<td>Increment</td>
<td>0.9±0.12</td>
<td>7.4±0.27**</td>
<td>13.9±0.54</td>
<td>14.5±0.42</td>
<td>4.7±0.19</td>
</tr>
<tr>
<td>ABA</td>
<td></td>
<td></td>
<td>39.4±1.20</td>
<td>60.4±2.78*</td>
<td>12.0±1.65</td>
</tr>
<tr>
<td>Harvest 1</td>
<td>39.4±1.20</td>
<td>60.4±2.78*</td>
<td>12.0±1.65</td>
<td>19.4±0.66*</td>
<td>29.6±1.06</td>
</tr>
<tr>
<td>Harvest 2</td>
<td>33.3±1.05</td>
<td>31.8±1.04</td>
<td>53.2±2.84</td>
<td>56.1±2.02</td>
<td>59.1±2.26</td>
</tr>
<tr>
<td>Increment</td>
<td>-6±0.42</td>
<td>-29±1.61**</td>
<td>41±1.56</td>
<td>37±1.54*</td>
<td>29±1.27</td>
</tr>
</tbody>
</table>

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
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 transposon 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. Sgro 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$_2$PO$_4^-$ 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* (Gamaliero 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
Rhizobacteria affect nutrient and ABA relations

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

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**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.
grown peas (Tietz, 1973), the ABA-deficient wilty 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 wilty 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, it logically focused on attenuating the growth-inhibitory effects of ethylene, other mechanisms such as improved nutrient uptake (Fig. 2A, 2B) and CO₂ 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 ACCd-containing PGPR to overcome the effects of soil drying (Dodd, 2009) requires re-evaluation.

### Acknowledgements

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### References


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**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.

<table>
<thead>
<tr>
<th>Xylem sap concentrations in internodes 1–4</th>
<th>Control</th>
<th>V. paradoxus 5C-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abscisic acid (ABA) (nM)</td>
<td>15.5 ± 1.76</td>
<td>12.6 ± 1.79</td>
</tr>
<tr>
<td>Calcium (Ca) (mM)</td>
<td>0.25 ± 0.03</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>Potassium (K) (mM)</td>
<td>1.35 ± 0.33</td>
<td>2.05 ± 0.33</td>
</tr>
<tr>
<td>Phosphorus (P) (mM)</td>
<td>0.14 ± 0.02</td>
<td>0.14 ± 0.05</td>
</tr>
<tr>
<td>Xylem sap concentrations in internodes 5–7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abscisic acid (ABA) (nM)</td>
<td>15.3 ± 1.67</td>
<td>9.20 ± 2.31</td>
</tr>
<tr>
<td>Calcium (Ca) (mM)</td>
<td>0.26 ± 0.05</td>
<td>0.36 ± 0.03</td>
</tr>
<tr>
<td>Potassium (K) (mM)</td>
<td>1.84 ± 0.26</td>
<td>2.49 ± 0.24</td>
</tr>
<tr>
<td>Phosphorus (P) (mM)</td>
<td>0.15 ± 0.02</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>Xylem sap ratios (entire shoot)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K/Ca</td>
<td>6.45 ± 0.72</td>
<td>8.17 ± 0.74</td>
</tr>
<tr>
<td>P/Ca</td>
<td>0.60 ± 0.07</td>
<td>0.62 ± 0.12</td>
</tr>
<tr>
<td>ABA/K</td>
<td>10.4 ± 1.39</td>
<td>6.0 ± 1.37*</td>
</tr>
</tbody>
</table>

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

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**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.


