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Rhizosphere bacteria containing 1-aminocyclopropane-1-carboxylate deaminase increase growth and photosynthesis of pea plants under salt stress by limiting Na^+ accumulation

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Abstract. Although plant salt tolerance has been improved by soil inoculation with rhizobacteria containing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (which metabolises ACC, the immediate precursor of the phytohormone ethylene), it is not always clear whether ion homeostasis and plant water relations are affected. When pea (*Pisum sativum* L. cv. Alderman) was grown with 70 and 130 mM NaCl, the ACC-deaminase containing rhizobacterium *Variovorax paradoxus* 5C-2 increased total biomass by 25 and 54% respectively. Nutrient flow modelling showed that *V. paradoxus* 5C-2 increased K uptake and root to shoot K flow, but decreased Na flow and increased Na deposition in roots. Thus, shoot K^+ : Na^+ ratio increased following *V. paradoxus* 5C-2 inoculation. At 70 and 130 mM NaCl, rhizobacterial inoculation decreased stomatal resistance by 14 and 31% and decreased xylem balancing pressure by 7 and 21% respectively. Furthermore, rhizobacterial inoculation improved photosynthetic efficiency (F_v/F_m) by 12 and 19% and increased maximal electron transport rate (ETR) by 18 and 22% at 70 and 130 mM NaCl respectively. Thus *V. paradoxus* 5C-2 mitigates salt stress by improving water relations, ion homeostasis and photosynthesis of pea plants, and may provide an economic means of promoting growth of plants exposed to salt stress.

Additional keywords: ion homeostasis, maximal electron transport rate, nutrient flow modelling, photosynthetic efficiency, water relations.

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Introduction

Salinity is a major factor responsible for reducing plant growth and productivity, causing the abandonment of land. The Food and Agriculture Organisation (FAO) reported that more than 800 million ha of land throughout the world were affected by salinity (FAO 2005). Most crops tolerate salinity to a threshold level, but exceeding this level decreases crop productivity due to osmotic and ion-specific effects.

For most glycophytes, salt stress often leads to an increased stomatal resistance, a significant inhibition of photosynthesis and specific ion (Na^+) toxicity (Rajasekaran *et al.* 1998; Munns and Tester 2008; Pandolfi *et al.* 2012). Salt stress increases external osmotic pressure (Munns 2002), which decreases leaf water potential and turgor, and ultimately causes stomatal closure (Munns and Tester 2008). Na^+ in the transpiration stream is transported to the leaves where excessive Na^+ accumulates (Munns 2002). Excessive Na^+ concentrations in plants result in K^+ deficiency and interrupt multiple physiological processes mediated by K^+, including protein synthesis, stomatal movement and photosynthesis (Adams and Shin 2014). Excessive accumulation of Na^+ and diminished K^+ status are the major characteristics of plants under salt stress (Maathuis and Amtmann 1999; Chen *et al.* 2007).

Unfortunately, transgenic approaches and molecular breeding programs for improving crop tolerance to salt stress have generally not brought promising results in farmers' fields (Wang *et al.* 2003; Bhatnagar-Mathur *et al.* 2008; James *et al.* 2008) with some notable exceptions (Munns *et al.* 2006). Therefore, alternative approaches such as the use of soil micro-organisms such as mycorrhizal fungi and plant growth-promoting rhizobacteria (PGPR) may be exploited (Dodd and Pérez-Alfocea 2012). The use of PGPR containing 1-aminocyclopropane-1-carboxylate (ACC) deaminase is one of the most promising approaches to promote growth of plants exposed to salt stress (Mayak *et al.* 2004; Glick 2014), by diminishing stress-induced ethylene production (Ali *et al.* 2014). These bacteria hydrolyse ethylene precursor ACC into ammonia and α-ketobutyrate (Glick *et al.* 1997). Since ethylene

inhibits plant growth via multiple mechanisms (Pierik *et al.* 2006), lowering the ethylene levels can increase growth of plants exposed to environmental stress (Mayak *et al.* 2004; Barnawal *et al.* 2012; Ali *et al.* 2014). However, there are alternative non-hormonal mechanisms by which ACC deaminase containing rhizobacteria can alleviate salt stress and increase growth of their host plants (Mayak *et al.* 2004; Ali *et al.* 2014).

Maintaining tissue water status is a key strategy to reduce the harmful impacts of salt stress on plant growth. Some ACC deaminase containing PGPR can increase osmolyte (e.g. proline) accumulation (Bharti *et al.* 2013) and water uptake (Belimov *et al.* 2009) under optimal conditions, which may enhance tolerance to salt stress. However, there are few measurements of plant water potential of salinised plants that have been inoculated with ACC deaminase containing PGPR (Mayak *et al.* 2004; Nadeem *et al.* 2007, 2010). Although these bacteria can increase stomatal conductance (Jiang *et al.* 2012) and maintain higher photosynthetic ability (F_v/F_m ratio) of plants grown under optimal conditions (Gamalero *et al.* 2008), their impacts on stomatal and non-stomatal limitation of photosynthesis of salinised plants are not clear. These rhizobacteria can also maintain ion homeostasis by improving plant N, P and K uptake (Jiang *et al.* 2012; Safronova *et al.* 2012) or enhancing the $K^+:Na^+$ ratio (Nadeem *et al.* 2009; Chang *et al.* 2014). However, previous measurements of the ionic status of salinised plants inoculated with ACC-deaminase containing PGPR have simply measured foliar Na^+ and K^+ concentrations (Nadeem *et al.* 2007, 2010), without considering elemental flows within the entire plant. When there is excessive Na^+ in the rhizosphere, the partitioning, cycling and recycling of K^+ and Na^+ between shoot and root are altered (Wolf *et al.* 1990). By using the nutrient modelling method, this communication between root and shoot can be studied.

Variovorax paradoxus 5C-2 was described as a root-associated bacterium containing ACC deaminase and promoting plant growth in the presence of toxic cadmium concentrations (Belimov *et al.* 2005). Previous work has shown that soil inoculation with *V. paradoxus* 5C-2 benefited plant growth, especially when plants were exposed to drying soil, by influencing multiple physiological processes including decreasing ACC concentrations in the rhizosphere of potato (Belimov *et al.* 2015) and xylem sap of pea (Belimov *et al.* 2009), and enhancing the nitrogen fixing symbiosis between pea and rhizobia (Belimov *et al.* 2009). Enhanced nutrient uptake by pea (Jiang *et al.* 2012) and decreased concentrations of abscisic acid in tomato seedlings (Belimov *et al.* 2014) were also observed in inoculated plants. However, whether *V. paradoxus* 5C-2 can protect plants from the effects of salt stress has not been tested.

To provide new insights into how ACCd-containing PGPR affect growth of salinised pea plants, we used a range of physiological techniques that hitherto have not been used to study plant–PGPR interactions under salt stress. Nutrient flow modelling (Wolf *et al.* 1990; Pate *et al.* 1979) was used to determine nutrient deposition, transport and flows in plants (rather than simple instantaneous measurements of nutrient content), while Mini-Pam was used to measure photosynthetic electron transport rates *in vivo* (Hoshida *et al.* 2000) and a whole-plant pressure chamber was used to determine xylem balancing pressure (Termaat *et al.* 1985). We hypothesised that decreased shoot Na^+ accumulation (due to increased Na^+ deposition in roots and decreased xylem Na^+ export from roots) benefited photosynthesis, thereby mitigating salt stress in pea plants.

Materials and methods

Bacterial culture and salt tolerance

The PGPR strain *Variovorax paradoxus* 5C-2 containing ACC deaminase (Belimov *et al.* 2005) was obtained from the Russian Collection of Agricultural Microorganisms (St Petersburg, Russian Federation) and maintained on Bacto-*Pseudomonas* F (BPF) medium as previously described (Belimov *et al.* 2005). Briefly, bacteria were incubated on agar BPF medium for 72 h at 28°C, cells were scraped from the agar surface (to minimise transfer of nutrient-rich agar to the pots) and suspended to a final concentration of 10^8 cells ml^{-1} in a nutrient solution (μM): KNO_3, 2800; $Ca(NO_3)_2$•$4H_2O$, 1600; $MgSO_4$•$7H_2O$, 1000; NH_4NO_3, 2000; NaH_2PO_4, 600; and microelements NaFeEDTA, 40; H_3BO_3, 10; $ZnSO_4$, 2; $MnSO_4$•$4H_2O$, 2; $CuSO_4$•$5H_2O$, 0.5; $Co(NO_3)_2$•$6H_2O$, 0.2; H_2MoO_4, 0.08.

To determine salt tolerance of *V. paradoxus* 5C-2, bacteria were incubated for 5 days at 28°C on agar BPF medium supplemented with increasing NaCl concentrations in steps of 20 mM. Threshold growth-inhibitory and threshold lethal concentrations of NaCl were estimated visually.

ACC deaminase activity of *V. paradoxus* 5C-2 in the presence of toxic NaCl concentrations was determined by monitoring the amount of α-ketobytyrate (αKB) generated enzymatically via hydrolysis of ACC (Saleh and Glick 2001) as previously described (Safronova *et al.* 2012). The protein concentration of disrupted cell suspensions was determined by the method of Bradford (1976) using the Bio-Rad protein reagent (Bio-Rad Laboratory, Hercules, CA, USA).

Plant culture and growth conditions

Pea (*Pisum sativum* L. cv. Alderman) seeds (Moles Seeds, Colchester, UK) were selected for homogeneity of seed weight, then surface-sterilised with 10% H_2O_2 for 10 min, rinsed carefully with sterile water, and germinated in well washed quartz sand and irrigated daily with distilled water. Seven-day-old seedlings were washed with sterile water to remove quartz sand from the roots. Afterwards, plants of similar size and developmental stage were transplanted individually (one plant per pot) into 4 L plastic pots (19 cm diameter, 14 cm height) containing carefully washed quartz sand. Each group was watered daily with half-strength nutrient solution for 5 days, then full-strength nutrient solution supplied. The experiment was carried out in a naturally lit glasshouse. The temperature was 12°C (night) and 27°C (day) and photosynthetically active radiation (at noon) was 1300 $\mu mol\ m^{-2}\ s^{-1}$. Ten days after transplanting, plants were divided into six groups. In groups 1–2 seedlings were irrigated daily with full-strength nutrient solution without NaCl. Seedlings in groups 3–4 and 5–6 were irrigated daily with full-strength nutrient solution containing 70 mM and 130 mM NaCl, respectively. Meanwhile, plants in groups 2, 4 and 6 were additionally supplied with 4 mL of a suspension of *V. paradoxus* 5C-2 (10^8 cells mL^{-1}) every 4 days,

and irrigated on intervening days with nutrient solutions at the required salt concentrations. In comparison, field experiments with this organism have applied single doses of 10^{10} cells mL^{-1} at transplanting (Teijeiro *et al.* 2011).

Leaf physiology

At the end of the study (30 days after transplanting, 20 days after inoculation of *V. paradoxus* 5C-2 and salt treatments), leaf stomatal resistance of fully expanded leaves was measured with a transient-time porometer (Model AP4, Delta-T Devices, Cambridge, UK) between 0900 and 1100 hours.

The same leaves were used to quantify components of chlorophyll fluorescence *in situ* with a portable modulated fluorometer (Mini-Pam Photosynthesis Yield Analyser; Walz, Effeltrich, Germany) (Bilger *et al.* 1995). Maximal fluorescence, F_m, was measured after a 0.8 s saturating white light pulse (2318 µmol m^{-2} s^{-1}) and the minimal fluorescence, F_0, was measured after 30 min dark-adaptation. Maximal variable fluorescence was calculated as $F_v = F_m - F_0$. The PSII photochemical efficiency was calculated as F_v/F_m. The efficiency of electron transport rate (ETR) was also calculated as previously described (Genty *et al.* 1989). Rapid light curves (RLCs) were measured by a software controlled protocol with 10s illumination times and intensities increasing in eight steps (Bilger *et al.* 1995).

Measurement of transpiration and xylem balancing pressure

Whole-plant transpiration was measured gravimetrically daily (before and after the daily addition of nutrient solution and drainage) by weighing pots of each treatment during the last six days of the study period (from 25 days after transplanting to 30 days after transplanting). Whole plant transpiration was summed during the last 6 days of the experiment, with corrections applied for the water loss from pots without plants (Jiang *et al.* 2001).

Xylem balancing pressure of 2–3 pea plants was measured (once per plant) each day during the last 10 days of the study period (from 21 to 30 days after transplanting). The entire pot (including the plant) was placed in the pressure chamber (25 cm in diameter and 16 cm in length) and external pressure applied until the appearance of the first drop of xylem sap on the cut surface of the youngest fully expanded leaf, which was recorded as the xylem balancing pressure (Liang *et al.* 1996). Since preliminary experiments had established that additional overpressure did not yield sufficient xylem sap for ionic analysis, the entire shoot was removed at the stem base to facilitate xylem sap collection from the roots. All xylem sap samples were immediately frozen in liquid nitrogen after collection and stored at −20°C.

Plant harvests and ion analysis

At the beginning and the end of the study (20 and 30 days after transplanting, 10 and 20 days after inoculation of *V. paradoxus* 5C-2 and salt treatments), FW was determined, then all samples were frozen in liquid nitrogen. Dry tissues were weighed after being lyophilised, then ground into powder and kept for further analyses. Ionic composition of different tissues (and xylem sap) were analysed using an ICP-OES (inductively coupled plasma-optical emission spectrometer - JY Plus, Division d' Instruments SA, Longjumeau, France).

Modelling plant internal flows

Based on the assumption that calcium is transported in the xylem only and mass flow occurs in the xylem, net xylem potassium flow and sodium flow (µmol $plant^{-1}$) from root to shoot ($J_{K,X}$) and ($J_{Na,X}$) were calculated from the ratio of potassium to calcium $(K:Ca)_X$ and $(Na:Ca)_X$ in xylem sap and the increment of calcium in the shoot, ΔCa (Armstrong and Kirkby 1979):

$$J_{K,X} = (K:Ca)_X \times \Delta Ca, J_{Na,X} = (Na:Ca)_X \times \Delta Ca. \quad (1)$$

Net potassium flow and sodium flow in the phloem ($J_{K,P}$) and ($J_{Na,P}$) were calculated from the difference between the potassium increment ΔK and sodium increment ΔNa in each organ and the net xylem import to the organ, $J_{k,X}$ and $J_{Na,X}$;

$$J_{K,P} = \Delta K - J_{K,X}, J_{Na,P} = \Delta Na - J_{Na,X}. \quad (2)$$

The content of each element in the organs in µmol $plant^{-1}$ and increments in moles per plant over the study period were then calculated from the element concentrations and the DW (Jiang *et al.* 2012).

Bacterial root colonisation

To determine the persistence of *V. paradoxus* 5C-2 on the root surface, bacterial colonisation was assayed at the beginning and the end of the study (20 and 30 days after transplanting, corresponding to 10 and 20 days after inoculation of *V. paradoxus* 5C-2 and salt treatments). Roots were removed from the pots in each treatment group and shaken gently to remove adhering sand particles. Main roots and lateral roots were homogenised in sterile tap water with a sterile mortar and pestle, the homogenates serially diluted in 10-fold steps, and 50 µL aliquots plated in duplicate on BPF agar supplemented with rifampicin 20 mg L^{-1}, kanamycin 30 mg L^{-1} (to which *V. paradoxus* 5C-2 shows resistance) and nystatin 40 mg L^{-1} (to prevent the growth of fungi). Then the numbers of colony forming units (CFU) were counted after incubation for 4 days at 28°C.

Statistics

Two-way analysis of variance (ANOVA) was performed to determine effects of salt, inoculation and their interactions, using SPSS version 19 (SPSS, Chicago, IL, USA). One-way ANOVA with Tukey's test ($P < 0.05$) was used to discriminate means. Analysis of correlations was made using the Microsoft Excel statistical software (Microsoft Corporation, Seattle, WA, USA).

Results

Incubating *V. paradoxus* 5C-2 on NaCl-supplemented agar showed that this strain possessed threshold growth-inhibitory and threshold lethal concentrations of 140 and 280 mM NaCl respectively. This is consistent with the salt treatments (70 and 130 mM NaCl) having no significant effects on root

colonisation of *V. paradoxus* 5C-2 (Table 1). Moreover, these salt concentrations had no inhibitory effect on ACC deaminase activity of *V. paradoxus* 5C-2 *in vitro*, but a higher salt concentration (260 mM) significantly decreased ACCd activity (Fig. 1). A residual amount of αKB was detected in the presence of 520 mM NaCl, probably due to the activity of a trace amount of ACC deaminase in the bacterial inoculum.

Salt stress significantly ($P < 0.05$) decreased whole plant biomass, by 34% and 60% at 70 and 130 mM NaCl respectively (Fig. 2). *V. paradoxus* 5C-2 improved plant growth significantly independently of salt treatment (no significant salt × inoculation interaction), with inoculation increasing whole plant biomass by 36% at 70 mM NaCl and by 58% at 130 mM NaCl (Fig. 2). Salinity stress inhibited shoot growth more strongly than root growth. Shoot growth of uninoculated plants was reduced by 36 and 62% when treated with 70 mM and 130 mM NaCl, respectively, but by only 30 and 48% in inoculated plants, respectively. Although 70 mM NaCl had no significant impact ($P < 0.05$) on root biomass, 130 mM NaCl decreased root biomass of non-inoculated plants by 29%. In contrast, root biomass of inoculated plants grown at 130 mM NaCl was not statistically different to uninoculated plants grown in the absence of salt.

Salt significantly decreased whole plant transpiration by 44 and 65% at 70 and 130 mM NaCl respectively (Table 2). Inoculation with *V. paradoxus* 5C-2 significantly increased transpiration by 31, 42 and 85% at 0, 70 and 130 mM NaCl respectively (Table 2).

The effect of salt on stomatal resistance was moderated by *V. paradoxus* 5C-2 inoculation, as indicated by a significant salt × inoculation interaction (Fig. 3*a*). Inoculation of *V. paradoxus* 5C-2 decreased stomatal resistance by 19, 14 and 31% when 0, 70 and 130 mM NaCl was applied

Table 1. Colonisation of pea roots by *Variovorax paradoxus* 5C-2
Values are means ± s.e. of three replicates. There were no significant ($P < 0.05$) differences between treatments. Different letters indicate significant differences ($P < 0.05$) between measurement occasions according to Tukey's test. Abbreviation: CFU, colony forming units

Days after transplanting	Number of bacteria (10^6 CFU g^{-1} FW)		
	Non-salt	70 mM NaCl	130 mM NaCl
20	1.42 ± 0.02a	1.33 ± 0.02a	1.36 ± 0.01a
30	1.81 ± 0.02b	1.73 ± 0.02b	1.77 ± 0.02b

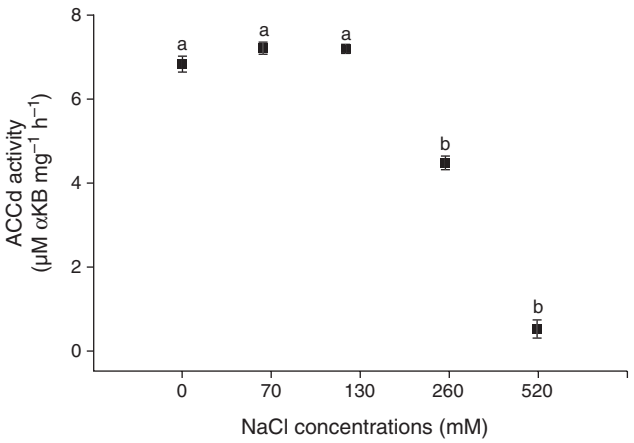

Fig. 1. ACC deaminase activity (μM αKB $mg^{-1} h^{-1}$) of *Variovorax paradoxus* 5C-2 *in vitro*. Different letters above the symbols indicate significant differences ($P < 0.05$; Tukey's test) between means.

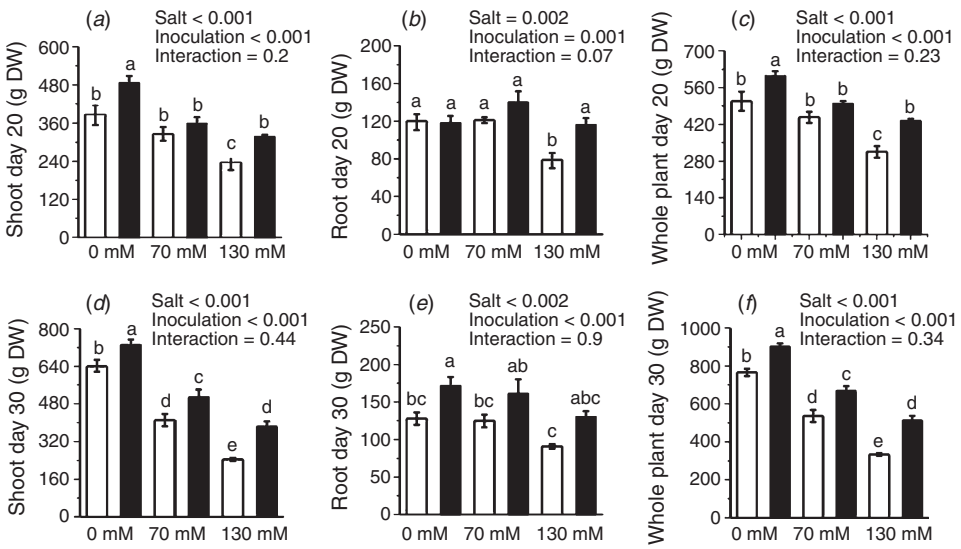

Fig. 2. Biomass (mg DW $plant^{-1}$) of pea plants with (closed bars) and without (open bars) inoculation with *Variovorax paradoxus* 5C-2 at the beginning (*a–c*) and the end (*d–f*) of the study period (20 days and 30 days after transplanting, 10 days and 20 days after inoculation and salt treatment). Data are means ± s.e. of four replicates. Different letters above the bars indicate significant differences ($P < 0.05$; Tukey's test) between means. *P*-values determined by two-way ANOVA for salt, inoculation and their interaction are shown.

Table 2. Transpiration and accumulations of K, Na, Ca, Mg and total P of pea plants between the beginning and the end of the study

The accumulation of nutrients within whole-plant equals to the uptake by roots over the study period. Values are means ± s.e. of four replicates. Within each column different letters indicate significant difference ($P < 0.05$) according Tukey's test. Then the significance (P-values reported) of the salt effects (Salt), inoculation with *Variovorax paradoxus* 5C-2 (Inoculation) and interaction of these factors (salt × inoculation interaction) on the measured parameters are shown, determined by two-way ANOVA

Treatments		Transpiration (mL)			Accumulation (μmol)							
			K		Na			Ca		Mg	P	
NaCl (mM)	*V. paradoxus* 5C-2	Shoot	Root	Whole plant	Shoot	Root	Whole plant	Shoot	Root	Whole plant	Whole plant	Whole plant
0	Non-inoculated	153 ± 2b	28 ± 3b	229 ± 10b	11 ± 1d	18 ± 1d	30 ± 1c	63 ± 3a	–4 ± 1c	59 ± 5ab	21 ± 4ab	47 ± 3a
	Inoculated	200 ± 2a	102 ± 7a	288 ± 11a	17 ± 1d	25 ± 2d	41 ± 2c	60 ± 3a	10 ± 1a	70 ± 3a	27 ± 2a	41 ± 2a
70	Non-inoculated	86 ± 1e	–32 ± 5e	89 ± 9c	326 ± 12b	129 ± 14c	455 ± 24b	39 ± 3c	–6 ± 1c	33 ± 4c	12 ± 2b	40 ± 3a
	Inoculated	122 ± 2c	–23 ± 7e	114 ± 21c	282 ± 19c	205 ± 27a	488 ± 44b	49 ± 5b	1 ± 0b	50 ± 6b	32 ± 4a	49 ± 6a
130	Non-inoculated	53 ± 2f	–20 ± 4d	0c	–20 ± 3e	462 ± 9a	116 ± 4c	578 ± 17a	2.0 ± 1.1d	–28 ± 2.7d	–26 ± 4e	–13 ± 3e	–4 ± 4c
	Inoculated	98 ± 1d	30 ± 7c	–10 ± 5d	20 ± 3d	435 ± 23a	155 ± 8b	590 ± 30a	4.4 ± 0.8d	–3.4 ± 0c	1 ± 0d	3 ± 0d	14 ± 3b
								P-values				
	Salt	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
	Inoculation	0.0001	0.05	0.0001	0.0006	0.0003	0.0002	0.0001	0.0001	0.0001	0.0001	0.03
	Salt × Inoculation Interaction	0.09	0.0013	0.0001	0.56	0.0001	0.013	0.002	0.06	0.123	0.12	0.06	0.01

respectively. In contrast, only salinity had a significant ($P < 0.05$) effect on xylem balancing pressure (Fig. 3*b*). Nevertheless, *V. paradoxus* 5C-2 inoculation decreased the xylem balancing pressure by 9, 7 and 21% when 0, 70 and 130 mM NaCl was applied respectively.

In all non-inoculated plants, salt stress significantly ($P < 0.05$) increased shoot and root Na^+ concentrations at both the beginning and the end of the study period (Fig. 4*a–d*). Over the same interval, *V. paradoxus* 5C-2 inoculation significantly decreased shoot Na^+ concentrations at both NaCl levels. Importantly, rhizobacterial effects on limiting shoot Na^+ concentration were greater with increasing salt concentration (as indicated by a significant salt × inoculation interaction).

Although salt stress significantly decreased both root and shoot K^+ concentrations ($P < 0.05$) when measured at the beginning and end of the study period, rhizobacterial inoculation had no significant effect. Averaged across both measurement occasions, 70 and 130 mM NaCl decreased shoot K^+ concentrations by 7 and 10%, and root K^+ concentrations by 30 and 45% respectively (Fig. 4*e–h*).

In both salt treatments, the $K^+ : Na^+$ ratio was decreased in both shoots and roots ($P < 0.05$) (Fig. 4*i–l*). In comparison, rhizobacterial inoculation significantly increased the $K^+ : Na^+$ ratios in shoot tissues (Fig. 4*i*, *j*) whereas no significant differences were observed in root tissues. In shoot tissues of salinised plants, the inoculated plants grown at 70 mM NaCl had the highest $K^+ : Na^+$ ratio and non-inoculated plants grown at 130 mM NaCl had the lowest.

The effects of salinity and the inoculation of *V. paradoxus* 5C-2 on K, Na, Ca, Mg and P accumulation are shown in Table 2. Two-way ANOVA showed that both salt and rhizobacterial inoculation had a significant ($P < 0.05$) effect on accumulation of total K, Na, Ca, Mg and P in whole plant (Table 2). Inoculation with *V. paradoxus* 5C-2 increased the accumulation of Ca, Mg and P in 70 mM NaCl-stressed plants by 52, 167 and 23% respectively (Table 2).

Rhizobacterial effects on nutrient budgets were investigated by constructing flow models of K and Na (Fig. 5). Salt stress decreased K flows from root to shoot in xylem by 5 and 92% in 70 and 130 mM NaCl, respectively (Fig. 5*a*, *c*, *e*). Rhizobacterial inoculation increased total K uptake by 26 and 28% at 0 and 70 mM NaCl (Fig. 5*b*, *d*). Rhizobacterial inoculation increased xylem K flows by 14, 42 and 220% and increased phloem K flows by 45, 59 and 19% at 0, 70 and 130 mM NaCl treatment respectively (Fig. 5*a–f*). Nevertheless, phloem export exceeded root K^+ deposition in the roots, indicating K recycling back to the shoot. Under salt stress, K^+ uptake and K^+ retrieved from roots were allocated to shoot. Interestingly, at 130 mM NaCl treatment, 20 μmol K was effluxed from the root of non-inoculated plants (Fig. 5*e*), which may be due to cell membrane damage caused by high salt stress (Cuin and Shabala 2005; Shabala and Cuin 2008).

In non-inoculated plants, salt clearly increased total Na uptake by 15- and 19-fold at 70 and 130 mM NaCl respectively (Fig. 5*g*, *i*, *k*). Even greater changes were detected in xylem sap Na^+ concentration, which increased from 0.35 to 6 to 197 mM at 0, 70 and 130 mM external NaCl respectively (Table 3). We noted that rhizobacterial inoculation increased root Na deposition and decreased Na flow from root to shoot by

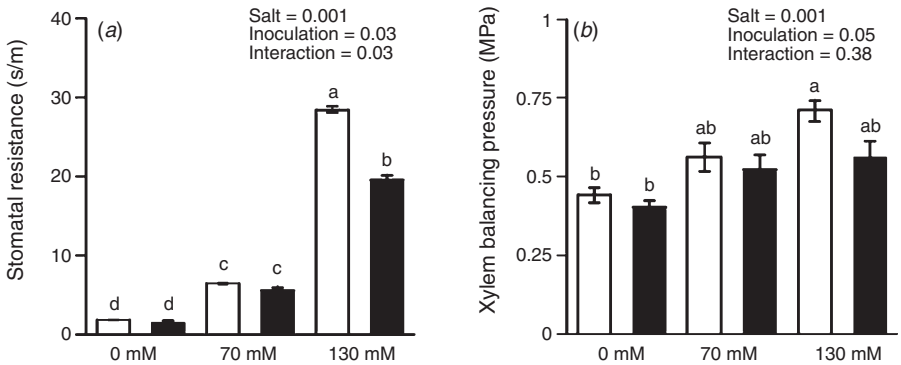

Fig. 3. Stomatal resistance (*a*) at the end of the study period (30 days after transplanting, 20 days after inoculation and salt treatment), xylem balancing pressure (MPa) (*b*) over 10 days period (21–30 days after transplanting) of pea plants with (closed bars) and without (open bars) inoculation with *Variovorax paradoxus* 5C-2. Data are means ± s.e. of four replicates. Different letters above the bars indicate significant differences ($P < 0.05$; Tukey's test) between means. *P*-values determined by two-way ANOVA for salt, inoculation and their interaction are shown.

17 and 9%. Moreover, such inoculation decreased shoot Na^+ deposition by 13 and 6% under 70 and 130 mM NaCl respectively (Fig. 5*i*, *j*), which may be related to enhanced performance in the presence of salt. Rhizobacterial inoculation had no statistically significant effect on xylem sap Na^+ concentration at 0 and 70 mM NaCl, but at 130 mM NaCl decreased it by 65% (Table 3).

All measured chlorophyll fluorescence variables were significantly, independently affected by both salt stress and rhizobacterial inoculation. Salt treatments increased F_0 accompanied by a decrease in F_m; however, inoculated plants had higher F_m with a lower F_0 (Fig. 6*a*, *b*). In uninoculated plants, F_v/F_m ratio significantly decreased ($P < 0.05$) by 12 and 24% when 70 and 130 mM NaCl was applied, whereas inoculation significantly increased the F_v/F_m ratio by 12 and 19%, respectively (Fig. 6*c*), at these salt concentrations.

Salt treatments decreased PSII ETR, especially at 130 mM NaCl. The maximal PSII ETR decreased by 16 and 64% when 70 and 130 mM NaCl was applied respectively. However, rhizobacterial inoculation increased the maximal PSII ETR by 18 and 22% of at 70 and 130 mM NaCl respectively (Fig. 7*a–c*). Even in non-stressed plants, rhizobacterial inoculation increased ETR.

Significant negative correlations ($P < 0.01$) were found between chlorophyll fluorescence parameters (F_v/F_m, maximal ETR) and stomatal resistance ($r = –0.8$, $–0.94$), shoot Na^+ concentration ($r = –0.81$, $–0.92$) and xylem balancing pressure ($r = –0.85$, $–0.76$) (Table 4). Stomatal resistance was significantly ($P < 0.01$) positively correlated with both shoot Na^+ concentration ($r = 0.93$) and xylem balancing pressure ($r = 0.75$). Xylem balancing pressure was also significantly positively correlated ($P < 0.01$) with shoot Na^+ concentration ($r = 0.8$) (Table 4).

Discussion

A recurrent question with the use of PGPR to enhance plant growth is whether their effects are consistent in a range of environments (Dodd and Ruiz-Lozano 2012), or are magnified under specific stresses. Although *V. paradoxus* 5C-2 enhanced plant growth similarly under all salt treatments (Fig. 2 - as indicated by a non-significant inoculation × salt interaction), it seems that the relative importance of some physiological mechanisms differed according to the salinity level. Notably, shoot Na^+ concentration was alleviated by *V. paradoxus* 5C-2 at high salinity (Fig. 4*a*, *b* – as indicated by a significant inoculation × salt interaction), as was the salinity-induced increase of stomatal resistance (Fig. 3*a*). Although the highly significant correlation between shoot Na^+ concentration and stomatal resistance (Table 4) suggests a potential regulatory mechanism whereby apoplastic Na^+ concentration causes stomatal closure (Perera *et al.* 1995), other factors may also regulate stomatal responses. Although stomatal closure acts to limit transpiration and maintain leaf water status, xylem balancing pressure (Fig. 3*b*) increased in response to salinity as reported previously (Termaat *et al.* 1985). Inoculation with *V. paradoxus* 5C-2 decreased xylem balancing pressure (Fig. 3*b*), which may result from increased root hydraulic conductance (*L*), since many rhizobacteria can increase *L* under drought and salinity (Groppa *et al.* 2012). This is especially likely in ACCd-containing rhizobacteria such as *V. paradoxus* 5C-2 that decrease root ethylene synthesis, since increased ethylene synthesis may limit hydraulic conductance by inhibiting aquaporin activity (Li *et al.* 2009).

Although the correlation of xylem balancing pressure with stomatal resistance (Table 4) may be causative, maintaining salinised plants at full turgor (xylem balancing pressure was set to ensure sap was on the verge of bleeding from an incision in the leaf) via root pressurisation did not alleviate salt-induced restriction of transpiration (Termaat *et al.* 1985), suggesting non-hydraulic regulation of stomatal responses. Indeed, well fertilised pea plants inoculated with *V. paradoxus* 5C-2 had significantly lower root ABA concentrations and enhanced ABA degradation in lower leaves, which likely explained the lower stomatal resistance of inoculated plants (Jiang *et al.* 2012). *V. paradoxus* 5C-2 also decreased shoot ABA concentrations of tomato seedlings grown *in vitro* (Belimov *et al.* 2014). Attenuation of liming-induced stomatal closure in the ABA-deficient *wilty* pea mutant (Rothwell *et al.*

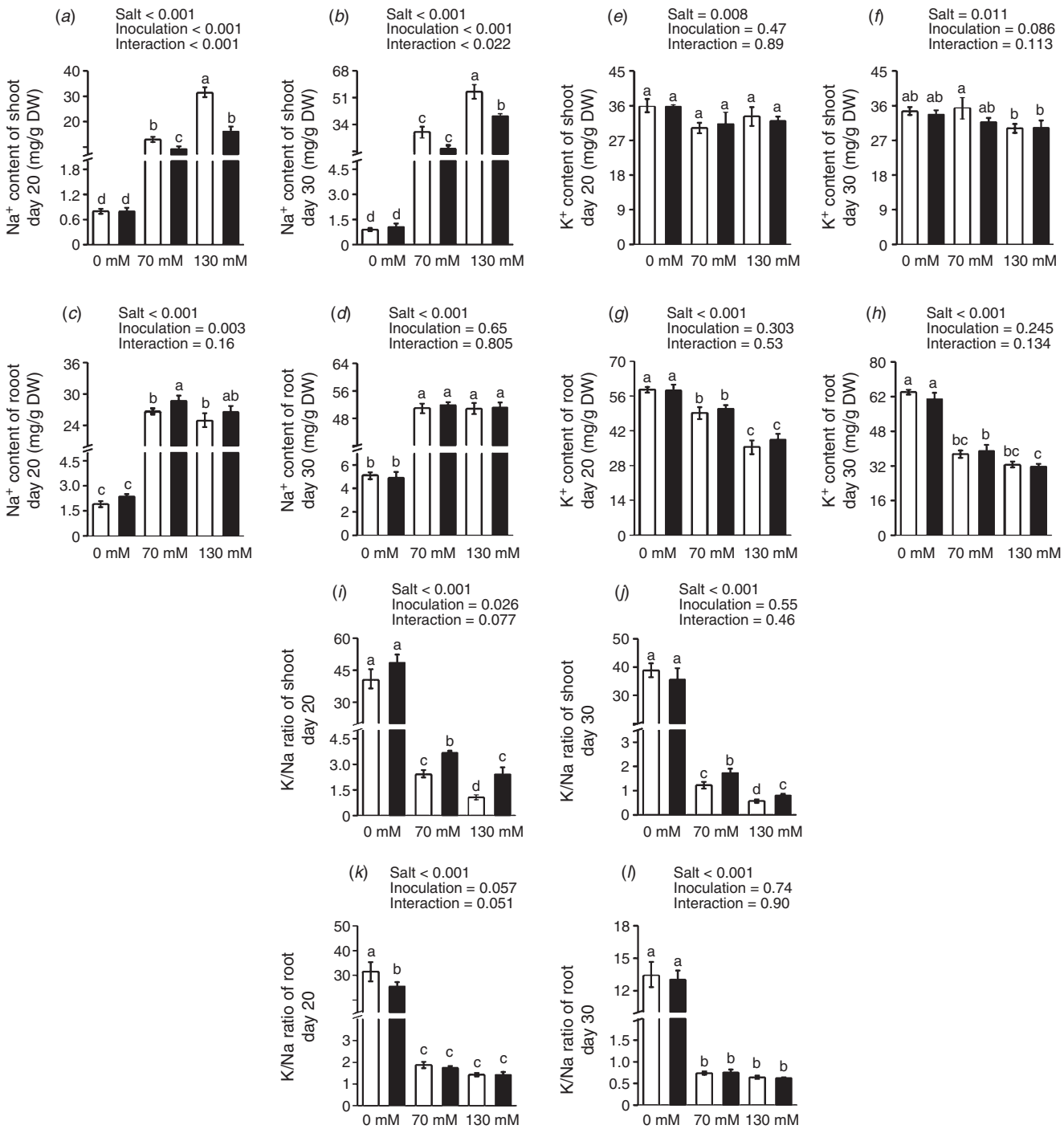

Fig. 4. Sodium concentrations (*a–d*), potassium concentrations (*e–h*) and the ratio of K^+ : Na^+ (*i–l*) in shoot (*a*, *b*, *e*, *f*, *i*, *j*) and root (*c*, *d*, *g*, *h*, *k*, *l*) of pea plants with (closed bars) and without (open bars) inoculation with *Variovorax paradoxus* 5C-2 at the beginning (*a*, *c*, *e*, *g*, *i*, *k*) and the end (*b*, *d*, *f*, *h*, *j*, *l*) of the study period (20 days and 30 days after transplanting, 10 days and 20 days after inoculation and salt treatment). Data are means ± s.e. of four replicates. Different letters above the bars indicate significant differences ($P < 0.05$; Tukey's test) between means. *P*-values determined by two-way ANOVA for salt, inoculation and their interactions are shown.

2015) suggests an important role for ABA in mediating stomatal responses of pea to changes in rhizosphere elemental status, requiring further studies in salinised plants. Nevertheless, distinguishing the relative importance of phytohormonal and nutritional effects on stomatal closure can be challenging (Rothwell *et al.* 2015).

Although increased shoot K^+ : Na^+ ratio in response to ACC-deaminase containing PGPR has been demonstrated previously

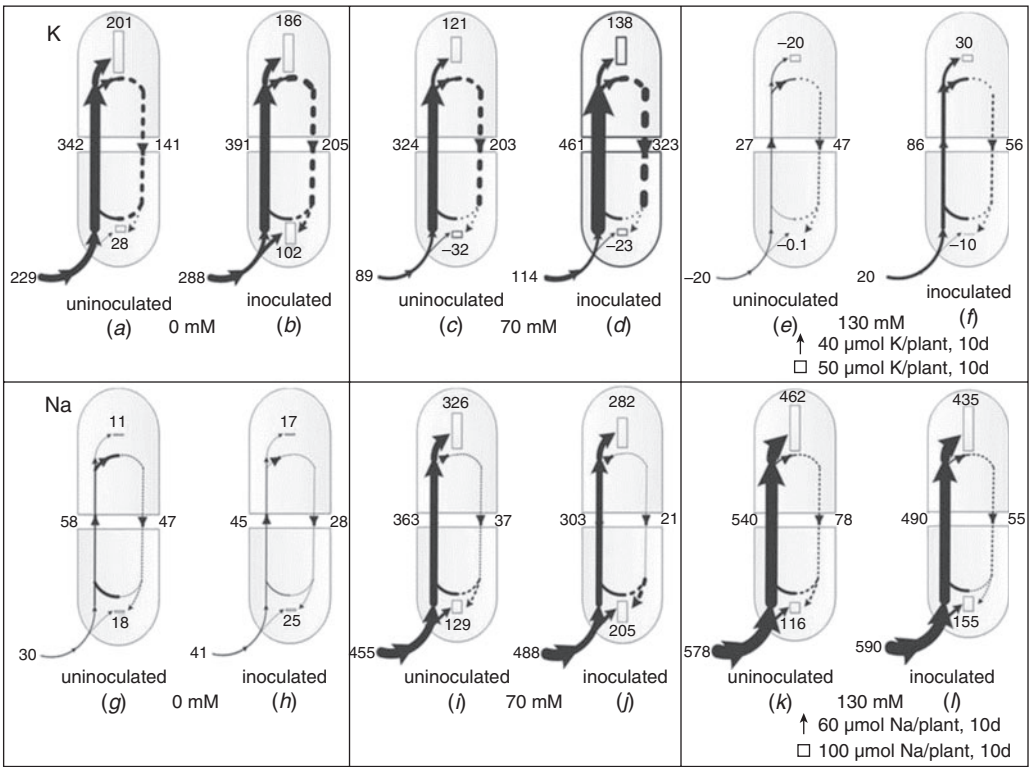

Fig. 5. Empirical models of the uptake, transport and utilisation of potassium (*a–f*) and sodium (*g–l*) of pea plants with and without inoculation with *Variovorax paradoxus* 5C-2 over the study period (20–30 days after transplanting). Arrow widths (net flows in xylem sap (black) or phloem (dotted)) and rectangle heights (deposition in each organ) are drawn in proportion to net flows and the magnitude of depositions respectively.

Table 3. The concentrations of potassium (K^+), sodium (Na^+), calcium (Ca^{2+}) and the ratios of K : Ca and Na : Ca in xylem sap
The statistical analysis is described in Table 2

Treatments		Concentrations in xylem sap (mM)			K : Ca	Na : Ca
NaCl (mM)	*Variovorax paradoxus* 5C-2	K	Na	Ca		
0	Non-inoculated	2.1 ± 0.38d	0.35 ± 0.03c	0.38 ± 0.01b	5.4 ± 0.2d	0.9 ± 0.1e
	Inoculated	2.2 ± 0.32d	0.25 ± 0.05c	0.33 ± 0.05b	6.5 ± 0.6d	0.8 ± 0.1e
70	Non-inoculated	5.4 ± 1.35cd	6 ± 0.7c	0.64 ± 0.06a	8.4 ± 0.8cd	9.4 ± 0.9c
	Inoculated	7.8 ± 1.70bc	5.1 ± 0.3c	0.82 ± 0.15a	9.5 ± 0.7c	6.2 ± 0.4d
130	Non-inoculated	10 ± 2ab	197 ± 6a	0.73 ± 0.1a	13.7 ± 1.2b	270 ± 12a
	Inoculated	12.1 ± 2.31a	69 ± 6b	0.62 ± 0.11a	19.5 ± 1.7a	111 ± 13b
				P-values		
Salt		0.0001	0.0001	0.0001	0.0001	0.0001
Inoculation		0.038	0.0001	0.577	0.025	0.0001
Salt × inoculation interaction		0.361	0.0001	0.079	0.127	0.0001

(Nadeem *et al.* 2007; Nadeem *et al.* 2010), our study showed that it was at least partially due to increased deposition of Na^+ in roots (Fig. 5). In contrast, although root K^+ uptake increased significantly (Table 2), inoculation did not greatly improve K^+ concentrations in plant tissues, likely due to the diluting effect of the increased plant biomass. Nutrient modelling (Fig. 5) has allowed the evaluation of dynamic flows and partitioning of K^+ and Na^+ via phloem and xylem during the study period. Rhizobacterial inoculation increased K^+ uptake by roots and xylem flows of K^+ from root to shoot, which provided a sufficient supply of potassium via xylem (Fig. 5*a–f*) to sustain K^+ deposition in the shoot and a higher growth rate. When plants were salt stressed, a negative K^+ accumulation in roots indicated that K was remobilised from roots into xylem and transported into shoots.

Salt stress increased Na^+ uptake by 15- and 19-fold at 70 and 130 mM NaCl treatments respectively. Although inoculation also increased Na^+ uptake slightly, the xylem stream became depleted in Na^+ due to increased Na^+ deposition in roots (Fig. 5*g–l*). The increased Na^+ deposition

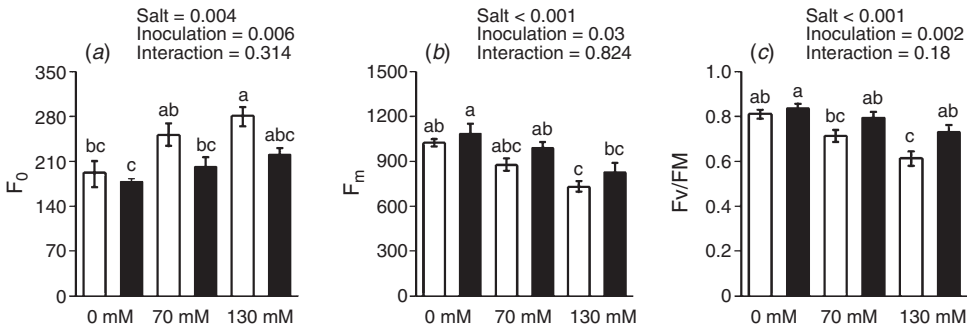

Fig. 6. Chlorophyll fluorescence parameters in leaves of pea plants with (closed bars) and without (open bars) inoculation with *Variovorax paradoxus* 5C-2 at the end of the study period (30 days after transplanting, 20 days after inoculation and salt treatment). F_0 was the minimal fluorescence, F_m was the maximal fluorescence and F_v/F_m was the PSII photochemical efficiency. Data are means ± s.e. of four replicates. Different letters above the bars indicate significant differences ($P < 0.05$; Tukey's test) between means. *P*-values determined by two-way ANOVA for salt, inoculation and their interaction are shown.

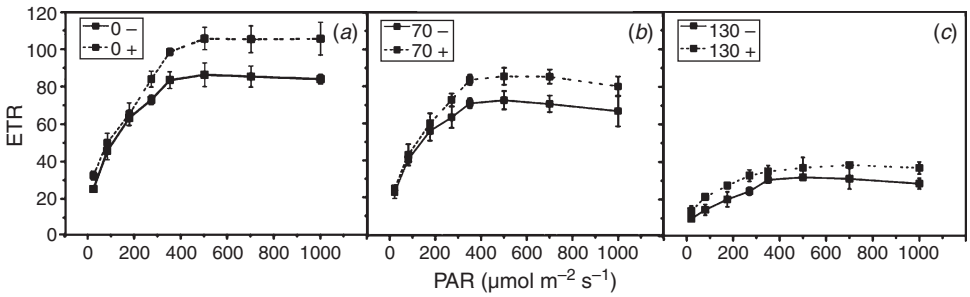

Fig. 7. Relative electron transport rate (ETR) of pea plants grown without salt (*a*) or under 70 mM NaCl (*b*) or 130 mM NaCl (*c*) with (dotted lines) and without (solid lines) inoculation with *V. paradoxus* 5C-2 at the end of the study period (30 days after transplanting, 20 days after inoculation and salt treatment). Data are means ± s.e. of four replicates.

Table 4. Linear correlations between chlorophyll fluorescence parameters, stomatal resistance, shoot Na^+ concentration and xylem balancing pressure

Significant differences are indicated: **, $P < 0.01$: – indicates that no correlation is possible

	F_v/F_m	Maximal ETR	Stomatal resistance	Na^+	Xylem balancing pressure
F_v/F_m	–	0.82**	–0.8**	–0.81**	–0.85**
Maximal ETR		–	–0.94**	–0.92**	–0.76**
Stomatal resistance			–	0.93**	0.75**
Na^+				–	0.8**
Xylem balancing pressure					–

in roots of inoculated plants may be due to changes in gene expression (such as the sodium transporters HKT1 and SOS1) (Shi *et al.* 2002; Zhang *et al.* 2008). Under NaCl stress, inoculation decreased Na^+ deposition in shoot tissue, which is simply due to the decreased xylem Na^+ flow from root to shoot, despite lower stomatal resistance. Future studies need to consider both the molecular regulation of Na^+ uptake and xylem loading of Na^+ in inoculated pea roots.

Besides the positive effects of *V. paradoxus* 5C-2 on the K and Na balance, the bacterium also enhanced the uptake of Ca, Mg and P (Table 2). Similarly, *Lotus edulis* plants inoculated with *V. paradoxus* 5C-2 and grown in heavy metal contaminated soil had increased Ca, Mg and P content and increased shoot accumulation of several nutrient elements including K (Safronova *et al.* 2012), which may be simply due to improved root growth. Improved uptake of K and other nutrients by the plants inoculated with *V. paradoxus* 5C-2 may enhance photosynthesis.

As is well known, salinity induced stomatal closure by an osmotic stress and displaced essential cations from the endomembrane structure and degraded thylakoid membrane proteins (Flowers and Yeo 1981), thereby causing photoinhibition. In our study, salt stress decreased photosynthetic efficiency (F_v/F_m) and ETR of pea plants while inoculation with *V. paradoxus* 5C-2 mitigated these declines (Figs 6, 7). Limitation of Na^+

transport into, and accumulation in, the shoot (Fig. 5) may also contribute to the enhanced photosynthesis by alleviating the damage of photosynthetic apparatus caused by excessive Na^+ (Seemann and Critchley 1985). Although chlorophyll fluorescence is correlated with both shoot Na^+ concentration and stomatal resistance, resolving the contributions of both stomatal and non-stomatal limitations to photosynthesis requires further work (Table 4).

Decreased photosynthesis in response to salt stress might be expected to decrease carbohydrate flow to the root system and root exudation, thereby limiting the establishment of symbiotic plant/microbe interactions in the rhizosphere. Although it is well known that high salinity can decrease nodulation of legumes by rhizobia (Steinborn and Roughley 1974; Steil *et al.* 2003; Barea *et al.* 2005), effects of salinity on associative PGPR inhabiting the rhizosphere may be variable. Although high salinity increased root colonisation of lettuce with PGPR *Pseudomonas mendocina* (Steil *et al.* 2003), pea root colonisation of *V. paradoxus* 5C-2 was independent of salinity level (Table 1), similar to experiments where soil drying had no effect on, or even increased, colonisation of *V. paradoxus* 5C-2 (Belimov *et al.* 2009). Salinity-induced increases of root ACC concentration (Albacete *et al.* 2008) may enhance root ACC efflux thereby increasing substrate availability for ACC-deaminase containing PGPR. Indeed, several such rhizobacteria were recently shown to decrease rhizosphere ACC concentration (Belimov *et al.* 2015) but separating the contributions of root ACC efflux from bacterial ACC utilisation remains challenging.

Conclusions

Although ACC-deaminase containing rhizobacteria have previously been shown to improve plant growth under salt stress (Mayak *et al.* 2004; Bal *et al.* 2013; Qin *et al.* 2014), this study is the first to investigate ion homeostasis using nutrient flow modelling, and to demonstrate both stomatal and non-stomatal (F_v/F_m and ETR) effects of ACC-deaminase containing rhizobacteria on photosynthesis. Although the relative importance of these different mechanisms remains to be established, *V. paradoxus* 5C-2 may be an important and economic means of decreasing the deleterious effects of saline soils on plant growth.

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