Abscisic acid-utilizing rhizobacteria disturb nitrogen-fixing symbiosis of pea *Pisum sativum* L.

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Abstract

Rhizosphere bacteria are capable of utilizing various phytohormones (particularly auxins) as nutrients and thereby affect plant growth, nutrition and interactions with symbiotic microorganisms. Here, for the first time we evaluated the effects of rhizosphere bacteria *Novosphingobium* sp. P6W and *Rhodococcus* sp. P1Y capable of utilizing abscisic acid (ABA) on growth and nitrogen-fixing symbiosis of pea (*Pisum sativum* L.) line SGE and its Cd-insensitive mutant SGECd^t using hydroponic culture. The plants were co-inoculated with the ABA-utilizing bacteria and nodule bacterium *Rhizobium leguminosarum* bv. *viciae* RCAM1066. Treatment with cadmium (Cd) was applied as an inducer of ABA biosynthesis in plants. In the presence of only nodule bacteria, Cd significantly inhibited the growth of roots and shoots and also decreased the nodule number and nitrogen-fixing activity in SGE peas, but not in the SGECd^t mutant. Inoculation with ABA-utilizing bacteria also inhibited biomass production, nodulation and nitrogen-fixation of Cd-untreated SGE plants. This negative effect of bacteria on the SGECd^t mutant was less pronounced. Contrary to this, ABA-utilizing bacteria had no effect on SGE plants treated with Cd, but decreased shoot biomass and nitrogen-fixing activity of the SGECd^t mutant. Inoculation with ABA-utilizing bacteria had no effect on shoot Cd and nutrient content of both pea genotypes, suggesting that bacterial effects on plants were not associated with the plant nutrient status. We propose that the bacteria counteracted the increased ABA concentrations in SGE roots caused by Cd due to utilization of this phytohormone. However, opposite processes aimed at inhibiting and stimulating growth and legume–rhizobia symbiosis can be caused by the ABA-utilizing bacteria.

Keywords: abscisic acid, cadmium, nitrogen fixation, nodulation, *Novosphingobium*, *Rhodococcus*, pea, phytohormones, PGPR, symbiosis

Introduction

The production of phytohormones (auxins, cytokinins, and gibberellins) by bacteria is one of the most important mechanisms of interaction between plant and bacterial associations (Frankenberger and Arshad, 1995; Dodd, Zinovkina, Safronova and Belimov, 2010). Most attention has been paid to the role of bacterial auxins in stimulating plant growth and nutrition, since the ability to synthesize the phytohormone indole-3-acetic acid (IAA) is widespread among bacteria (Spaepen, Vanderleyden and Remans, 2007). The ability to synthesize the phytohormone abscisic acid (ABA) has been described in various phytopathogenic fungi (Frankenberger and Arshad, 1995; Syrova et al., 2019) and in plant growth-promoting bacteria (PGPB) such as *Azospirillum brasilense* (Perrig et al., 2007; Cohen et al., 2009), *Achromobacter xylosoxidans* (Forchetti et al., 2007), *Brevibac-*

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terium halotolerans and several *Bacillus* species (Sgroy et al., 2009). It was also shown that inoculation with ABA-producing bacteria can change the content of this hormone in plants (Cohen et al., 2009).

Symbiotic bacteria can not only synthesize, but also destroy phytohormones, in particular by utilizing them as a nutrient source, and thereby they have a significant effect on plant metabolism (Dodd, Zinovkina, Safronova and Belimov, 2010). The ability of bacteria to utilize IAA has been known for a relatively long time, and the bacterial genes and enzymes involved in this process in bacteria are known (Frankenberger and Arshad, 1995). The important role of PGPB containing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase in plant growth stimulation due to modulation of phytohormone ethylene biosynthesis is well documented (Glick, Cheng, Czarny and Duan, 2007; Belimov and Safronova, 2011; Nascimento et al., 2014). In particular, ACC-utilizing bacteria increased plant resistance to abiotic and biotic stresses, and also improved the formation of nitrogen-fixing symbiosis of leguminous plants with nodule bacteria.

Microorganisms that degrade other phytohormones remain scarcely studied. It is only known that the bacterium *Serratia proteamaculans* metabolized the artificial cytokinin N-benzyladenine using the enzyme xanthine dehydrogenase (Taylor et al., 2006). The ability to degrade gibberellins (GA20 glycosides) was described for *Azospirillum lipoferum* (Cassan, Bottini, Schneider and Piccoli, 2001). Bacteria of the genus *Pseudomonas* hydrolyzed salicylic acid with the formation of catechol (Yen and Serdar, 1988). The soil bacterium *Corynebacterium* sp. isolated from soil was capable of decomposing ABA with the formation of dehydrovomifoliol (Hasegawa, Poling, Mayer and Bennett, 1984). But the ecological role of these bacteria and their interactions with plants have not been studied. Recently, ABA-utilizing rhizosphere bacteria *Novosphingobium* sp. P6W and *Rhodococcus* sp. P1Y were characterized and were able to decrease ABA content and alter plant growth in inoculated rice and tomato seedlings (Belimov et al., 2014). It should be mentioned that the studied strains are the only rhizosphere bacteria described to date as ABA utilizers. However, the effect of such bacteria on nitrogen-fixing legume–rhizobia symbiosis has not been studied.

The phytohormone ABA is intensively synthesized in plants under osmotic stress (Davies and Zhang, 1991). Treatments with Cd decreased stomatal conductance in plants, probably due to increased ABA concentrations (Poschenrieder, Gunse and Barcelo, 1989). A dramatic increase in xylem ABA concentration was observed in Cd-treated pea line SGE, whereas it was scarcely affected in the xylem of its Cd-tolerant mutant SGECd^t (Belimov et al., 2015). ABA is known to be a negative regulator of legume root nodule formation in various plant species (Suzuki et al., 2004; Ding et al., 2008; Tominaga et al., 2010; Liu et al., 2018) including peas (Phillips, 1971). Cadmium also had a more pronounced negative effect on nodulation and nitrogen fixation of pea SGE as compared to the SGECd^t mutant (Belimov et al., 2019). These observations allowed us to propose that ABA-utilizing rhizosphere bacteria *Rhodococcus* sp. P1Y and *Novosphingobium* sp. P6W may affect the formation of legume–rhizobia symbiosis. To test this hypothesis, a model system based on the Cd-insensitive SGECd^t mutant processed with Cd as an ABA inducer was applied.

Materials and methods

Seeds of wild-type pea (*Pisum sativum* L.) line SGE and its Cd-tolerant mutant SGECd^t (Tsyganov et al., 2007) were surface sterilized and scarified by treatment with 98 % H_2SO_4 for 30 min, rinsed with sterile tap water and germinated on filter paper in Petri dishes for three days at 25 °C in the dark. Seedlings were transferred to plastic pots (three pots with 3 seeds per genotype) containing 800 mL of aerated nutrient solution (μmol L^{-1}): KH_2PO_4, 400; KNO_3, 1200; $Ca(NO_3)_2$, 60; $MgSO_4$, 250; KCl, 250; $CaCl_2$, 60; Fe-tartrate, 10; H_3BO_3, 2; $MnSO_4$, 4; $ZnSO_4$, 3; NaCl, 6; Na_2MoO_4, 0.06; $CoCl_2$, 0.06; $CuCl_2$, 0.06; $NiCl_2$, 0.06; pH = 5.5. The nutrient solution was supplemented or not with 0.5 μmol L^{-1} $CdCl_2$ and with nodule bacterium *Rhizobium leguminosarum* bv. *viciae* RCAM1066 and/or with rhizobacteria *Rhodococcus* sp. P1Y and *Novosphingobium* sp. P6W in the amount of 10^8 cells L^{-1}. For this purpose the bacteria were cultivated on agar yeast extract mannitol (YM) agar (Vincent, 1970) for 5 days and then suspended in nutrient solution. The plants were cultivated in a growth chamber for 45 days with 400 μmol quanta m^{-2} s^{-1}, 12 h photoperiod with minimum/maximum temperatures of 18 °C/23 °C respectively. Nutrient solution was changed and supplemented with Cd and bacteria every 5 days. In the end of the experiment the roots were collected, the nodules counted and the nitrogen fixation activity on the roots was measured by the acetylene-reduction method (Turner and Gibson, 1980) using a gas chromatograph GC-2014 (Shimadzu, Japan).

The dried plant shoots were ground to a powder and total nitrogen content was determined using a Kjeltec 2300 Auto Distillation unit (FOSS Analytical, Denmark). To determine Cd and nutrient (Ca, K, Mg, Mn, S, and P) contents, the ground shoot samples were digested in a mixture of concentrated HNO_3 and 38 % H_2O_2 at 70 °C using DigiBlock digester (LabTech, Italy). The elemental content of digested plant samples was determined using an inductively coupled plasma emission spectrometer ICPE-9000 (Shimadzu, Japan).

Statistical analysis of the data was performed using the software STATISTICA version 10 (TIBCO Software Inc., USA). Variance analysis and Fisher's LSD test were used to evaluate differences between means.

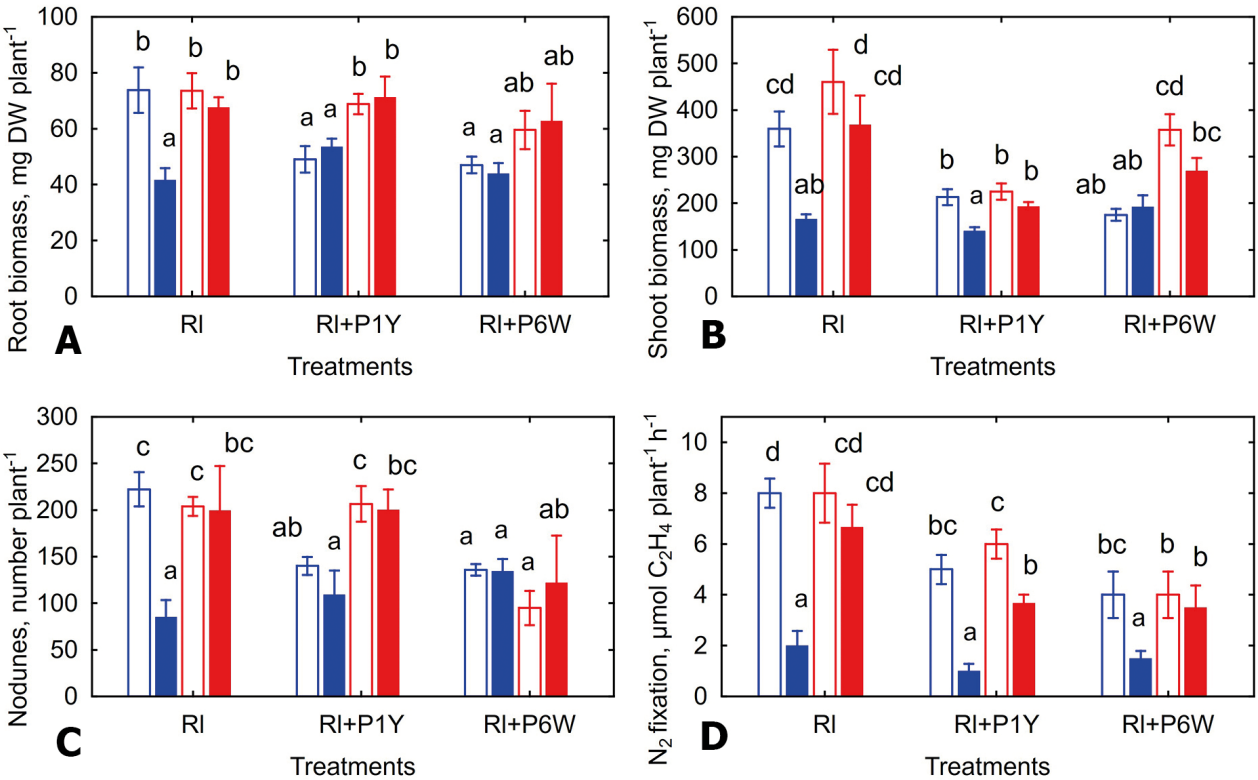

Fig. 1. Effect of rhizobacteria and cadmium on root (A) and shoot (B) biomass, nodulation (C) and nitrogen fixation (D) of pea. Bacterial strains: RI — *Rhizobium leguminosarum* bv. *viciae* RCAM1066; P6W — *Novosphingobium* sp. P6W; P1Y — and *Rhodococcus* sp. P1Y. Blue boxes — wild type pea SGE. Red boxes — pea mutant SGECdt. Empty boxes — Cadmium-untreated plants. Filled boxes — plants treated with 0.5 μmol L^{-1} $CdCl_2$. Different letters show significant differences between treatments and genotypes (Fisher's LSD test, $P < 0.05$).

Results and discussion

In the presence of only nodule bacteria, Cd significantly inhibited the growth of roots (Fig. 1A) and shoots (Fig. 1B), and also decreased the nodule number (Fig. 1C) and nitrogen-fixing activity (Fig. 1D) in SGE peas, but not in the SGECd^t mutant. This result is in line with our previous reports about increased Cd tolerance of the SGECd^t mutant (Tsyganow et al., 2007; Malkov, Zinovkina, Safronova and Belimov, 2012; Belimov et al., 2019). Additional inoculation with either strain of ABA-utilizing bacteria inhibited biomass production, nodulation and nitrogen-fixation of Cd-untreated SGE plants (Fig. 1). Such negative effect on the SGECd^t mutant was evident only on shoot biomass of plants inoculated with *Rhodococcus* sp. P1Y (Fig. 1B) and on nodulation and nitrogen-fixation of plants inoculated with *Novosphingobium* sp. P6W (Fig. 1C, D). Contrary to this, ABA-utilizing bacteria had no effect on SGE plants treated with Cd. In the presence of Cd, the strain *Rhodococcus* sp. P1Y decreased shoot biomass, and both ABA-utilizing strains had an inhibitory effect on the nitrogen-fixing activity of the SGECd^t mutant (Fig. 1B, D). The results showed that Cd treatment changed the response of pea plants to inoculation with ABA-utilizing bacteria and these changes (namely the elimination of negative effects of bacteria) were more pronounced in wild type SGE as compared to the SGECd^t mutant. Indeed, the negative effects of bacteria on Cd-treated SGECd^t plants were partially retained.

The SGECd^t mutant showed increased Cd content in shoots (Table 1), supporting previous findings about its increased ability to accumulate this toxic element (Tsyganow et al., 2007; Belimov et al., 2015). Cd-treated SGECd^t tended to have increased shoot N content by 18 % as estimated by average values for all treatments (Table 1). Shoot content of nutrient elements (K, Mg, Mn, S, and P) was not affected by Cd or pea genotype (data not shown). The only exception was Ca content in shoots of SGECd^t being 12 % higher as compared to wild type SGE (Table 1). Treatment with Cd tended to decrease Ca in SGE shoots. Previously we showed that maintenance of nutrient homeostasis is one of the mechanisms involved in Cd tolerance of SGECd^t, and Ca plays an important role in this trait (Tsyganow et al., 2007). It is well known, that changes in the uptake of nutrient elements by plants is an important mechanism of plant–PGPB interactions (Pii et al., 2015). Here we showed that inoculation with ABA-utilizing bacteria had no effect on shoot Cd and nutrient content of both

Table 1. Cadmium and nutrients content in pea shoots

Treatments and genotypes	Cd, μg g^{-1} DW		N, mg g^{-1} DW		Ca, mg g^{-1} DW	
	Control	Cd treated	Control	Cd treated	Control	Cd treated
R. leguminosarum bv. *viciae* RCAM1066						
SGE	ND	7.5 ± 0.9 a	22 ± 2 a	17 ± 2 a	12.7 ± 0.3 a	12.1 ± 0.2 a
SGECd^t	ND	12.3 ± 1.5 b	22 ± 3 a	22 ± 2 a	13.0 ± 0.3 a	13.2 ± 0.3 b
R. leguminosarum bv. *viciae* RCAM1066 + *Rhodococcus* sp. P1Y						
SGE	ND	7.1 ± 0.8 a	21 ± 2 a	17 ± 3 a	12.5 ± 0.4 a	12.2 ± 0.4 ab
SGECd^t	ND	13.5 ± 2.0 b	21 ± 1 a	19 ± 2 a	13.2 ± 0.3 a	13.3 ± 0.3 ab
R. leguminosarum bv. *viciae* RCAM1066 + *Novosphingobium* sp. P6W						
SGE	ND	7.3 ± 1.1 a	19 ± 3 a	18 ± 2 a	12.7 ± 0.5 a	11.7 ± 0.3 a
SGECd^t	ND	13.1 ± 2.1 b	18 ± 3 a	20 ± 3 a	12.5 ± 0.5 a	13.0 ± 0.5 b
Averages for all treatments						
SGE	ND	7.3 ± 0.1	21 ± 1	17 ± 1	12.6 ± 0.1	12.0 ± 0.1
SGECd^t	ND	13.0 ± 0.3 *	20 ± 1	20 ± 1 *	12.9 ± 0.2	13.2 ± 0.1 *

ND stands for not detected.
The data are means ± SE.
Different letters show significant differences between treatments and genotypes, asterisks show significant differences between average values for pea genotypes (Fisher's LSD test, $P < 0.05$).

pea genotypes, suggesting that bacterial effects on plants were not associated with the plant nutrient status.

The studied ABA-utilizing bacteria had negative effects on pea growth and symbiosis with rhizobia in the absence of toxic Cd. Previously we demonstrated inhibition of root elongation of rice and tomato seedlings by *Novosphingobium* sp. P6W (Belimov et al., 2014). However, other information about interactions between ABA-utilizing bacteria and plants is limited. In the presence of Cd the negative effects of the bacteria on wild type SGE were completely eliminated, whereas they were partially retained on the SGECd^t mutant having the Cd-insensitive phenotype. Our previous observation showed that Cd treatments increased ABA concentration by several times in xylem sap of SGE only (Belimov et al., 2015). Therefore we assume that Cd might increase ABA concentrations in SGE roots and the observed genotypic difference in response to ABA-utilizing bacteria might be related to modulation of the plant ABA status. This is in line with information about induction of ABA biosynthesis by Cd (Poschenrieder, Gunse and Barcelo, 1989) and negative effects of elevated ABA concentrations on the development and function of legume–rhizobia symbiosis (Phillips, 1971; Suzuki et al., 2004; Ding et al., 2008; Tominaga et al., 2010; Liu et al., 2018).

It may be proposed that the bacteria counteracted the increased ABA concentrations in SGE roots caused by Cd due to utilization of this phytohormone. Such effect was much less pronounced in SGECd^t, which does not respond to Cd by the increase in ABA concentration (Belimov et al., 2015). The results also suggest that negative effects of the studied bacteria on plants in the absence of toxic Cd (when root ABA concentration was presumably low) were not associated with their ability to utilize ABA. More likely it was caused by the release of some unknown growth-inhibiting compounds. For example, the strain *Novosphingobium* sp. P6W was characterized as an IAA hyper-producer (Belimov et al., 2014). However, bacterial ABA utilization became important for restoring hormonal balance in plants in the presence of toxic cadmium. Thus, opposite processes aimed at inhibiting and stimulating growth and symbiosis can be caused by the ABA-utilizing bacteria. To test this hypothesis, further study using bacterial mutants unable to utilize ABA and monitoring ABA concentrations in plants is needed.

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