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ACC deaminase-producing rhizosphere bacteria modulate plant responses to flooding

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Summary

1. Flooding events are predicted to increase over the coming decades, calling for a better understanding of plant responses to submergence. Specific root-associated microbes alter plant hormonal balance, affecting plant growth and stress tolerance. We hypothesized that the presence of such microbes may modulate plant responses to submergence.

2. We tested whether root-associated bacteria producing the enzyme ACC (1-aminocyclopropane-1-carboxylate) deaminase affect submergence responses in *Rumex palustris*, a flood-tolerant riparian plant. Ethylene is a key plant hormone regulating flood-associated acclimations, and ACC deaminase activity of bacteria may decrease ethylene levels in the plant. *Rumex palustris* plants were inoculated with *Pseudomonas putida UW4* or an isogenic mutant lacking ACC deaminase, and subsequently exposed to complete submergence.

3. Submergence triggered ethylene-mediated responses, including an increase in leaf elongation and shoot fresh weight. Flood responses, including post-submergence ethylene production, were reduced in plants inoculated with ACC deaminase-producing wild type bacteria, as compared to plants inoculated with the ACC deaminase-negative mutant.

4. *Synthesis.* We demonstrate that root-associated bacteria can alter plant response to environmental stress by altering plant hormonal balance. Plant–microbe interactions may thus be an overseen driver of plant life-history strategies that should be taken into account when assessing plant ecological adaptations such as abiotic stress resistance.

Key-words: ACC deaminase, ecophysiology, ethylene, PGP-bacteria, plastic response, *Rumex palustris*, submergence

Introduction

Flooding is a major stress for plants, and extreme flooding events are predicted to increase in many parts of the world (Hirabayashi *et al.* 2013). Thus, better understanding of how plants adapt to flooding may be crucial to mitigate the effects of increasing flooding frequency on natural and cultivated ecosystems.

Flooding reduces oxygen and light availability for plants, resulting in an energy and carbohydrate crisis (Voesenek & Bailey-Serres 2015). Another consequence of flooding is the rapid accumulation of ethylene within flooded plant organs due to restricted outward diffusion of this gas underwater. Two general contrasting flooding survival strategies have been described in plants: quiescence and escape (Bailey-Serres & Voesenek 2008). These survival strategies restrict growth to conserve energy or stimulate growth to outgrow the flood water respectively. Ethylene is an important regulator of both these strategies (Sasidharan & Voesenek 2015).

Interestingly, specific soil microbes can interfere with ethylene levels and thus signalling, potentially affecting plant phenotypes. For instance, root-associated bacteria producing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase can degrade the ethylene precursor ACC, thereby reducing ethylene levels inside plants (Grichko & Glick 2001a; Penrose & Glick 2003; Barnawal *et al.* 2012; Glick 2014). Until now, these bacteria have mainly been studied in the context of crop plants, where they prevent the accumulation of deleteriously high ethylene levels (Mayak, Tirosh & Glick 2004; Jalili *et al.* 2009; Chen *et al.* 2013; Glick 2014). However, the same enzyme may also cause reduced ethylene levels in plants that require ethylene for adaptive growth responses. This could affect the survival of these plants for instance under flooded conditions, when proper ethylene-based signalling is required. ACC deaminase-producing bacteria are very common in the rhizosphere (Ma *et al.* 2003; Duan *et al.*

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2008). For instance, 12% of all isolated bacteria from 30 different sites in Canada were shown to carry this enzyme (Duan *et al.* 2008). We therefore propose that ACC deaminase-producing bacteria in the rhizosphere may be important in the ultimate development of adaptive flooding-induced plant traits, thereby affecting plant fitness.

We tested this hypothesis using *Rumex palustris Sm.*, a widespread temperate plant species tolerant to waterlogging and submergence that has been used as a model plant for submergence research for decades (Peeters *et al.* 2002). In the vegetative phase of its life cycle, this species responds to complete submergence by accelerated petiole elongation, resulting in longer leaves. This growth response allows the leaf tips to emerge above water if the flood is not too deep, thereby ensuring inward diffusion of oxygen and improved survival. Ethylene accumulation in flooded petioles of *Rumex* has been shown to be critical for the rapid elongation of leaves when submerged (Voesenek & Blom 1989).

To test our hypothesis, we inoculated *R. palustris* plants with an ACC deaminase-producing strain, *Pseudomonas putida UW4* (later 'WT'), or an isogenic ACC deaminase-deficient mutant (later referred to as 'mutant'). We then subjected plants to short- and long-term submergence. We recorded ethylene production rates, plant elongation and biomass production after 3 and 17 days of full submergence. We expected that (i) plant ethylene levels would increase under submergence, triggering elongation of the aerial parts and that (ii) bacteria producing ACC deaminase would interfere with ethylene accumulation thereby impairing the adaptive response of *R. palustris* to submergence.

Materials and methods

PLANT MATERIAL

Seeds of *Rumex palustris Sm.* were collected (2014) from plants grown in a common garden on the Utrecht University campus, the Netherlands. Seeds were germinated on polyethylene beads (Elf Atochem, Marseille, France) floating on water in a transparent container (12 h of light, 25 °C, 70 μmol m^{-2} s^{-1} photon flux density and 12 h of dark, 10 °C). Twelve-day-old seedlings were transplanted into small plastic pots (70 mL), filled with a mixture (ratio 2 : 1) of potting soil (Jonkind, the Netherlands, autoclaved at 90 °C) and sand (autoclaved at 130 °C), enriched with 0·14 mg of MgOCaO (17% w/w of each MgO and CaO in compound, Vitasol BV, Stolwijk, The Netherlands) per pot, and saturated with 20 mL of nutrient solution containing: 7·50 mM $(NH_4)_2SO_4$, 15·00 mM KH_2PO_4, 15·00 mM KNO_3, 86·35 μM Fe-EDTA, 4·27 μM $MnSO_4$, 1·81 μM $ZnSO_4$, 0·32 μM $CuSO_4$, 42·67 μM H_3BO_3 and 0·53 μM Na_2MoO_4 (Analytic grade, Merck, Darmstadt, Germany). Pots were placed on irrigation mats (Brinkman Agro BV, s'-Gravenzande, The Netherlands) in a walk-in-controlled growth chamber (20 °C; 70% relative humidity, 16 h of light; 200 μmol m^{-2} s^{-1} photon flux density) and watered automatically with tap water to saturation twice a day.

BACTERIAL STRAINS

Pseudomonas putida UW4 wild type strain (hereafter referred to as WT) and an isogenic ACC (1-aminocyclopropane-1-carboxylate) deaminase-deficient mutant (hereafter referred to as mutant) were obtained from Prof. Bernard Glick, Department of Biology, University of Waterloo, Waterloo, ON, Canada. *Pseudomonas putida UW4* is a bacterial model for the studies of ACC deaminase production, and it has been shown to reduce ethylene levels in plants (Glick 2014). The ACC deaminase-deficient mutant was obtained by insertion of a tetracycline resistance gene in the ACC deaminase gene-coding region (Li *et al.* 2000). Bacteria were kept as frozen stocks at −80 °C. Prior to experiments, one single colony was grown overnight on DF (Dworkin & Foster 1958) salt minimal medium supplemented with ACC (to isolate WT ACC deaminase containing bacteria) or $(NH_4)_2SO_4$ (to isolate mutant bacteria lacking ACC deaminase) as nitrogen sources (Penrose & Glick 2003). Bacteria were harvested by centrifugation (6000 *g*, 10 min) and washed three times with 10 mM $MgSO_4$ and adjusted to an OD_{600} (optical density on 600 nm) of 0·5 before inoculation. A total of 250 μL of bacteria suspension was inoculated to the base of each plant after seedling transfer.

EXPERIMENTAL DESIGN, GROWTH CONDITIONS AND MEASUREMENTS

Plants were inoculated with one of three bacterial treatments (uninoculated control, WT, mutant) 7 days after the transfer of the seedlings to the pots. After 2 weeks, plants with four leaves were selected based on homogeneity of developmental stage. The youngest leaf (petiole + leaf blade) size (leaf no. 4) and petiole size (leaf no. 3 and 2) were measured before and after 72 h of complete submergence. This developmental stage was selected because *R. palustris* shows strong ethylene-mediated responses to complete submergence at this stage (Cox *et al.* 2004). Plants (except non-submergence controls) were exposed to a submergence treatment by total immersion in tap water in climate chamber [20 °C, 16 h daytime at PPFD: 100–130 μmol m^{-2} s^{-1} (Philips TDL 58 W/84 and 400-W SON-T sodium lamps) and 8 h of darkness], for 72 h (short-term submergence) and 17 days (long-term submergence). After 72 h, for each bacterial treatment, eight plants stayed completely submerged for 17 days, and eight plants were de-submerged and immediately young leaf length (petiole + leaf blade; leaf 4), petiole length (leaf 3 and 2) and total shoot fresh weight were measured. Shoots were separated from the roots with a razor blade and de-submergence-induced ethylene production was measured using Chrompack Packard gas chromatograph model 438 A, with a Poropack Q column (length 100 cm, packed to 0·34 g cm^{-3}) at 60 °C based on standard procedure (Voesenek *et al.* 2003b). Root-associated bacterial population densities were measured (next section), and shoot and root dry weights were determined after drying at 70 °C for 48–72 h until a constant weight was obtained. After 17 days (long-term submergence), fresh weight and dry weight of roots and shoots were also measured. The plant height was measured every day from images taken using a Nikon D90 (Tokyo, Japan), and final plant heights were used for analysis.

BACTERIAL DENSITY ON PLANT ROOTS

We assessed the density of the WT bacterial strain in the short-term submergence treatment. Briefly, roots were gently shaken to remove adhering soil, and bacteria were recovered by shaking the roots in 10 mM $MgSO_4$ for 30 min at 200 min^{-1}, sonicating them for 20 s and vortexing for 30 s. Re-suspended bacteria were enumerated on DF salt minimal medium (Dworkin & Foster 1958) supplemented with ACC as the sole nitrogen source (Penrose & Glick 2003). The ACC deaminase-deficient mutant strain was enumerated by drop

plating on DF salt minimal medium (Dworkin & Foster 1958) supplemented with $(NH_4)_2SO_4$ as nitrogen source and 100 μg mL^{-1} tetracycline.

STATISTICAL ANALYSIS

Independent effects of 72 h and 17 days of submergence and bacterial treatments on petiole size, leaf size, dry weight, fresh weight, plant height and post-submergence ethylene production rates were evaluated using separate one-way ANOVAs. If the *F*-test of variance ratio was significant ($P < 0{\cdot}05$), individual means were compared by post hoc Duncan's multiple range test.

The correlation between bacterial density on rhizoplane and the post-submergence ethylene production rate was evaluated with one-way ANOVA on the log-transformed bacterial abundance (continuous predictor). All analyses were performed in SPSS (V. 22).

We used a one-way ANOVA with contrasts to assess the effects of bacterial inoculation (WT and mutant vs. control), ACC deaminase enzyme (WT vs. mutant) and effect due to the genetic background of the bacteria independently of ACC deaminase activity (mutant vs. control). The confidence levels were adjusted based on the number of contrasts.

Results

EFFECTS OF BACTERIAL INOCULATION ON *R. PALUSTRIS* RESPONSES TO SHORT-TERM SUBMERGENCE

Post-submergence ethylene production

Post-submergence ethylene production increased significantly in submerged *R. palustris* from 7·82 pmol g^{-1} Fresh Weight (FW) h^{-1} (non-submerged control) to 288·64 pmol g^{-1} FW h^{-1} (submerged) ($F_{1,15} = 236{\cdot}10$, $P < 0{\cdot}001$; Fig. 1a). Inoculation of *R. palustris* with ACC (1-aminocyclopropane-1-carboxylate) deaminase-producing bacteria (WT) decreased significantly ($F_{2,23} = 6{\cdot}50$; $P = 0{\cdot}006$) the post-submergence ethylene production rate compared to inoculation with the ACC deaminase-deficient mutant (mutant) from 353·94 to 238·29 pmol g^{-1} FW h^{-1} (Fig. 1b).

Plant inoculation with the ACC deaminase-negative mutant bacterial strain increased post-submergence ethylene production ($t_{20} = 2{\cdot}01$; $P = 0{\cdot}06$) in *R. palustris* compared to the bacteria-free control treatment, suggesting positive effects of other bacterial traits on this factor in submergence condition (Fig. 1b).

There was a strong negative correlation ($R^2 = -0{\cdot}58$) between the WT population density on the rhizoplane and post-submergence ethylene production in *R. palustris* associated with WT bacteria ($F_{1,8} = 11{\cdot}54$; $P = 0{\cdot}01$), indicating that ACC deaminase-producing bacteria decreased the post-submergence ethylene production rate under submergence conditions (Fig. 2).

Rumex palustris leaf morphology

The length of the youngest leaf (leaf no. 4) of *R. palustris* increased significantly from 7·25 mm to 66·22 mm after 72 h submergence ($F_{1,15} = 500{\cdot}65$; $P < 0{\cdot}001$; Fig. 1a). Submergence also resulted in significantly larger older petioles: leaf 2 ($F_{1,15} = 89{\cdot}96$; $P < 0{\cdot}001$) and leaf 3 ($F_{1,15} = 191{\cdot}47$; $P < 0{\cdot}001$) from 1·15 to 20·35 mm and 0·75 mm to 12·40 mm, respectively (Fig. 1a).

The effects of the root-associated bacterial strains (WT and ACC deaminase-deficient mutant) on young leaf size and older petiole elongation of submerged *R. palustris* were investigated by separate one-way ANOVA tests complemented with contrast analysis. WT bacterial inoculation decreased elongation of the youngest leaf ($F_{2,23} = 6{\cdot}32$; $P = 0{\cdot}007$) and of the older third leaf petiole ($F_{2,23} = 2{\cdot}08$; $P = 0{\cdot}15$) compared to ACC deaminase-negative mutant bacterial inoculation after 72 h submergence (Fig. 1b).

Total shoot fresh weight of *R. palustris* increased significantly after 72 h submergence from 1·06 g to 2·13 g ($F_{1,15} = 23{\cdot}94$, $P < 0{\cdot}001$; Fig. 1a). WT bacterial inoculation decreased *R. palustris* shoot fresh weight significantly compared to the ACC deaminase-deficient mutant bacterium ($F_{2,23} = 7{\cdot}28$; $P = 0{\cdot}004$) from 3·01 g to 2·56 g. This points to negative effects of ACC deaminase activity on ethylene-mediated shoot fresh weight gain as well as on young leaf and petiole elongation in *R. palustris* subjected to submergence (Fig. 1b).

The effects of bacterial inoculation (WT and mutant) on young leaf elongation, petiole elongation and fresh weight were evaluated by separate one-way ANOVAs test complemented with contrast analyses. Bacterial treatments improved submergence-induced young leaf elongation ($t_{24} = 2{\cdot}49$; $P = 0{\cdot}02$) compared to the no bacteria treatment (Fig. 1b). This factor also increased significantly ($t_{24} = 3{\cdot}16$; $P = 0{\cdot}005$) for plants inoculated with the ACC deaminase-negative mutant strain as compared to no bacteria submergence treatment (Fig. 1b). Bacterial strain (WT and mutant) inoculation ($t_{24} = 3{\cdot}21$; $P = 0{\cdot}004$) and mutant inoculation ($t_{24} = 3{\cdot}81$; $P = 0{\cdot}001$) increased *R. palustris* shoot fresh weight significantly compared to the no bacteria treatment based on contrast analysis, indicating positive effects of other undetermined traits of *P. putida UW4* traits (e.g. auxin, siderophores) on fresh weight, young leaf length and older petiole elongation length.

Shoot dry weight of non-inoculated plants did not change after 72 h of submergence (see Table S1, Supporting Information), implying that no extra biomass was produced while the plant was submerged ($F_{1,15} = 2{\cdot}08$; $P = 0{\cdot}17$). However, bacterial inoculation increased ($F_{2,23} = 5{\cdot}42$; $P = 0{\cdot}01$) shoot dry weight significantly compared to the no bacterial treatment (Fig. 3). There was no significant difference between WT and ACC deaminase-negative mutant inoculation with respect to this factor ($t_{24} = 0{\cdot}71$; $P = 0{\cdot}48$; see Table S2).

Similarly, the root fresh weight ($F_{1,15} = 0{\cdot}06$; $P = 0{\cdot}80$) and dry weight ($F_{1,15} = 2{\cdot}53$; $P = 0{\cdot}13$) of *R. palustris* did not change significantly during 72 h of submergence. Bacterial inoculation did increase both root fresh weight ($F_{2,23} = 3{\cdot}95$; $P = 0{\cdot}03$) and root dry weight ($F_{2,23} = 6{\cdot}31$; $P = 0{\cdot}007$) significantly compared to the no bacteria treatment during the short-term submergence (Fig. 3).

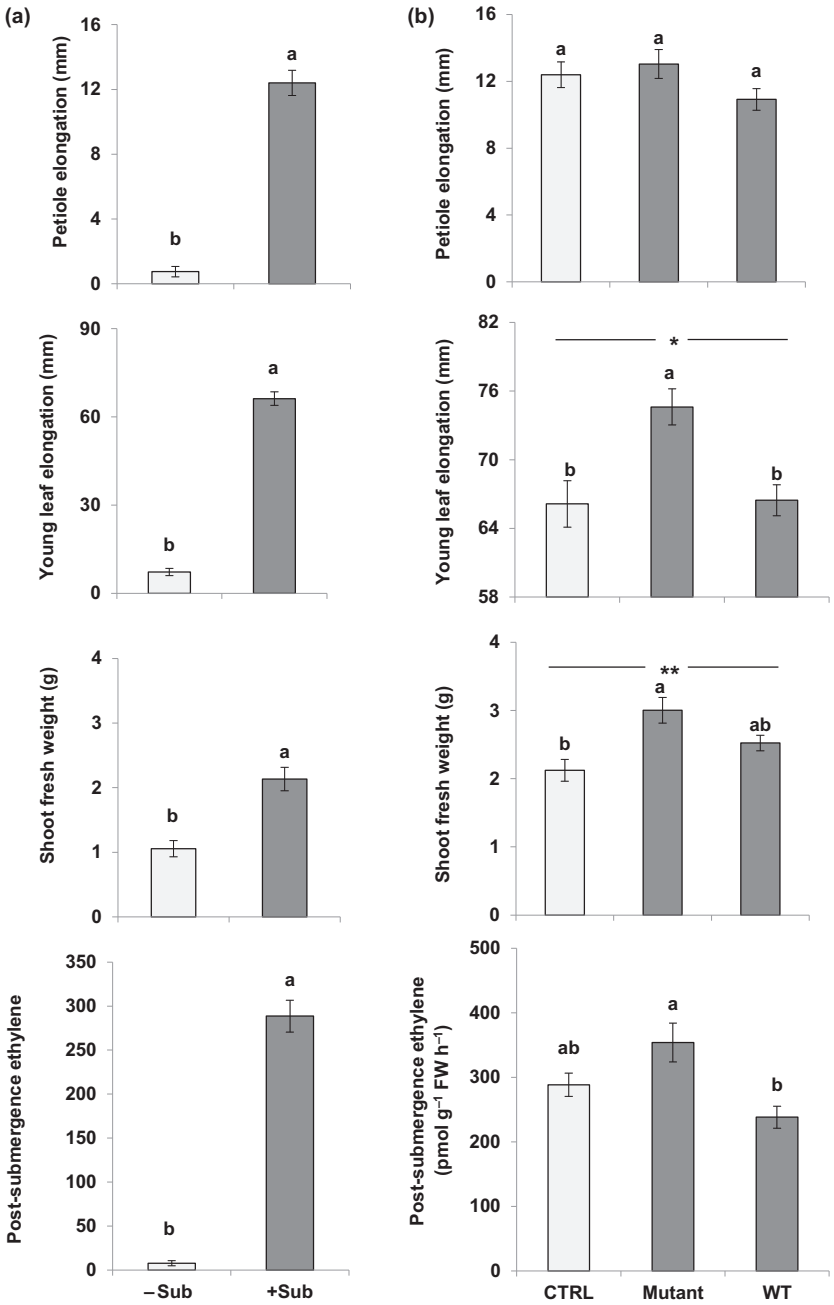

Fig. 1. (a) Effects of 72 h of complete submergence on post-submergence ethylene production and morphology of *Rumex palustris*. −Sub; no submergence (air-control), +Sub; short-term (72 h) submergence. (b) Effects of ACC (1-aminocyclopropane-1-carboxylate) deaminase-producing bacteria *Pseudomonas putida UW4* (WT), its isogenic ACC deaminase-deficient mutant (mutant), and a bacteria-free control (CTRL) on *R. palustris* ethylene-mediated responses (young leaf, petiole elongation and fresh weight) to short-term submergence. In (b), the differences between CTRL (group 1) vs. bacterized (WT and Mutant) plants (group 2) were analysed by contrast analysis and are indicated by horizontal lines. Significant effects based on contrast analysis are shown by asterisks (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$). Different letters above each bar indicate statistically significant differences. Error bars show ± SE.

EFFECTS OF BACTERIA ON THE RESPONSES OF *R. PALUSTRIS* TO LONG-TERM SUBMERGENCE

To examine the impacts of a more severe long-term submergence treatment, we evaluated the effects of bacteria on *R. palustris* responses to 17 days of complete submergence.

Bacterial inoculation (WT and mutant) increased shoot fresh weight ($t_{20} = 2{\cdot}34$; $P = 0{\cdot}02$), root fresh weight ($t_{20} = 3{\cdot}43$; $P = 0{\cdot}003$) and final plant height ($t_{20} = 2{\cdot}61$; $P = 0{\cdot}02$) compared to the no bacteria treatment (Fig. 4). Furthermore, inoculation with the WT bacterial strain significantly decreased root fresh weight ($F_{2,22} = 8{\cdot}56$; $P = 0{\cdot}002$), root dry weight ($F_{2,22} = 7{\cdot}05$; $P = 0{\cdot}005$) and final plant height ($F_{2,22} = 6{\cdot}08$; $P = 0{\cdot}009$) compared to plants inoculated with the ACC deaminase-negative mutant (Fig. 4; see Table S3). Together, these results implied a negative effect of the ACC deaminase enzyme on root characteristics and plant height, in addition to positive effects of other undetermined bacterial traits on *R. palustris* response to long-term submergence.

Discussion

Plants continuously adjust their physiology to adapt to environmental stresses and this plasticity is seen as a key determinant of plant survival. Most plant ecophysiology studies in submergence context have considered plants independently of

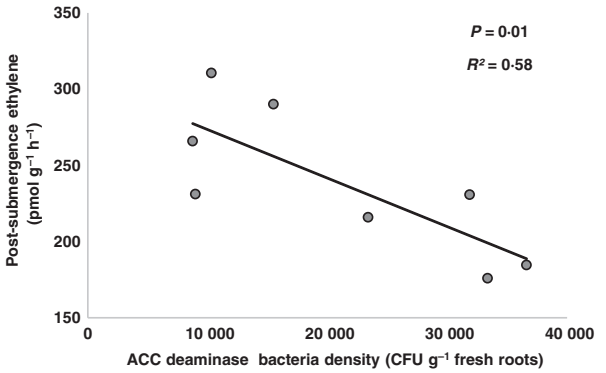

Fig. 2. Relationship between the density of ACC (1-aminocyclopropane-1-carboxylate) deaminase-producing bacteria on the roots of *Rumex palustris* and post-submergence ethylene production (pmol g^{-1} FW (Fresh Weight) h^{-1}).

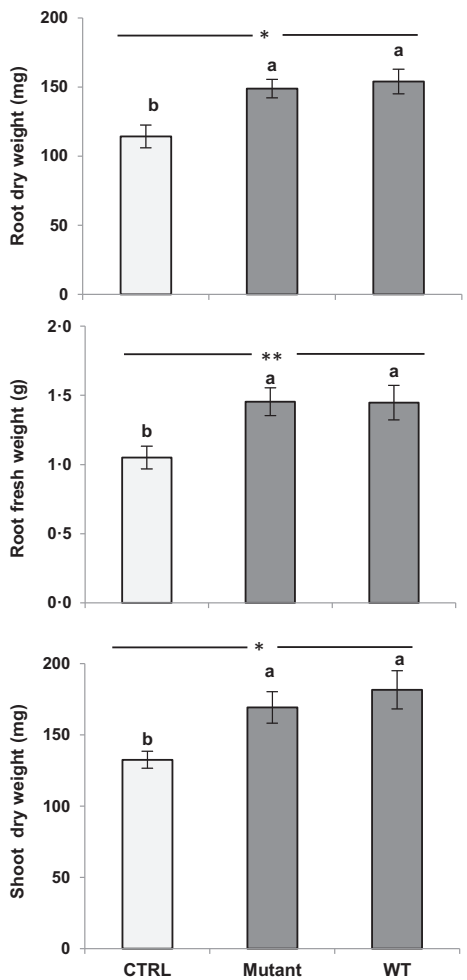

Fig. 3. Shoot fresh weight, shoot dry weight, root fresh weight and root dry weight of *Rumex palustris* in the three experimental treatments after 72 h of complete submergence. Plants were inoculated with a bacteria-free control (CTRL), *Pseudomonas putida UW4* (WT) or its ACC deaminase-deficient mutant (Mutant). The differences between CTRL (group 1) vs. bacterized (WT and Mutant) plants (group 2) were analysed by contrast analysis and are indicated by horizontal lines. Significant effects based on contrast analysis are shown by asterisks (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$). Different letters above each bar indicate statistically significant differences. Error bars show ± SE.

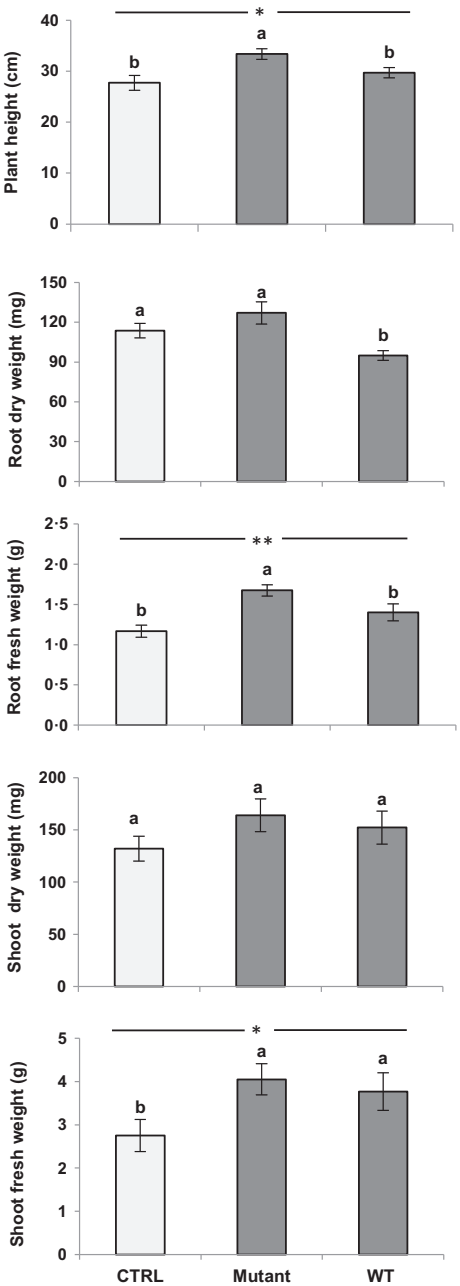

Fig. 4. Shoot dry weight, root dry weight, shoot fresh weight, and root fresh weight and plant final height of *Rumex palustris* in the three experimental treatments after 17 days of complete submergence. Plants were inoculated with a bacteria-free control (CTRL), *Pseudomonas putida UW4* (WT) or its ACC deaminase-deficient mutant (Mutant). The differences between CTRL (group 1) vs. bacterized (WT and Mutant) plants (group 2) were analysed by contrast analysis and are indicated by horizontal lines. Significant effects based on contrast analysis are shown by asterisks (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$). Different letters above each bar indicate statistically significant differences. Error bars show ± SE.

their interactions with associated microbes. At the same time, there is growing evidence that bacteria can influence plant physiology during submergence stress (Grichko & Glick 2001a,b; Barnawal *et al.* 2012; Glick 2014). However, the results of these different studies are often contradictory, calling for a unifying interpretation.

We focus, in this study, on the effects mediated by the modulation of ethylene response by root-associated bacteria. The volatile plant hormone ethylene accumulates rapidly within flooded plant organs due to restricted gas exchange underwater and is a major regulator of numerous flood-adaptive traits. Various studies demonstrated the importance of ethylene for flood tolerance in different plants (Voesenek & Blom 1989; Cox *et al.* 2003; Millenaar *et al.* 2005; Sasidharan & Voesenek 2015). At the same time, several studies on plant–microbe interactions base on the assumption that reducing ethylene levels enhances plant stress tolerance (Grichko & Glick 2001a,b; Barnawal *et al.* 2012; Glick 2014). In the present study, we bridge these two fields of investigations and attempt to assess the importance of plant–microbe interactions as the regulator of plant response to submergence stress.

We assessed the impact of ACC deaminase-producing bacteria, which can reduce plant ethylene levels, on submergence responses of the riparian plant *R. palustris*. This plant shows a remarkable plasticity (fast shoot elongation) in response to submergence stress, making it a convenient model to study the adaptive response of riparian plants to submergence (Cox *et al.* 2003). In order to assess the contribution of ACC (1-aminocyclopropane-1-carboxylate) deaminase enzyme on the plant stress response, we compared the impact of inoculation by *P. putida UW4* strain (WT) and an isogenic ACC deaminase-deficient mutant (mutant). This mutant is otherwise identical to the WT, allowing us to distinguish between effects mediated by ACC deaminase and other traits.

Our results showed that ACC deaminase activity in the rhizosphere inhibited *R. palustris*'s ethylene-mediated response to submergence. Inoculation of *R. palustris* with ACC deaminase-producing bacteria decreased post-submergence ethylene production compared to inoculation with the ACC deaminase-negative mutant strain. The level of post-submergence ethylene gives good estimates of ACC accumulation in roots, as ACC is rapidly converted to ethylene during the first hours of de-submergence (Voesenek *et al.* 2003b). During submergence, ACC levels increase in *R. palustris* due to a combined induction of the ACC synthase gene, *RP-ACS1* (Rieu *et al.* 2005), and inhibition of the ACC oxidase gene, *RP-ACO1* (Vriezen *et al.* 1999), at reduced O_2 levels (Bradford & Yang 1980; Voesenek *et al.* 1997). This results in a large pool of ACC in the roots that ultimately diffuses outside the roots. ACC deaminase activity around the roots will keep ACC concentration low, increasing its diffusion and reducing its concentration within the plant root, thereby contributing to a decrease in submergence and post-submergence ethylene levels.

Ethylene is a key regulator of submergence responses (Cox *et al.* 2003; Millenaar *et al.* 2005; Sasidharan & Voesenek 2015), and it is essential for flood tolerance of many semi-aquatic plants. Inhibition of the ethylene signalling pathway results in strongly reduced submergence responses (Voesenek *et al.* 2003a; Cox *et al.* 2004) and may reduce survival rates (Voesenek *et al.* 1992). Ethylene synthesis upon de-submergence requires a high flux of ACC from the root to aerial parts (Bradford & Yang 1980). By affecting ACC levels in roots, ACC deaminase-producing bacteria can decrease overall ethylene levels in the plant (Glick 2014), which has previously been shown to improve crop plant growth under certain stress conditions. We come to a contrasting conclusion: In our study, reduction in ethylene levels impeded the ability of *R. palustris* to express its normal morphological adaptation to flooding. This goes in line with past findings that plant flood response and survival can be improved by addition of ACC (Voesenek *et al.* 2003a; Cox *et al.* 2004). Thus, reducing plant ethylene levels may have contrasting impacts on plant survival depending on the ecological context. Plant flooding responses are species dependent. In the tested species, *R. palustris*, ethylene promotes submergence escape by stimulating leaf elongation. ACC deaminase-producing bacteria reduced this leaf extension thereby hindering this flooding escape strategy. Other plant species, such as some lowland rice varieties (Hattori, Nagai & Ashikari 2011) opt for an alternative, quiescent strategy that is also mediated by ethylene. In this case, limited growth and energy expenditure during submergence facilitates endurance of extended periods of flooding. It is possible that in these species, ACC deaminase bacterial modulation of the ethylene response would interfere with the normal quiescence strategy.

The contrasting results between our work and other studies on ACC deaminase-producing bacteria may come from the experimental design. Available studies consistently show that plant inoculation with ACC deaminase-producing bacteria enhanced plant growth under submergence (Grichko & Glick 2001a; Barnawal *et al.* 2012; Glick 2014). However, in the absence of functional mutant, they could not separate the effect of ACC deaminase from other bacterial traits. Here, we take advantage from a recently published ACC deaminase isogenic mutant to disentangle potential mechanisms. Our main conclusion is that in addition to the negative effect of ACC deaminase activity on plant submergence response, other bacterial traits may also have affected plant morphology and growth. In order to assess this, we performed a contrast analysis comparing the two treatments with bacterial inoculation vs. the control treatment. This revealed that bacterial inoculation increased *R. palustris* elongation during both short- and long-term submergence (Figs 1b and 4), but that ACC deaminase activity reduced bacterial influence on plant morphology. Most ACC deaminase-producing bacteria, including the model bacterial strain used in this study, also harbour other traits that may stimulate growth, such as the production of the phytohormone auxin (Duan *et al.* 2013). We propose that these traits, rather than the ACC deaminase activity, may be responsible for the often reported beneficial effects of bacteria on plant growth under stress.

The current experimental set up, with two separate controls, allowed us to disentangle two main effects: (i) The effect of ACC deaminase (comparison of WT and ACC deaminase-negative mutant treatment), (ii) The effect of bacterial inoculation independent of ACC deaminase activity, for instance due to other traits impacting plant growth

(comparison of non-inoculated control with ACC deaminase-negative mutant treatment). Further studies investigating in detail collections of functional mutants may help elucidate the relative importance of these different bacterial traits for plant responses to submergence.

ACC deaminase-producing bacteria are common in soils (Duan *et al.* 2008), suggesting that our results may be of widespread relevance under field conditions: Given the high abundance of ACC deaminase-producing bacteria around plant roots (Duan *et al.* 2008), they may have a significant impact on plant ecophysiology in different ecosystems, including wetlands.

This study expands the view of plant–microbe interactions in the context of stress tolerance. In contrast to common views on ACC deaminase-producing bacteria, which have to date mainly been investigated in relation to their ability to promote plant growth under inhibitory ethylene concentrations (Glick *et al.* 2007; Glick 2014), we show that these bacteria may be a drawback for aquatic and riparian species by hampering development of key adaptations to flooding. This highlights ethylene's roles as a key regulator that fine-tunes several plant traits. Shoot elongation upon submergence is critical to re-establishing leaf area above the water to increase aerial photosynthesis and improve inward diffusion of O_2 (Voesenek *et al.* 2003b). Hence, soil microbes should be taken into account as essential modulators of plant–environment interactions. From an evolutionary perspective, shoot elongation may be beneficial if the costs of elongation are outweighed by the benefits derived from gas exchange, energy generation and carbon production (Pierik, van Aken & Voesenek 2009). Hence, this elongation is particularly useful in shallow and prolonged floods (Pierik, van Aken & Voesenek 2009; Bailey-Serres & Voesenek 2010) where it contributes to better survival and seed production (Voesenek *et al.* 1992). Fresh weight and dry weight of shoots and roots are key factors improving plant survival in submergence-resistant and -sensitive rice cultivars (Singh, Mackill & Ismail 2014). The observed bacteria-mediated increase in root and shoot biomass of submerged plants inoculated with bacteria may thus open new avenues for enhancing plant flood tolerance.

Ethylene and auxin are two key hormones regulating plant morphology during submergence (Cox *et al.* 2004) and both hormones can be affected by soil microbes. ACC deaminase enzyme may reduce ethylene levels (Glick 2014), and exogenous auxin production may shift hormonal balance (Spaepen, Vanderleyden & Remans 2007; Duan *et al.* 2008). *Pseudomonas Putida UW4* produces both bacterial traits (Duan *et al.* 2013). These traits are also widespread in plant-beneficial bacteria and should be considered when predicting plant responses and should be integrated into wetlands community models, although, this should not necessarily be restricted to these two traits. To date, very few studies have focused on the interaction of soil microbial traits and wetland plants. We conclude that soil microbes may be an overlooked modulator of plant flood tolerance and may also be used as a tool to mitigate predicted impacts of climate change such as extreme and irregular water fluctuations.

Authors' contributions

A.J., M.R., L.V., R.S. and G.K. contributed to designing the research and writing of the manuscript. M.R. was mainly responsible for establishment of the experiments, chemical analyses and collecting data, with substantial contributions from A.J. A.J., M.R., L.V. and R.S. contributed to analysis and interpretation of data. M.R. and A.J. drafted manuscript. A.J., L.V., R.S., G.K. and M.R. edited and revised manuscript and gave final approval for publication.

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Data accessibility

Data available from the Dryad Digital Repository http://dx.doi.org/10.5061/dryad.17h1b (Ravanbakhsh *et al.* 2016).

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Supporting Information

Details of electronic Supporting Information are provided below.

Table S1. Effects of 72-h complete submergence on *Rumex palustris* response compared to non-submerged *R. palustris* (control) using one-way ANOVAs.

Table S2. Effects of ACC (1-aminocyclopropane-1-carboxylate) deaminase-producing bacteria *Pseudomonas putida UW4* (WT), its isogenic ACC deaminase-deficient mutant (mutant) and bacteria-free control (CTRL) on the growth of submerged *Rumex palustris* after 72 h of submergence (short-term submergence).

Table S3. Effects of ACC (1-aminocyclopropane-1-carboxylate) deaminase-producing bacteria *Pseudomonas putida UW4* (WT), its isogenic ACC deaminase-deficient mutant (mutant), and bacteria-free control (CTRL) on submerged *Rumex palustris* after 17 days of submergence (long-term submergence).