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ORIGINAL ARTICLE

Does drought stress modify the effects of plant-growth promoting rhizobacteria on an aboveground chewing herbivore?

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Abstract Soil microbes have important effects on the interactions of plants with their environment, by promoting plant growth, inducing resistance to pests or by conferring tolerance to abiotic stress. However, their effects are variable and the factors responsible for this variation are mainly unknown. Our aim was to assess how drought stress modifies the effect of the nonpathogenic rhizobacterium *Pseudomonas simiae* WCS417r on plant growth and resistance against the generalist leaf-chewing caterpillar *Mamestra brassicae*. We studied *Arabidopsis thaliana* Col-0 plants, as well as mutants altered in the biosynthesis of the phytohormones jasmonic acid (JA) and abscisic acid (ABA). Caterpillars did not prefer rhizobacteria-treated plants, independently of drought stress. Rhizobacteria colonization had a variable effect on caterpillar performance, which ranged from positive in one experiment to neutral in a second one. Drought had a consistent negative effect on herbivore performance; however, it did not modify the effect of rhizobacteria on herbivore performance. The effect of drought on herbivore performance was JA-mediated (confirmed with the use of the *dde2-2* mutant), but it was still present in the ABA-deficient mutant *aba2-1*. Plant biomass was reduced by both drought and herbivory but it was enhanced by rhizobacterial colonization. *Pseudomonas simiae* WCS417r is able to promote plant growth even when plants are suffering herbivory. Nevertheless, the microbial effect on the herbivore is variable, independently of drought stress. To get the best possible outcome from the rhizobacteria-plant mutualism it is important to understand which other factors may be responsible for its context-dependency.

Key words abiotic stress; abscisic acid; crosstalk; induced systemic resistance; jasmonic acid; *Pseudomonas simiae*

Introduction

In nature, plants are involved in negative interactions with e.g. pathogens and herbivorous insects, but also in positive ones such as those with beneficial microorganisms and beneficial insects (Dicke & Baldwin, 2010; Philippot *et al.*, 2013). In addition, plants have to cope with abiotic stresses, such as drought. Plant growth is reduced by herbivory and drought and importantly, both stresses are predicted to increase in frequency and intensity in the coming years (Massad & Dyer, 2010; Zhao & Running, 2010). However, despite the importance of understanding plant responses to simultaneous biotic or abiotic stresses, these are only recently starting to be unraveled (Mattson

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& Haack, 1987; van Oosten *et al.*, 2008; Tariq *et al.*, 2013; Coolen *et al.*, 2016; Davila Olivas *et al.*, 2016; Nguyen *et al.*, 2016; Pineda *et al.*, 2016). Nonpathogenic soil microbes that live around plant roots may play an important role in plant responses to their biotic and abiotic environment, since they can induce plant tolerance to both types of stresses (Bennett *et al.*, 2006; Sugio *et al.*, 2014; Timmusk *et al.*, 2014). In addition, they can promote plant growth (Paré *et al.*, 2011; Zamioudis *et al.*, 2013).

Importantly, the effect of root-associated microbes on herbivorous insects varies from positive (D'Alessandro *et al.*, 2014; Megali *et al.*, 2014) to negative (van Oosten *et al.*, 2008; Pangesti *et al.*, 2016a; Zebelo *et al.*, 2016) and to date we do not fully understand the factors responsible for this variation. Biotic factors such as feeding guild and degree of specialization of the insects have been shown to affect the outcome of plant–microbe interactions. Root-associated microorganisms have generally a positive or neutral effect on phloem feeders and specialist chewers and a negative effect on mesophyll feeders and generalist chewers (Koricheva *et al.*, 2009; Pineda *et al.*, 2010). In contrast, it is less well understood how abiotic factors modulate microbe–plant–insect interactions (Pineda *et al.*, 2013). For instance, soil composition (ratio of sand and peat) and more specifically iron availability seem to be among the factors that affect the enhanced immunity that microbes confer to plants (Pangesti *et al.*, 2014; Zamioudis *et al.*, 2014). Drought was also shown to increase the direct effect that grass endophytes had on herbivores (Vidal, 1996). However, whether drought influences the plant-mediated effects of microbes on herbivores is not yet known and the underlying mechanisms have not been studied.

Plant responses to biotic and abiotic stresses are regulated by signal-transduction pathways under the control of several phytohormones. Feeding by piercing-sucking insects and biotrophic pathogens mainly activates salicylic acid (SA)-dependent responses, whereas jasmonic acid (JA) signaling is mainly activated upon damage by chewing insects and necrotrophic pathogens (Erb *et al.*, 2012; Pieterse *et al.*, 2012). In addition, abscisic acid (ABA) has been known for decades as key regulator of plant responses to abiotic stress, and especially to drought (Fujita *et al.*, 2006; Yoshida *et al.*, 2014). However in the last years it is becoming evident that ABA is also involved in plant responses to herbivory (Vos *et al.*, 2013; Hillwig *et al.*, 2016).

Plants fine-tune their defense responses to multiple stresses through crosstalk between phytohormonal signaling pathways (Pieterse *et al.*, 2012). In *Arabidopsis* the MYC2-branch of the JA-signaling pathway is activated upon chewing insect feeding resulting in the transcription of the JA-responsive genes *VSP1* and *VSP2* (Bodenhausen & Reymond, 2007; Verhage *et al.*, 2011; Vos *et al.*, 2013). Interestingly, this MYC2-branch is positively regulated by ABA, and, for instance, the herbivore-induced levels of *VSP1* are not observed in the ABA-deficient mutant *aba2-1* (Vos *et al.*, 2013). However, how drought affects the cross-talk between ABA and JA signaling pathways and the consequences for herbivores still needs to be unraveled.

Besides its role in plant defenses against herbivorous insects, JA is also involved in induced systemic resistance that is mediated by beneficial rhizosphere bacteria (van der Ent *et al.*, 2009; Pozo *et al.*, 2015). Root-associated microorganisms can induce resistance in systemic tissues (ISR) against pathogens and herbivorous insects through a priming mechanism (Pieterse *et al.*, 2014). Priming is characterized by a faster or stronger activation of plant defenses upon pathogen or insect attack, with lower fitness costs than induced resistance (Conrath *et al.*, 2006; Martinez-Medina *et al.*, 2016). Most cases of microbial-ISR require intact JA- and ET-signaling pathways (Pieterse *et al.*, 2014). However, it is not fully understood whether a functional ABA signaling pathway is required for ISR.

The objective of our study was to assess whether drought modifies the effect of the rhizobacterium *Pseudomonas simiae* WCS417r (previously known as *Pseudomonas fluorescens* WCS417r) on *Arabidopsis thaliana* growth and defenses against the generalist insect herbivore *Mamestra brassicae*. In addition, we aimed at unraveling the role of JA and ABA in these interactions. The bacterial strain *P. simiae* WCS417r has been shown to promote plant growth, lateral root formation and root hair formation in *Arabidopsis* Col-0 plants grown in agar (Zamioudis *et al.*, 2013). Therefore, we expected *P. simiae* WCS417r to have a positive effect on plant growth in potting soil as well (Pangesti *et al.*, 2016b). However, since rhizobacteria-induced resistance against *M. brassicae* was variable in our previous studies (Pangesti *et al.*, 2014; Pangesti *et al.*, 2015; Pangesti *et al.*, 2016b), we expected to observe again a variable effect. Additionally, based on previous evidence showing that (1) *P. simiae* WCS417r–mediated ISR requires an intact JA signal-transduction pathway (Pangesti *et al.*, 2014; Pieterse *et al.*, 2014), (2) that drought induces ABA (Fujita *et al.*, 2006; Yoshida *et al.*, 2014), and (3) that ABA has a synergistic effect on the MYC2-branch of the JA pathway against chewing insects (Vos *et al.*, 2013), we expected drought to strengthen the negative effect of rhizobacteria on herbivore performance.

Materials and methods

Preparation of plants, microbes, and insects

Plants were grown from seeds in a mixture of potting soil and sand (1 : 1, v : v). We used the wild type *Arabidopsis thaliana* accession Columbia (Col-0). In addition we studied the mutant *delayed-dehiscence2-2* (*dde2-2*), defective in the *ALLENE OXIDE SYNTHASE* gene (*AOS*), encoding one of the enzymes in the JA biosynthetic pathway (von Malek *et al.*, 2002). Finally we used the ABA biosynthetic mutant *aba2-1*, whose gene product *ABA2* is implicated in the conversion of xanthoxin to ABA-aldehyde (Pieterse *et al.*, 2016). The soil mixture was autoclaved twice at 121 °C for 20 min with a 24 h interval. All seeds were sterilized by 3 h exposure to chlorine gas, and subsequently sown in Petri dishes on water-saturated filter paper and kept for 3 d at 4°C (van de Mortel *et al.*, 2012).

The rifampicin-resistant nonpathogenic plant growth-promoting rhizobacterial strain *Pseudomonas simiae* WCS417r (*P. s.* WCS417r, previously known as *Pseudomonas fluorescens* WCS417r) (Berendsen *et al.*, 2015) was used for induction of induced systemic resistance (ISR). Bacteria were grown on King's B (KB) medium agar plates containing rifampicin (25 μg/mL) (Pieterse *et al.*, 1996) for 48 h at 28 °C. Bacterial cells were collected, resuspended in 10 mmol/L $MgSO_4$ and adjusted to a cell density of 1×10^9 cfu/mL ($OD_{660} = 1.0$). For the rhizobacterial treatment the soil was inoculated with 100 mL of the suspension of *P. s.* WCS417r per kilogram of soil. An equal amount of 10 mmol/L $MgSO_4$ was added to the soil for the control treatment. Seeds were sown on the inoculated soil in individual pots and 10 d after sowing, plants were re-inoculated with 1 mL/plant of a suspension with the same bacterial density as described above, and the control plants with 1 mL of 10 mmol/L $MgSO_4$.

Plants were cultivated in a growth chamber under 8 h : 16 h light : dark at 21 ± 1 °C and 60% ± 10% relative humidity (RH) and watered with 20 mL water 3 times a week, keeping the soil water content at 100%. From the second week onwards, plants were fertilized once a week with 10 mL Hyponex® per plant. One week before the bioassays, plants were assigned randomly to the drought treatment, previously named "high drought" (Pineda *et al.*, 2016). In the control treatment, plants were watered 3 times per week. For the drought treatment plants were not watered during a week after which plants were rewatered to 60% of soil water content (Pineda *et al.*, 2016).

Mamestra brassicae L. (Lepidoptera: Noctuidae; Cabbage moth) was reared on *Brassica oleracea* var. *gemmifera* cv. Cyrus (Brussels sprouts) in a climate chamber at 22 ± 2 °C, 40%–50% RH, 16 h : 8 h light:dark. Neonate larvae without feeding experience were used in the experiments.

Experiment 1: Effect of rhizobacterial colonization and drought on performance of a generalist insect and expression of marker genes

A. thaliana plants were randomly assigned to different treatments. Plants were treated with rhizobacteria or not, water-stressed or normally watered and inoculated with neonate larvae of *Mamestra brassicae* or not. All these combinations led to 8 treatments.

(a) Performance of the generalist herbivore *M. brassicae* and plant performance Three neonate larvae of *M. brassicae* were transferred to 5-week-old *A. thaliana* Col-0 plants using a fine paintbrush. All plants were individually confined in plastic containers covered with insect-proof mesh cloth and closed with elastic bands. Larvae were weighed to the nearest 0.001 mg using a microbalance (CP2P, Sartorius AG, Germany) 9 d after infestation. To address the effect of the treatments on plant performance, fresh aboveground plant biomass was measured using an analytical balance. A total of 20 infested plants per treatment were evaluated, whereas 10 plants per uninfested treatment were arranged to assess plant biomass. Bioassays were performed in a growth chamber under 21 ± 1 °C, 60%–70% RH, 16 h : 8 h light:dark.

(b) Expression of defense-related genes The same treatments as described above were used to evaluate the transcript levels of several marker genes of the JA- and ABA-signaling pathways on wild-type Col-0 plants. A total of 12 plants per treatment were prepared, and fully expanded leaves of all the treatments were harvested at 24 h after infestation. For each treatment, 4 biological replicates were used, each consisting of 9 damaged leaves (3 leaves from 3 plants). Leaf samples were immediately frozen by immersion in liquid nitrogen and stored at –80 °C for further RNA extraction. An extra set of plants was prepared to assess leaf damage 48 h after infestation, using transparent paper with square millimeter raster and counting by eye the number of mm² removed by the insect.

Total RNA was extracted and purified as indicated by the protocol RNA plant Kit (Bioline, London, UK). Measurement of RNA quality and synthesis of cDNA was performed as explained in Pangesti *et al.* (2014). Transcript levels of the JA/MYC2-regulated gene *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) and of the ABA and drought stress-responsive gene *RAB18* were quantified. As reference genes, *GLYCERALDEHYDE-3-*

PHOSPHATE DEHYDROGENASE (*GAPDH*) and *F-BOX FAMILY PROTEIN1* (*FBOX1*) were used. Sequences of gene-specific primers for qRT-PCR are included in supplemental materials. The quality of each primer was determined before qRT-PCR analysis, which was completed following the same procedure as Pangesti *et al.* (2014). All qRT-PCR experiments were performed in duplicate (technical replicates) and average values were used in the analyses. Thermal cycling conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 and 45 s at 60 °C. A normalization factor was calculated by geometrically averaging the threshold cycle (Ct) values of the constitutively expressed genes *GAPDH* and *F-BOX*. Then the transcript level for each tested gene was calculated relative to the normalization factor using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Experiment 2: Corroborating the role of JA/ABA phytohormone signaling pathways in the rhizobacteria–insect–drought interaction

To assess the role of JA- and ABA-signaling pathways on the effect of *P. s.* WCS417r and drought on the generalist herbivore *M. brassicae*, 3 neonate larvae were inoculated on *A. thaliana* mutants that are defective in the JA (*dde2-2*) or ABA (*aba2-1*) signaling pathways. All mutants used in the experiment have the Col-0 background, hence larvae fed on the wild-type Col-0 plants were used as a control to compare herbivore performance. Caterpillars were weighed at 9 d after infesting the plants as explained above. Additionally, leaf damage on Col-0 plants was assessed visually at 3 d postinfestation by using millimeter raster paper. Plants were divided into 2 groups (half of the replicates in each group), so the full experiment was done in 2 consecutive days following exactly the same methodology. A total of 20 infested plants were evaluated, whereas 10 plants per uninfested treatment were arranged to measure plant biomass.

Experiment 3: Preference of M. brassicae larvae

Two-choice experiments were performed by setting 2 plants in a 1 L open container, and placing 10 neonate larvae in the middle of the container. All experiments were conducted in a climatized room (21 ± 2 °C) without windows, and artificial light was provided from above by 4 high-frequency fluorescent tubes (TL-D 58 W Philips, Eindhoven, The Netherlands) at an intensity of 60 ± 5 μmoL photons/m^2/s (8 h : 16 h light : dark). The position of the containers and of the plants inside these, was randomized. The preference of the neonate larvae was assessed, by counting how many larvae were on each plant after 48 h. The following combinations of plants were used (14–16 replicates for each pair): (1) control versus rhizobacteria-treated plants; (2) control versus drought-stressed plants; (3) rhizobacteria-treated plants versus rhizobacteria-treated plants under drought stress; and (4) drought-stressed plants versus rhizobacteria-treated plants under drought stress.

Statistical analysis

Residuals were first inspected to confirm that the assumptions of linear models were met. To analyze the effect of *P. s.* WCS417r and drought on herbivore performance, linear mixed models (LMM) were used. Plants were set as a random factor, whereas rhizobacteria and drought treatment were set as fixed factors. To evaluate the effect of *P. s.* WCS417r and drought on the relative expression of defense-related genes and on plant biomass, 3-way ANOVA was used. Herbivory, drought, and rhizobacterial colonization were set as fixed factors. In order to assess the effect of the treatments on leaf damage, 2-way ANOVA was used, setting drought and rhizobacteria as fixed factors. To analyze preference in the 2-choice tests, we calculated a response ratio for each replicate ([#control]/[#control +#treatment]). This was tested against a null hypothesis value of 0.5 in a 1 sample *t*-test. Data were analyzed with Genstat (14th Edition, VSN Int., UK).

Results

Rhizobacterial colonization does not affect larval preference but enhances larval performance independently of drought stress

To unravel the effect of drought and rhizobacterial treatments on the preference of a generalist herbivore, we performed several 2-choice experiments with *A. thaliana* Col-0 (wild type).

When plants were not colonized by rhizobacteria, caterpillars preferred control over drought-stressed plants ($t_{11} = 0.034$). However, when plants were treated with rhizobacteria, caterpillars showed no preference ($t_{19} = 0.093$). Caterpillars did not show any preference between control and rhizobacteria-treated plants under normal watering conditions ($t_{12} = 0.893$) nor under drought conditions ($t_{17} = 0.524$).

In a first performance experiment with Col-0 plants (Part a in Materials and methods section), we evaluated the effect of rhizobacteria, drought and the combination of both on the insect herbivore. *M. brassicae* caterpillars

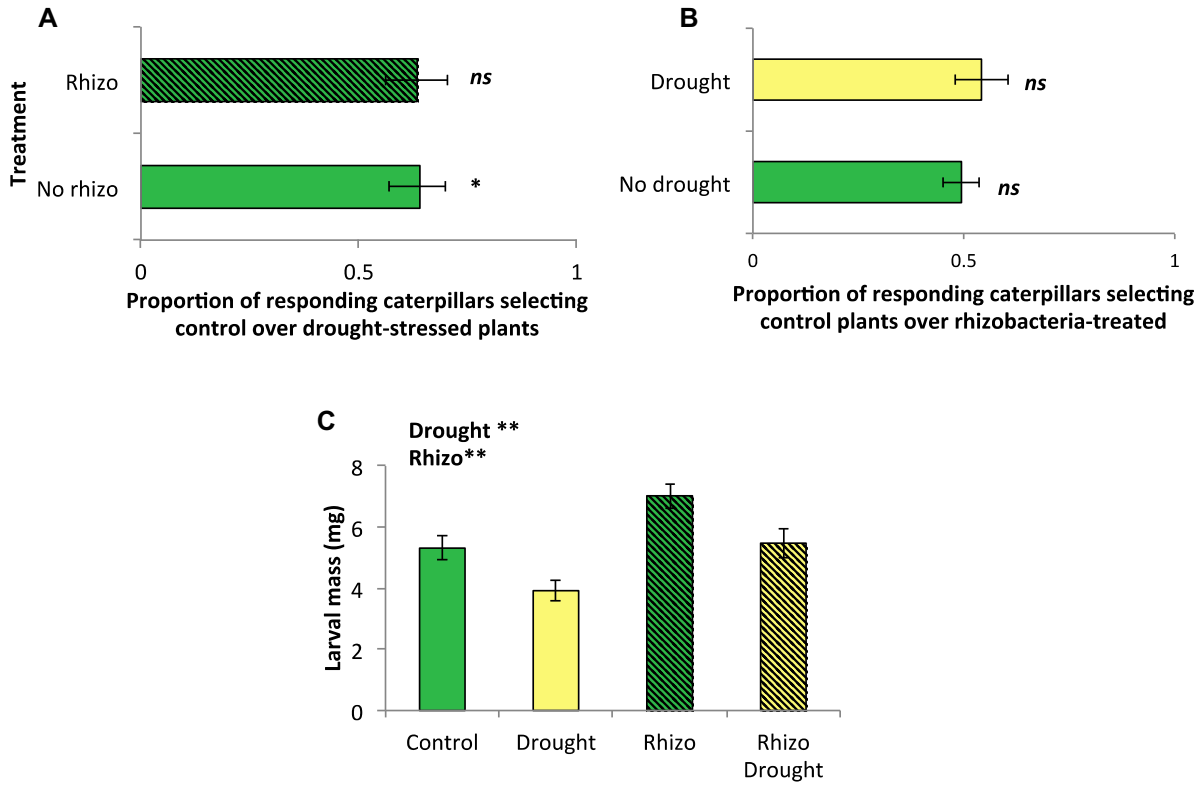

Fig. 1 Preference of *Mamestra brassicae* larvae in a 2-choice setup 48 h after releasing 10 larvae (n = 14–16). Bars represent means ± SE of ratio of larvae choosing control plants (A) over drought-stressed plants when both plants were colonized by rhizobacteria or remained untreated, and (B) over rhizobacteria-treated plants, when choices were drought-stressed or no drought. Asterisks indicate a significant preference in the 2-choice set-up (1 sample t-test, $P < 0.05$); *ns* = nonsignificant ($P > 0.05$). (C) Effect of the treatments on larval mass of *M. brassicae* at 9 d postinfestation. *Arabidopsis thaliana* Col-0 plants were infested with 3 first instar larvae. Bars represent means ± SE. Only significant effects are shown; $^{**}P < 0.01$ (2-way LMM, $n = 20$ plants).

had a reduced performance on drought-treated plants at 9 d after infestation (LMM; $F_{1,74} = 8.73$, $P = 0.004$; Fig. 1C). In contrast, rhizobacteria colonization had a positive effect on larval mass of the caterpillars (LMM; $F_{1,74} = 10.78$, $P = 0.002$; Fig. 1C). This effect was independent of drought stress, since there was no interaction between the main factors drought and rhizobacteria treatment on larval performance (LMM; $F_{1,74} = 0.00$, $P = 0.984$).

Drought negatively affects larval performance in a JA-dependent manner but independent of ABA, and does not affect the variable rhizobacterial effects

In a second performance experiment (Experiment 3 in Materials and methods section), we studied wild type Col-0 plants together with JA- and ABA-impaired mutant plants to assess the effect of these signaling pathways on the observed effects. Drought again negatively affected the performance of the generalist herbivore *M. brassicae* when feeding on wild type Col-0 plants at 9 d after infestation (LMM; $F_{1,163} = 8.88$, $P = 0.003$; Fig. 2). The same negative effect was observed in the ABA-impaired mutant *aba2-1* (LMM; $F_{1,159} = 23.96$, $P < 0.001$; Fig. 2). However, there was no negative effect of drought on larval performance when the caterpillars fed from the JA-impaired mutant *dde2-2* (LMM; $F_{1,192} = 1.28$, $P = 0.258$; Fig. 2). In contrast to the first experiment, in this experiment rhizobacteria colonization of Col-0 plants had no effect on *M. brassicae* performance (LMM; $F_{1,165} = 0.38$, $P = 0.538$; Fig. 2) nor did it modify the effect of drought (LMM, interaction; $F_{1,167} = 0.01$, $P = 0.926$). A similar lack of effect was observed in the mutants *dde2-2* and *aba2-1* (LMM; *aba2-1*: $F_{1,158} = 3.66$, $P = 0.058$; *dde2-2*: $F_{1,192} = 1.28$, $P = 0.258$; Fig. 2), although in the mutant *aba2-1* there was a trend towards rhizobacteria inducing resistance to *M. brassicae*. There was also no interaction between rhizobacteria and drought (LMM,

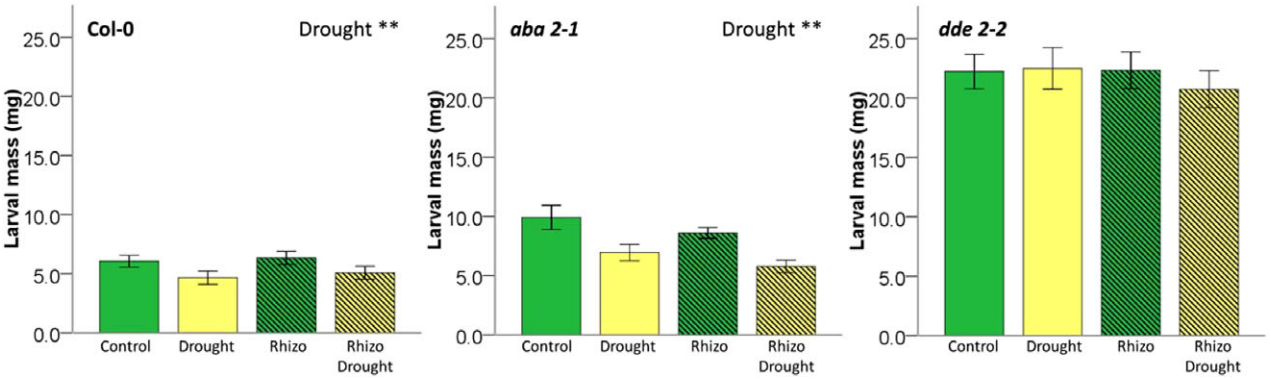

Fig. 2 Larval weight of *Mamestra brassicae* feeding on *Arabidopsis thaliana* wild type Col-0 (left), ABA-impaired mutant *aba2-1* (centre) and JA-impaired mutant *dde2-2* (right), at 9 d postinfestation. Bars represent means ± SE. Only significant effects are shown. $^{**}P < 0.01$ (2-way LMM, $n = 25$ plants).

interaction; *aba2-1*: $F_{1,158} = 0.21$, $P = 0.645$; *dde2-2*: $F_{1,190} = 1.34$, $P = 0.249$).

Rhizobacterial colonization enhances plant growth and decreases plant damage at an early time point

Both drought and herbivory had a negative effect on plant biomass (3-way ANOVA; herbivory: $F_{1,113} = 9.490$, $P = 0.003$; drought: $F_{1,113} = 119.799$, $P < 0.001$; Fig. 3A). In contrast, colonization with *P. s.* WCS417r enhanced plant growth (3-way ANOVA; $F_{1,113} = 18.996$, $P < 0.001$; Fig. 3A). Interestingly, the decrease in plant biomass of plants suffering herbivory was much stronger when plants were not stressed than when plants were exposed to drought (3-way ANOVA; interaction herbivory × drought: $F_{1,113} = 31.435$, $P < 0.001$).

In a first experiment (Experiment 2 in Materials and methods section), caterpillar feeding damage at 2 d after infestation was smaller under either drought or rhizobacterial treatment, however, there was no interaction between the factors (2-way ANOVA: drought: $F_{1,47} = 11.96$, $P = 0.001$; rhizobacteria: $F_{1,47} = 11.42$, $P = 0.002$; interaction: $F_{1,47} = 1.15$, $P = 0.289$; Fig. 3B left). Interestingly, plants that were colonized by rhizobacteria and suffered drought stress had the lowest level of leaf damage (Fig. 3B left). In a second experiment (Experiment 3 in Materials and methods section), leaf damage at 3 d postinfestation was also reduced by drought, however, not by rhizobacteria colonization and there was also no interaction between the factors (2-way ANOVA: drought: $F_{1,39} = 137.73$, $P < 0.001$; rhizobacteria: $F_{1,39} = 0.58$, $P = 0.450$; interaction: $F_{1,39} = 0.21$, $P = 0.647$; Fig. 3B right).

Herbivore-induced transcription of the JA-responsive gene VSP2 is neither primed by rhizobacterial colonization nor by drought stress

Herbivore feeding by the generalist insect *M. brassicae* induced expression of the defense-related gene *VSP2* (3-way ANOVA: $F_{1,31} = 39.03$, $P < 0.001$; Fig. 4 left). However, *P. s.* WCS417r did not prime *A. thaliana* plants for an enhanced expression of *VSP2*, as the expression levels of *VSP2* were the same in both control and *P. s.* WCS417r-treated plants (3-way ANOVA: $F_{1,31} = 2.04$, $P = 0.166$; Fig. 4 left). Drought had no effect on the expression levels of *VSP2* either ($F_{1,31} = 0.05$, $P = 0.82$; Fig. 4 left). There was no interaction between any of the factors (data not shown).

Expression of the ABA- and drought-regulated gene *RAB18* was induced by drought (3-way ANOVA: $F_{1,31} = 7.61$, $P = 0.011$; Fig. 4 right). Herbivory and the rhizobacterial treatment did not affect the expression levels of *RAB18* (3-way ANOVA: herbivory: $F_{1,31} = 3.14$, $P = 0.089$; rhizobacteria: $F_{1,31} = 0.00$, $P = 0.967$; Fig. 4 right). The interaction between the factors was not significant (data not shown).

Discussion

Root colonization with beneficial microbes has a differential effect on insects that ranges from positive to negative and to date we do not fully understand the modulators of this variability (Partida-Martinez & Heil, 2011; Pineda *et al.*, 2013; Pangesti *et al.*, 2014). Here we show that, contrary to our expectations, drought is not one of the factors modulating the outcome of the interaction between

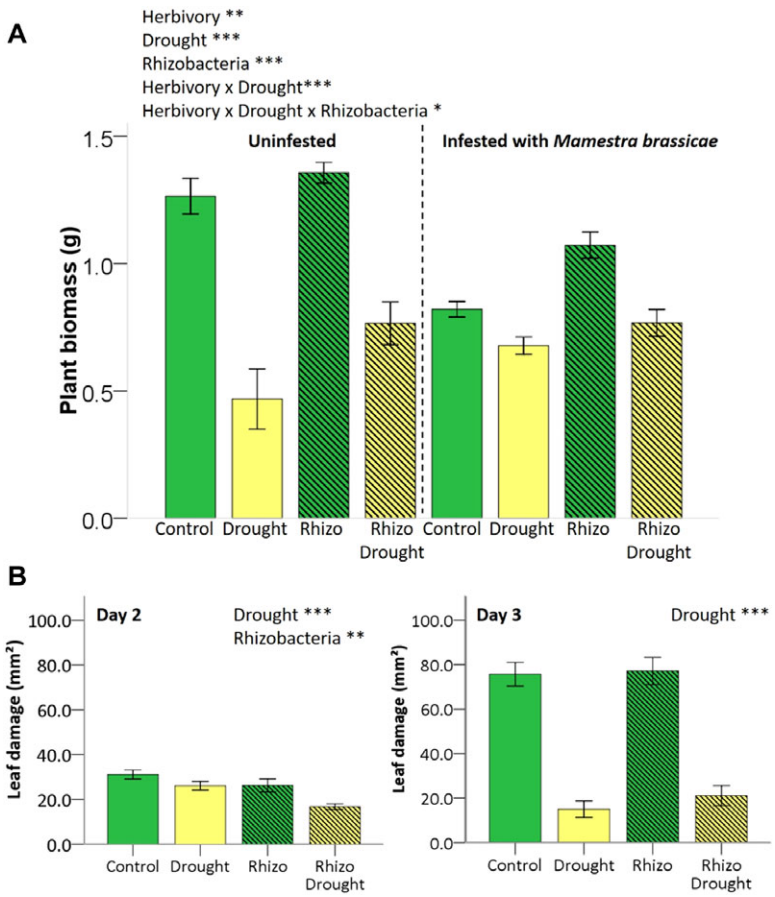

Fig. 3 (A) Fresh aboveground plant biomass (g) of *Arabidopsis thaliana* Col-0 (wild type) of the plants used in the first performance experiment. Bars represent means ± SE. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (3-way ANOVA, $n = 10$ for the uninfested plants; $n = 20$ for the infested plants). (B) Leaf damage (mm^2) of the *Arabidopsis thaliana* Col-0 plants used in the first performance experiment at 2 d postinfestation (right) and second performance experiment at 3 d postinfestation (left). The plants used for the first experiment were used for gene expression analysis. Bars represent means ± SE. $**P < 0.01$, $***P < 0.001$ (2-way ANOVA, $n = 10$–12 plants).

A. thaliana plants, the rhizobacterium *P. simiae* WCS417r, and the leaf herbivore *M. brassicae*. Variability in results is common when studying ecological interactions, especially when not all factors that influence these interactions are understood (Heil, 2014). In our previous studies, we observed a high consistency in a closed system of sterile plate assays, but not in an open system with soil (Pangesti *et al.*, 2014; Pangesti *et al.*, 2015; Pangesti *et al.*, 2016a). In these studies, we always used the same population of *A. thaliana* Col-0 plants, of the herbivore *M. brassicae*, and the same rhizobacterial strain. Now, after excluding drought as main driver of variability in our system, we propose that the microbial communities (and factors affecting those) that are present in the soil in the open system are probably responsible for the variability we observed in our studies. More studies unraveling what biotic and abiotic factors cause this variability are needed to be able to predict the final outcome of microbe–plant–insect interactions.

Rhizobacterial colonization increased both plant growth and herbivore performance in the first experiment, independently of whether plants were suffering from drought stress. However, when we repeated the experiment rhizobacteria did not affect caterpillar performance. Despite the general pattern suggesting that generalist leaf chewers are negatively affected by beneficial microbes (Koricheva *et al.*, 2009; Pineda *et al.*, 2010), examples of microbes inducing susceptibility to generalist leaf chewers are accumulating (D'Alessandro *et al.*, 2014; Megali *et al.*, 2014; Pangesti *et al.*, 2015). For example, larvae of the generalist insect *Spodoptera littoralis* had a higher mass and decreased mortality when feeding from tomato plants that were colonized by a mixture of beneficial bacteria, explained by a reduction of induced

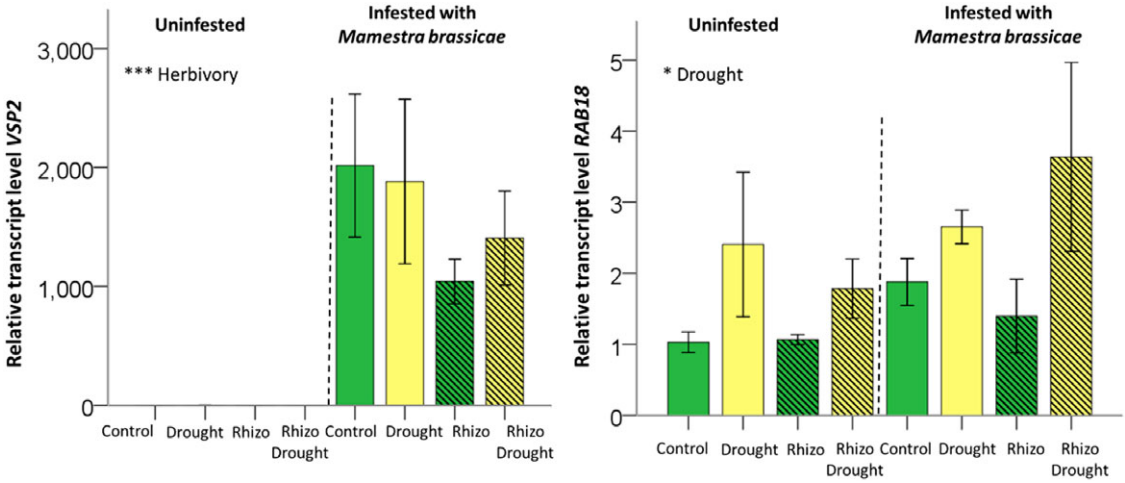

Fig. 4 Relative transcript levels of the JA-marker gene *VSP2* (left) and of the ABA-marker gene *RAB18* (right) in damaged leaves of *Arabidopsis thaliana*, 24 h after infestation. Data represent means ± SE. For *VSP2*, induction level in absence of herbivory was lower than 5. Values were normalized relative to the reference genes *GAPDH* and *FBOX* and quantified relative to control plants. $*P < 0.05$, $***P < 0.001$ (3-way ANOVA, $n = 4$).

plant defenses (JA and the toxic glycoalkaloid tomatine) in microbe-treated plants (Megali *et al.*, 2014). In contrast, most studies on *A. thaliana* have shown that plant growth-promoting rhizobacteria lead to an increase in the defensive compounds glucosinolates and an associated induced resistance against generalist leaf chewers (van de Mortel *et al.*, 2012; Aziz *et al.*, 2016; Pangesti *et al.*, 2016a). Although we did not investigate plant chemistry in this study, based on those previous studies, the induced susceptibility is more likely to be a result of the microbial-enhanced plant quality rather than to a suppression of defensive compounds.

The expression of the JA/ABA-responsive gene *VSP2* and the drought-responsive gene *RAB18* was upregulated after herbivore damage and drought respectively. However, *P. s.* WCS417r colonization did not prime the expression of either *VSP2* or *RAB18*. Our result for *VSP2* expression contrasts with previous findings. In other studies, root colonization by *P. s.* WCS417r primed *A. thaliana* plants for an enhanced expression of JA-responsive genes such as *VSP2*, *LOX2*, *PDF1.2* and *HEL* after pathogen or insect challenge (Pozo *et al.*, 2008; van Oosten *et al.*, 2008; Pangesti *et al.*, 2014). Interestingly, all those studies also observed microbial-induced systemic resistance, whereas in our study we did not observe ISR. The logical question then, is whether whenever microbial-ISR is not observed, plants are also not primed for an enhanced defensive response. The fact that we did not observe priming of defense-related genes in the colonized plants might be linked with the slightly reduced leaf damage and consequently less induction on the rhizobacteria-colonized plants. Experiments with an inducer such as MeJA or mechanical damage plus oral secretions could shed more light on this aspect without incorporating differences in induction as a result of insect behavior.

Interestingly, the negative effect of drought on larval performance disappears in the JA-impaired mutant *dde2-2* but not in the *aba2-1* mutant, which confirms that the effect of drought on herbivores is JA-mediated and not ABA-mediated. The plant response to osmotic stress involves ABA-dependent and ABA-independent signaling (Yoshida *et al.*, 2014), so our results suggest that the plant response to drought that negatively affects herbivores is mainly regulated by ABA-independent mechanisms. In contrast, JA has been proposed to be the core signal that regulates plant resistance to herbivory (Erb *et al.*, 2012), and here we show that it is also a crucial hormone regulating plant resistance under drought stress. Regarding the role of JA and ABA in plant–rhizobacteria–insect interactions, we could not provide evidence for this because rhizobacteria did not affect the herbivore on the wild-type Col-0 plants when analyzing the mutants. Therefore, the question of whether a functional ABA-mediated response is required to mount rhizobacteria-mediated ISR still remains open.

Plant growth-promoting rhizobacteria are able to promote plant growth and plant defenses. Nevertheless, the outcome of the interaction is variable and drought does not seem to be one of the factors explaining this variability. We first hypothesized that microbes may "help" plants against

insects only when plans "need help," drought being one scenario where plants would need such help. We could not provide support for this hypothesis here, a possible reason being that our system included a plant accession and a rhizobacterial strain originating from areas where drought is not common (Lamers *et al.*, 1988; Anastasio *et al.*, 2011). Further work with other systems that have co-evolved in areas suffering from drought stress may find completely different results. Studies addressing additional aspects such as the timing of the interaction or intensity of drought stress would shed light on how the modulators of microbe-plant-insect-interactions function. In addition, plant roots associate not only with 1 bacterium but with a diverse microbial community, the so-called microbiome. There is scant research on the ecological functions of the rhizosphere microbiome, especially on how it modulates the interactions of plants with their biotic and abiotic environment (Philippot *et al.*, 2013). This fundamental knowledge will allow us to advance at using of microbial inoculants in sustainable agricultural practices.

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Disclosure

The authors declare that they have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Primer sequences used in qRT-PCR.