# Manipulation of plant ethylene balance by soil microbiota: a holobiont perspective to stress tolerance

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# Manipulation of plant ethylene balance by soil microbiota: a holobiont perspective to stress tolerance

**Het manipuleren van de ethyleen balans in planten door bodem microben: een holobiont perspectief op stress tolerantie**

(met een samenvatting in het Nederlands)

Proefschrift

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door

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# 1

# General Introduction

Mohammadhossein Ravanbakhsh

## Introduction

Plants have evolved intricate pathways to adapt their behavior according to environmental conditions. This does not occur in isolation, but in presence of a complex microbiome that helps to shape plant fitness and evolution. Understanding the interplay of plant and microbes in determining plant health and performance represents an essential area of study in the light of future needs for increased crop production, nature conservation and a decreased environmental cost. Here, I focus on potential impacts of plant associated microbiota on ethylene, a central plant regulator functioning as an integrator of environmental stress and plant development.

## Ethylene regulation of plant stress responses

Plants can face multiple stressors during their life cycles, including submergence, drought or exposure to heavy metals. Being sessile, plants cannot physically escape stressful conditions. Instead, they have developed refined adaptations to cope with stress. These adaptations encompass a range of mechanical and physiological alterations that all come at some cost. Stress responses may divert resources from growth and development or have negative effects on other components of plant physiology. The widespread trade-offs between stress tolerance and other life history traits make an accurate regulation of the relative investment into growth and resistance critical for plant fitness. This implies the ability to perceive environmental constraints and prioritize resource allocation along the different physiological necessities required to maximize fitness. Ethylene is the central plant hormone functioning as an integrator of environmental stress and plant development (Abeles *et al.*, 2012). It coordinates several aspects of plant growth and development, throughout the whole plant life cycle, from germination to senescence (Bleecker & Kende, 2003). In addition, this hormone is essential for regulating stress responses and is required for stress tolerance (Sasidharan & Voesenek, 2015; Thao *et al.*, 2015). Stress induces increased levels of ethylene in plants, which in turn triggers adaptive responses and influences other hormonal signalling pathways (Pieterse *et al.*, 2009). Due to the multiple effects of ethylene on plant phenotype, increased ethylene levels induce a range of pleiotropic effects, such as growth inhibition and late flowering (Scarpeci *et al.*, 2017), in addition to the specific intended target response (Roux *et al.*, 2006). Precisely controlling cellular ethylene levels is thus a key aspect of plant physiology (Roux *et al.*, 2006). In this thesis, I approach ethylene as a coordinator balancing different life history traits to reach the best possible phenotype given the present constrains, thereby maximizing reproductive fitness. High and low ethylene levels may shift plant phenotype along trade-offs determined by the limitations

imposed by plant physiology. Depending on ethylene levels, a given plant genotype may, for instance, produce less biomass but flower early, or induce the opposite effect of attaining higher biomass with late flowering (Scarpeci *et al.*, 2017), (Fig. 1). The exact trait combination maximizing fitness will depend on the environment, meaning that small variations in ethylene signaling might have relatively large impacts on plant fitness or, in an agricultural context, yield. Thus, variation in ethylene signaling depends on an interplay between the plant genotype and the environment. The next section will examine how this interplay is also profoundly impacted by microbiota, and I argue that the plant microbiome is critical in the co-regulation of ethylene signaling.

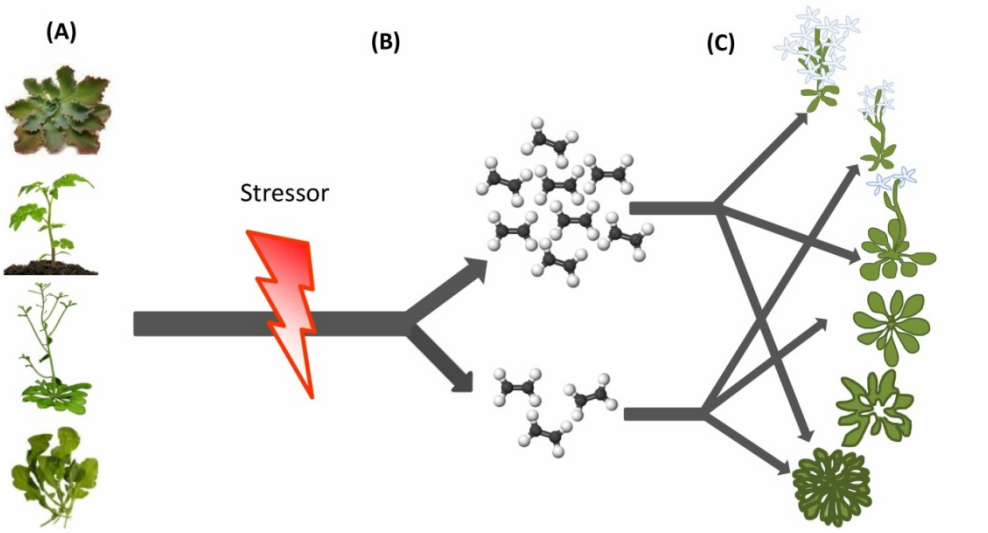



Fig. 1. Variability in stress perception, ethylene signal transduction and ethylene-based stress responses across plant species. Different plant species (A) may respond to the same stress with different levels of ethylene (B). In turn, this variation in ethylene levels will lead to a species-specific phenotype, illustrated here by a shift of resource allocation from vegetative to reproductive parts.

**Importance of microbiota as co-regulators of stress responses**

Plants are associated with a multitude of microbes that collectively manipulate plant physiology and fitness. In the context of stress, plant responses are not only be determined by the plant genome, but also by microbiota that co-regulate the plant stress response. Plant-associated microbiota can play an important role in

the entire plant life history, especially plant stress responses. One of the most studied examples of manipulation of plant stress response by microbiota is the reduction of ethylene by ACC deaminase-producing microorganisms. Several bacteria and fungi harbor this enzyme, which degrades the ethylene precursor ACC (1-Aminocyclopropane-1-carboxylic acid) and provides a nitrogen source (Glick, 2014). As a consequence, microbes with this enzyme may reduce ethylene concentrations in the plant and thereby potentially altering downstream cascades triggered by this hormone. Possession of an ACC deaminase enzyme by bacteria has typically been viewed as a plant growth-promoting trait across a wide range of environments and plant genotypes. This is rather surprising given the accepted role of ethylene as a central regulator of plant stress responses.

In this thesis, I examine two representative and widespread plant stressors, heavy metal contamination and flooding. Numerous soils worldwide experience heavy metal contamination often constituting a constant, sublethal stress for plants. For plants, flooding represents an intensive, potentially life-threatening pulse stress, and the importance of flood tolerance is increasing with continuing intensification of extreme weather event and global warming (Hirabayashi *et al.*, 2013). I examined the importance of ethylene signalling and its manipulation by ACC deaminase- producing bacteria as related to the ability of plant to cope with these stressors.

**Research question and approach**

Ethylene plays an essential role in plant responses to a range of abiotic and biotic stressors (Abeles *et al.*, 2012). The effect of ethylene is dose-dependent, and inappropriate responses are generated if ethylene levels are too high or too low (Voesenek *et al.*, 2015). ACC deaminase activity in bacteria has been considered as a plant growth-promoting trait, due to the notion that bacterial production of this enzyme can reduce the negative effects of high ethylene levels elicited by acute stressors. Hence, this microbial trait has generally been assumed to be beneficial for the plant across a wide range of environmental condition (Glick, 2014). However, this general notion of positive plant effects is somewhat at odds with the crucial role of ethylene for plant stress tolerance. In cases where increased ethylene levels lead to an adaptive response to stress, ACC deaminase activity will actually yield negative effects for the plant. In this thesis, I argue that this discrepancy is chiefly be attributed to two causes. Firstly, the general acceptance for ACC deaminase activity as a plant growth-promoting trait may be due to the search for plant growth-promoting bacteria. The growth conditions used and the isolates examined are typically designed to select for plant growth promoting traits such as ACC deaminase, auxin, Siderophore (Penrose &

Glick, 2003). Hence, these researches find what they are looking for, plant growth promoting bacteria producing ACC deaminase enzyme. Conditions for which growth inhibition might occur or ACC deaminase bacteria without growth promoting generally not considered or studied further. Secondly, most studies examining the effects of ACC deaminase activity fail to isolate ACC deaminase activity as the actual cause of plant growth promotion. As demonstrated in this thesis, the actual impact of bacterial ACC deaminase can best be examined by the use of functional mutants defective in ACC deaminase activity. Several past studies assessing the role of ACC deaminase-producing bacteria have described uncharacterized bacteria producing ACC deaminase enzyme, but without a ACC deaminase negative control. As such, it has until recently not been possible to disentangle the effects of ethylene modulation from other microbial traits that impact plant growth (Fig. 2) (Grichko & Glick, 2001; Belimov *et al.*, 2005; Ramadoss *et al.*, 2013). In the following chapters, I consider alterations of ethylene signaling by microbiota in the context of the recent developments in the fields of plant ecophysiology and plant-microbe interactions. Specifically, I seek to examine how microbial activity impacts plant tolerance to the stresses imposed by heavy metal contamination and submergence. This examination also includes the impacts of other bacterial traits that may affect plant growth and physiology and thereby mask or increase the effects of ACC deaminase. As an extension of this perspective, the effects of ACC deaminase are also assessed in relation to the bacterial genetic background (chapter 6).

**Effect of ethylene reduction by microbiota on heavy metal resistance**

Heavy metal contamination of soils is a worldwide problem. This can cause both extreme stress and harm to plants and also poses a serious food security concern due to heavy metal uptake by plants. Widespread heavy metal contamination of agricultural lands has been well documented in Europe and North America (Lewandowski *et al.*, 2006) (Fig. 3). Some plants have efficient resistance mechanisms that can either prevent heavy metal uptake or convert such contaminants into less harmful forms. In the context of food safety, preventing heavy metal entry into the plant represents a central goal to human prevent exposure to these toxic compounds. Ethylene is one of the key hormones coordinating plant physiology under heavy metal stress, and recent studies have revealed that ethylene is essential for plant heavy metal tolerance (Thao *et al.*, 2015). Ethylene increases plant tolerance to heavy metal stress by triggering the expression of systems for scavenging reactive oxygen species and chelating heavy metals into less toxic forms (Keunen *et al.*, 2016). Surprisingly, ethylene can increase expression of transporters linked to heavy metal uptake (Wong

& Cobbett, 2009; Lee & An, 2009) and potentially could be involved in heavy metal uptake (Waters et al., 2007). Ethylene-mediate plant heavy metal tolerance depends on the plant species and homeostasis mechanism, plant growth stage, metal specific properties, the level of plant exposure, the method of applying metals, and the experimental design (Keunen et al., 2016; Thao et al., 2015). Thus, I hypothesize that alterations of this cascade by the plant or its microbiota will have far reaching repercussions on plant growth and heavy metal content.

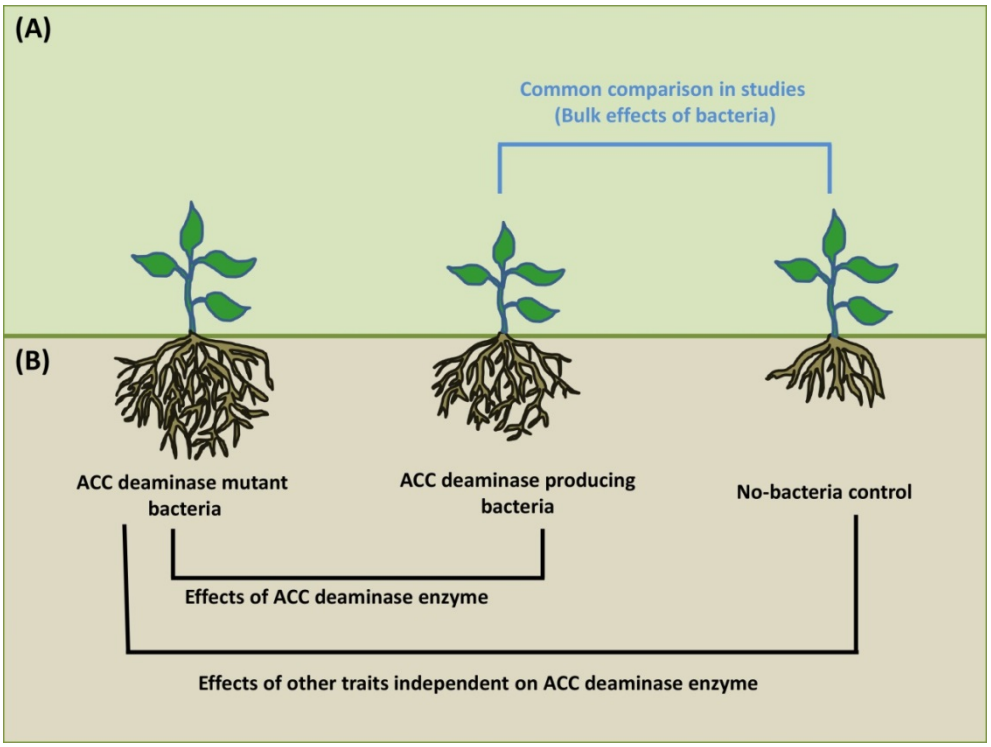



**Fig. 2.** Importance of experimental design for assessing the role of ACC deaminase on plant stress response. Improper design may lead to inaccurate or even incorrect interpretations. Most studies assessing the role of ACC deaminase-producing bacteria on submergence and heavy metal stress have lacked a control treatment to separate the effect of ethylene modulation from other microbial traits. Most experiments have compared inoculation with an ACC deaminase-containing bacterial strain with a non-inoculated control (A). In the present thesis, a more complete experimental design is used in which three treatments are considered: In addition to the reference bacteria producing ACC deaminase and the a no-bacteria control, I also include a treatment with an isogenic mutant bacteria unable of producing ACC deaminase. The

comparison of ACC deaminase-producing and -deficient bacteria zooms in on the actual importance of ACC deaminase, while the comparison between the ACC deaminase-deficient mutant and the no-bacteria control accounts for effects on plant growth that are independent of this enzyme (B).

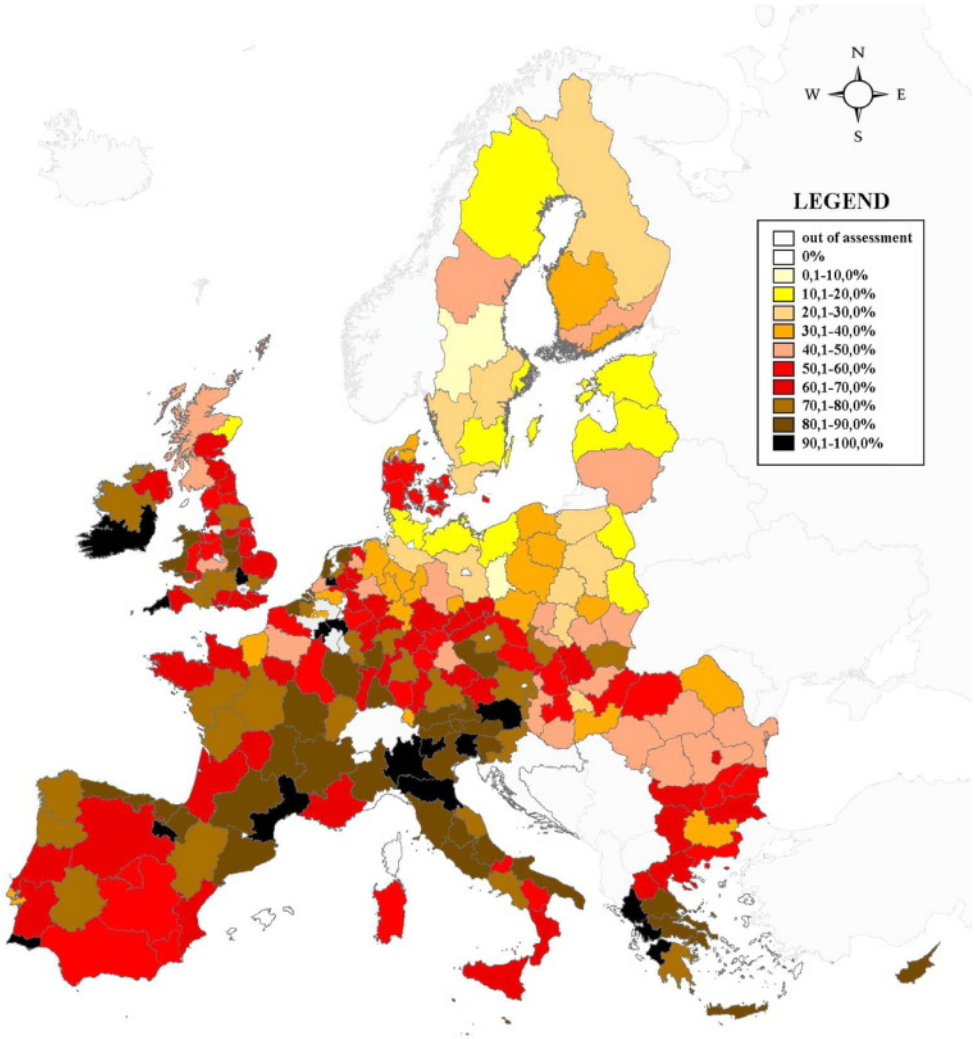



**Fig. 3.** Heavy metal contamination levels of agriculture lands in Europe. Colors correspond to percentage of samples with concentrations (for one or more heavy metals) above the threshold value of standardized guideline (MEF, Finland, 2007). Source; (Tóth *et al.*, 2016b).

As stated above, plants are associated with numerous microbes that have the potential to impact the ethylene balance of the plant. This thesis therefore seeks to focus on the joint role of the plant genetic background and ACC deaminase-producing bacteria as regulators of ethylene signalling and heavy metal stress response. Microbial ACC deaminase activity can reduce plant ethylene levels. Given the well-described effects of ethylene on traits regulating nutrient uptake and transport in plants, I assessed the effects of ethylene reduction by bacteria on cadmium uptake in three different plants varying in cadmium tolerance (chapter 3). I then studied the role of ethylene reduction by bacteria on plant response to cadmium, and show in chapter 4 that ethylene reduction by bacteria actually decreases a plant's ability to cope with cadmium, which is in sharp contrast to the commonly held dogma put forth in the literature. Finally, I show that the effect of ACC deaminase-producing microbes on plant stress tolerance is highly variable and depends on the bacterial strain examined and environmental conditions (chapter 6).

**Roles of ethylene and ACC deaminase-producing bacteria in the response to flooding**

Extreme climate events that cause flooding events have increased over the past decades and this trend is expected to continue into the future. Plants subjected to flooding suffer from low oxygen and light availability and a severe restriction in gas exchange (Voesenek & Bailey-Serres, 2015). Flooding thus represents a major stress for plants, and plant species have evolved different strategies to deal with flooding. Ethylene is the main hormone coordinating plant responses to submergence. This gaseous hormone readily accumulates under water-logged conditions and triggers different plant adaptive responses, including shoot and petiole elongation, aerenchym formation, and lateral root extension (Sasidharan & Voesenek, 2015). Plant species generally follow one of two different strategies in response to submergence: escaping it by growing tall shoots, or remaining quiescent, saving resources until the flood has past. As both strategies are ethylene dependent, alteration of ethylene levels by root-associated microorganisms may pose a serious threat to plants, or alternatively may offer an adaptive opportunity to help optimize the flooding response. In chapter 5, I show that ACC deaminase-producing bacteria can indeed inhibit a plant's response to submergence. I suggest that root-associated bacteria represent an overseen driver of plant life-history strategies that should be taken into account when assessing plant ecological adaptations, such as abiotic stress resistance.

**Outline of this thesis**

In this thesis, I examine how root-associated bacteria alter plant response to environmental stress by modulating plant hormonal balance. This effect is demonstrated to mainly deleterious to the plants under stressful conditions, which challenges the previously held view that ethylene reduction is a beneficial plant-microbe interaction. In chapter 2, I established a model system with plants varying in their resistance to heavy metal stress. I selected *Alcea aucheri*, a lead- and zinc-tolerant plant, *Rumex palustris*, a submergence-resistant plant, and *Arabidopsis thaliana,* as model organism to investigate the underlying molecular mechanisms. In chapter 3, I study the importance of the ACC deaminase enzyme as biotechnological tool to enhance or reduce cadmium uptake in plants and show that bacteria harboring this enzyme can reduce cadmium uptake in an ethylene-dependent manner. In chapters 4 and 5, I assess the effects of ethylene reduction by bacteria on plant stress tolerance (flooding and cadmium). In chapter 6, I then examine context dependent effects of bacterial ACC deaminase activity, using four pairs of ACC deaminase bacteria and their isogenic ACC deaminase-deficient mutants in interaction with three *A. thaliana* genotypes varying in ethylene production and sensitivity. In chapter 7, I review the evolutionary implications of microbial modulation of ethylene on plants life history.

**Chapter 2. Evaluation of model hyperaccumulator plants**

In this first section, I screened for plants able to take up high amount of heavy metals in aerial parts. I selected two plants for future experiments, one from a dryland area and one from riparian zones subject to repeated flooding. This first section of this chapter was performed at the Ecology and Biodiversity group of the Utrecht University and at the Shiraz University, Iran.

**Screening for the next generation of heavy metal hyperaccumulators for dryland decontamination**

In this section, I examine candidate plants for phytoremediation in arid climates. Sixteen dominant plant species were selected from a mining area naturally polluted with high Pb-Zn and Cd concentration. *Alcea aucheri* was the most promising of these species, accumulating more than 4 mg $g^{-1}$ Pb in shoots. I confirmed this ability with a greenhouse experiment in soil spiked with different Pb and Cd concentrations. I propose *A. aucheri* as model hyperaccumulator due to its ability to grow in adverse

conditions, produce high biomass, and surpass heavy metal accumulation reported for other plants.

***Rumex palustris*, a riparian wetland plant with high ability for Cd accumulation**

The widespread European riparian plant *Rumex palustris* was found to show very high cadmium shoot concentrations and translocation factors in contaminated soil. This plant showed high levels of Cd accumulation as well as the ability to grow across a wide range of wetland habitats. These results indicated that *R. palustris* could be an effective model plant for monitoring heavy metal contamination for a wide range of environments.

**Chapter 3. Reduction of ethylene levels by rhizosphere-associated bacteria can reduce cadmium uptake in three phylogenetically different plant species**

In chapter 3, I demonstrate that alteration of plant ethylene signalling by root-associated bacteria can reduce the uptake and accumulation of cadmium, a major pollutant and global concern for food safety. Plants were inoculated with bacteria producing the enzyme ACC deaminase, which degrades the precursor of ethylene. I tracked effect of bacterial inoculation on plant growth and cadmium uptake on a cadmium-contaminated soil. Results shed new light on the importance of plant-microbes interactions in adjusting plant hormonal balance. Microbes can prevent heavy metal uptake into plant material, making plants safe for consumption, despite their being grown on heavy-metal polluted soils.

**Chapter 4. Effect of ethylene reduction by associated bacteria along a stress gradient**

In chapter 4, I show that ethylene reduction by bacteria might have different effects on plant growth depending on the stress level. Reduction of ethylene by bacteria provides some advantage for plant in absence of stress, but reduced plant growth higher under cadmium stress. I conclude that, instead of always being beneficial to the plant as commonly accepted, ACC deaminase-producing bacteria can actually impair plant responses to cadmium.

**Chapter 5. Effect of ACC deaminase-producing bacteria on submergence tolerance**

ACC deaminase-producing bacteria are commonly accepted as having plant growth-promoting effects in the presence of stressors including submergence, by decreasing the levels plant ethylene. Under flooding stress conditions however,

ethylene accumulation is important. Limited underwater diffusion causes rapid ethylene accumulation, which then triggers morphological adaptations critical to survival. In chapter 5, I assessed whether phenotypically plastic plant responses that are essential for survival under flooding stress can be altered by ACC-deaminase producing bacteria. I found that plant responses to flooding are determined by both the plant and as well as its associated microbiota. Treatment of *Rumex palustris* with ACC deaminase-producing bacteria interfered with the ethylene-mediated response required for plant adaptation. It is therefore argued that soil microbes can modify plant life history strategies and need to be taken into account as an integral component of plant flood tolerance.

**Chapter 6. The effect of ACC deaminase-producing microbes on plant growth and stress resistance depends on the bacterial genetic background**

Ethylene reduction by different associated bacteria has been intensively investigated as a way of host stress alleviation in different plant species. However, ACC deaminase-producing bacteria able to reduce ethylene also carry other functional genes that may alter plant physiology. In chapter 6, I further show that the effect of ethylene reduction by bacteria on plant growth depends on the bacterial genetic background.

**Chapter 7. Microbial modulation of plant ethylene signalling: ecological and evolutionary consequences**

In this chapter, I summarize the results and concepts developed during my thesis in form of a perspective article. I propose to revise the concept of plant-growth-promoting-bacteria and instead consider microorganisms as matchmakers between plant genotype and the phenotypic requirements needed under a given set of environmental conditions. The central focus of this chapter is an examination of how interactions between plants and their associated microbiomes impact the evolution of ethylene-mediated plant responses to stress. Several microbiota are able to modulate ethylene levels by different traits, which depends on an intricate interaction between plant genotype and these associated microbiota. I propose that ethylene-mediated stress responses are the result of a co-evolutionary process between plants and microbes. Ethylene signaling forms a complex regulatory network allowing plants to respond to a range of stressors. Due to pleiotropic effects, evolution of precise ethylene signaling by the plant itself may be constrained. However, fine-tuning of ethylene regulation by microbes may help the host achieve the correct phenotypic response appropriate for the given environmental conditions, provided that there is a

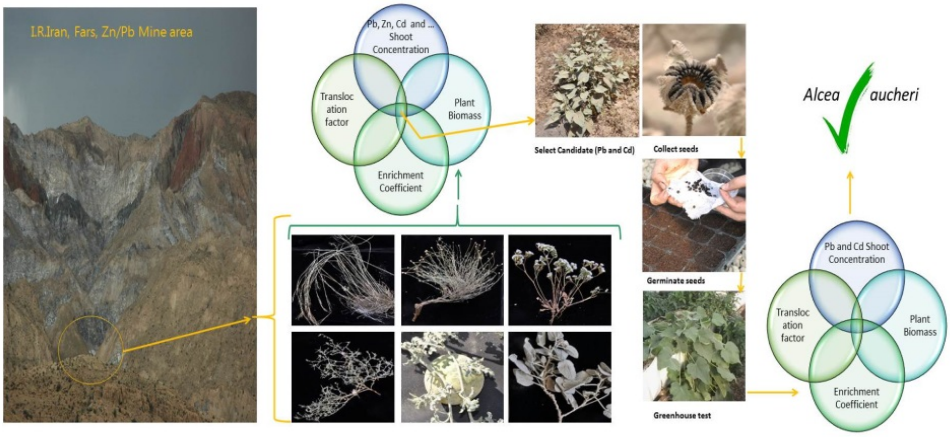
good match between the host plant and its associated microbes. In addition, associated microbiota offer a more dynamic and precise ability for the plant to match the response to environmental change. In contrast, a mismatch between hosts and microbes may result in either too high or too low levels of ethylene, resulting in a suboptimal phenotype.

**Chapter 8. Summarizing discussion**

Chapter 8 provides a brief summarizing discussion of the main results, applications and perspectives of the thesis.

# 2

# Screening for the next generation heavy metal hyperaccumulators for dryland decontamination.

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**Abstract**

Heavy metal removal by plants bears a great potential to decontaminate soils. A major challenge remains to find plant species that accumulate heavy metal, harbor a sufficient biomass and grow in the desired environmental conditions. Here we present candidate plants for phytoremediation in arid climates. We sampled sixteen dominant plants from mining area naturally polluted with high Pb-Zn and Cd concentration. Plants were assessed for their ability to accumulate Zn, Pb and Cd and six species were selected on the base of their heavy metal concentration in shoots and leaves, enrichment coefficient and translocation factor. Out of all the tested species in field study, *Alcea aucheri* was the most promising one which accumulated over than 460 and 4089 µg/g Pb in the roots and shoots, respectively. We confirmed this ability with a greenhouse experiment on soil spiked with different Pb and Cd concentrations. Concentration of Pb and Cd in aerial parts of *A. aucheri* were more than 1700 and 345 µg/g in 2400 and 200 mg/kg Pb and Cd soil treatment respectively. We propose that *A. aucheri* as model hyperaccumulator able to live in adverse condition, producing high biomass, and supersede heavy metal accumulation reported to other plants, making of this species one of the best Pb hyperaccumulator reported to date.

**Keywords**: *Alcea aucheri*, hyperaccumulator, phytoremediation, Pb, Cd.

**Introduction**

Contamination of air, water and soil with heavy metals is a major environmental concern in many parts of the world. In absence of better alternatives, polluted zones are often treated by common method such as removal and burial of the contaminated soil. These methods are still expensive and inefficient, making them hard to apply on large area (Kumar *et al.*, 1995). Phytoremediation, the removal of pollutants by plants, has often been proposed as an alternative and more practicable and environmental friendly strategy to restore soils. However, attempts to find new promising remediation methods have to date shown unsatisfactory results. Its low costs and disturbance coupled to its applications to a wide range of pollutants, makes phytoremediation very attractive (Kumar *et al.*, 1995). However, a couple of challenges must still be addressed if we are to use large scale phytoremediation strategies. The interest of phytoremediation depends on the ability to extract high amounts of heavy metals from soil by plants. This requires both a high uptake of heavy metals per unit of plant weight and a high aboveground biomass of candidate plants

(Ebbs *et al.*, 1997a; McGrath & Zhao, 2003), which is limited especially in case of Pb, Cu and Cr (McGrath & Zhao, 2003).

So far, more than 450 hyperaccumulator species are reported (McGrath & Zhao, 2003c), with new species added every year. A promising strategy to discover new species is to sample the vegetation of heavily polluted zones, such as those around mines (Yanqun *et al.*, 2004; Bech *et al.*, 2012). Tolerant or accumulating may be at an advantage so that dominant species will likely cope well with heavy metals (Sun *et al.*, 2008). Discovery of new plants is particularly needed for dry areas, where plants must cope with climatic extremes in addition to the heavy metal stress.

Here we sampled contaminated area in Iran to discover new hyper-accumulating plants. Iran is climatically located on Afro-Asian desert belt and consequently faced with high evapotranspiration and low precipitation. Screening semi-desertic area in order to find wild metallophytes for phytoextraction of contaminated drylands is a promising way to discover species suitable for large scale phytoremediation in difficult field conditions (Ghaderian *et al.*, 2012).

We sampled plants growing in a mine- contaminated area in Iran. We identified the dominant plant species and assessed their ability to take up Pb, Cd and Zn in their aerial parts. We report the results of six of the most promising plants with a special emphasis on *Alcea aucheri*, a new species that appears to accumulate heavy metals far beyond any other reported dryland plants and is especially efficient to remove non-mobile elements such as Pb in calcareous soils. We then validated heavy metal uptake of *A. aucheri* in controlled conditions in a separate greenhouse experiment.

## Method and materials

### Sampling Site description

We sampled plants in an area enriched in Pb, Zn and Cd close to an active Pb and Zn mine in the Fars province, Iran. This area is located on the Pb-Zn ore deposit of the Zagros folded belt between UTM coordinate of 640300 and 3158136 with the 603 m attitude and 640426 and 3158230 with the 982 m attitude. Lead and zinc in the form of carbonate and sulfate are the main ores. Total area of site was about 80 ha with an average annual temperature of 24°C and annual rainfall of 210 mm.

### Soil and plant sampling

Plant samples and related rhizosphere were collected from January to March 2014. We selected two different location, a 20 ha and a 60 ha area, near two main ores

with Pb concentration more than 500 mg/kg. Plant species and their abundance were surveyed by systematic sampling method. The dominant plants were recorded using 2 (10m × 10m) plots per hectare and identified. We assessed the six most abundant plants in relation to their ability to take up heavy metals uptake in roots and shoots. We then compared the heavy metal concentration to the surrounding soils to retrieve the translocation factor (transfer of heavy metals from roots to shoots), the enrichment coefficient (heavy metal concentration in plant relative to the surrounding concentration in soil).

**Plant and soil analysis**

Heavy metal content was determined separately in shoots and roots using standard procedures (Kalra, 1997) with a few modifications. Briefly, plants were washed with deionized water and surface-adsorbed heavy metals were removed by immersing roots in 20 mM $Na_2$–EDTA for 15 min. Plant samples were dried at 60°C, ground and sieved at 2 mm. Concentration of Pb, Zn and Cd were measured by atomic absorption spectrophotometer (AA-670 Shimadzu, Japan) after dry digestion (Kalra, 1997). The total concentration of Zn, Pb and Cd in soil were determined by standard method (McGrath & Cunliffe, 1985).

**Enrichment coefficient and translocation factor**

Enrichment coefficient, the capability of plants for adsorbing heavy metals from soil and accumulate in their roots was defined for each single heavy metal as follows:

Enrichment coefficient = Heavy metal concentration in plant above ground/Total heavy metal concentration in soil

The translocation factor, the ability of plants to translocate heavy metals from roots to shoot was defined as follows

Translocation Factor = Heavy metal concentration in shoots/ Heavy metal concentration in roots.

**Greenhouse experiment**

The ability of *Alcea aucheri* to take up Pb and Cd was validated in greenhouse experiment with five levels of soil heavy metal concentration (0, 300, 600, 1200 and 2400 $mg.kg^{-1}$ Pb or 0, 50, 100, 150 and 200 $mg.kg^{-1}$ Cd, respectively). Metal level was replicated with or without EDTA (0.5 $g.kg^{-1}$ soil), a chelating agent widely use to increase the bioavailability of micro nutrient and heavy metals in phytoremediation of Pb and Cd (Luo *et al.*, 2005). Two separate experiments were performed for Pb and

Cd. Three replicates were set up per treatment. Plants were grown for 48 days in pots filled with 1 Kg fine, mixed, mesic, fluventic calcixerepts soil at 28±2 °C. Lead (Pb) and cadmium (Cd) were added in the form of $Pb(NO_3)_2$ and $Cd(NO_3)_2$. The amount of extra nitrate from metal treatment was calculated and added to control to avoid side effects of nitrate on treatment effects. After 48 days, plants were harvested. We measured shoot and root dry weight as well as Pb and Cd concentration in shoots, roots and soil.

**Statistical analysis**

**Field sampling analysis**

The effect of plant species on heavy metal uptake, translocation factors and enrichment coefficient was evaluated using separate way ANOVA tests. Individual means were compare by Duncan's multiple range test. Cluster analysis was applied to identify different plants group, based on similarity in shoot concentration, translocation factor and enrichment coefficient. This analysis was undertaken by Ward-algorithmic method.

**Greenhouse experiment analysis**

The interactive effects of soil heavy metal concentration (five levels) and EDTA (two levels) in the green house experiment was assessed with two way ANOVA tests. Separate analyses were performed for Pb and Cd.

**Results and discussion**

**Dominant plant species in study area**

In two different areas with average Pb concentration higher than 500 mg/kg, 16 different plant species were identified and recorded by systematic sampling method (supplementary materials). Six dominant plants were selected and compared based on shoot concentration, translocation factors and enrichment coefficient.

**Similarity of plants based on shoot concentration, translocation factor and enrichment coefficient**

We selected six more frequent plant species in high Zn and Pb contaminated soils and classified them in two different clusters based on multivariate analysis of Zn, Pb and Cd Shoot Concentration, translocation Factors, Enrichment Coefficient, meaning plant in each cluster should have similar uptake, distribution characteristics in general for these three metals.

We observed two important clusters. The most promising one from a phytoremediation perspective comprised *Alcea Aucheri* and *Centaurea Bruguierana,* two plants showing a similarity level in their heavy metal accumulation patterns of more than 83%. This similarity was due to the high Zn, Pb and Cd shoot concentration, translocation factor, and enrichment coefficient in this cluster which makes this as a suitable candidate for phytoextraction. This cluster has this ability to uptake large amounts of metals with roots and translocates them efficiently to aerial part without limitation.

*Limonium Thounii*, *Citrullus Colocynthis*, *Hyparrhenia Hirta* and *Platychaete Aucheri* are belong to second cluster with similarity level more than 93% and difference with clusters 1 in terms of enrichment coefficient ($F_{1,69}$ = 15.7, $p < 0.001$) indicating significant difference between cluster one (M = 1.5, SE = 0.24) and cluster two (M = 0.3, SE = 0.17), translocation factors ($F_{1,70}$ = 7.1, $p < 0.001$) indicating significant difference between cluster one (M = 2.2, SE = 0.28) and cluster two (M = 1.2, SE = 0.20) by Duncan's Multiple Range Test. Shoot concentration in first cluster was 522.66 µg/g (SE = 98.71) compare to 335.43 µg/g (SE = 69.79) without statistical difference between two plants ($F_{1,70}$ = 2.4, $p$ = 0.13). Plants in cluster 2 mainly retrieved from soil with high level of Pb, Zn and Cd, and accumulate different and high concentration of heavy metals in roots (comparable with first cluster), but constant and relatively lower concentration in shoots, suggesting that exclusion was main survival mechanism of this cluster. Although this cluster may not be suitable for phytoextraction, it may prove interesting for other applications such as phytoremediation based on stabilization (Yanqun *et al.*, 2004; Wei *et al.*, 2005). *Alcea aucheri* appears as the most suitable plant species for heavy metal phytoextraction due to high concentration of metals in roots and ability to translocate it to aerial parts compared to other evaluated species.

**Criterion for hyperaccumulator plants**

We classified plants as hyperaccumulator when they 1) accumulated Cd, Pb and Zn in the plant aerial parts more than 100, 1000 and 10000 mg/kg, respectively, 2) show an enrichment coefficient and translocation factors above 1, demonstrating an active heavy metal transfer from roots to aerial parts (Yanqun *et al.*, 2004; Sun *et al.*, 2008).

Mean concentrations of Zn, Pb and Cd in usual plants are 100, 5 and 1 mg/kg, respectively (Yanqun *et al.*, 2004). All studied plants (except *L. Thounii* for Zn) accumulated heavy metals more than usual plants. Concentration of Pb in all six plants was 30-1100 times more than non-accumulator plants. Among six evaluated plants, *A.*

*aucheri* and *C. bruguierana* meet the criteria to be considered as Pb hyperaccumulator. They accumulate more than 5782.67 µg/gr and 2329.95 µg/gr Pb in aerial parts, respectively meaning 1156 and 93 times higher than non-accumulator plants. A typical Cd and Zn hyperaccumulator plants should has this ability to accumulate more than 100 and 10000 µg/g in the aerial parts. Although, based on field data, none of the studied plants could accumulate these amounts of Zn and Cd (Fig 1), we show that *A. aucheri* show this accumulation at greenhouse experiment and has this potential to evaluate as Cd accumulator.

Plants with translocation factor and enrichment coefficient more than one are suitable for phytoextraction process (Pilon-Smits, 2005). Lead translocation factor and enrichment coefficient in this two plants were 6.06, 1.50 and 5.38 and 2.19 respectively (Fig. 1). As *A. aucheri* shows a 30 times higher biomass than *C. bruguierana*, we selected it as main candidate for greenhouse experiment.

**Dose-dependent Pb and Cd accumulation in *A. aucheri* under greenhouse conditions**

The concentrations of Pb in the shoot of *A. aucheri* increased significantly ($F_{4,20}$ = 408.3, $p$ < 0.001) with increasing Pb concentration is soil with two ways ANOVA test from 4.69 µg/g in control to 1080.62 µg/g at higher applied Pb (2400 mg/kg) in the soil after 48 day exposure (table 1).

These concentrations also increased significantly ($F_{1,20}$ = 458.2; $p$ < 0.001) with application of EDTA from 147.85 to 557.23 µg/g (Fig. 2). The interaction effects of Pb level and EDTA application on Pb concentration in shoot was also significant ($F_{4,20}$ = 180.3, $p$ < 0.001). There were no visual symptoms of toxicity in this plant even at highest level of Pb.

Cadmium concentrations in the shoot of *A. aucheri* increased significantly ($F_{4,20}$ = 112.5, $p$ < 0.001) with increasing applied Cd in soil with two ways ANOVA test from 0.453 µg/g in control to 188.04 µg/g in higher applied Cd concentration (200 mg/kg) in the soil after 48 day exposure (table 1). These concentrations also increased significantly (p<0.001) in soil treated with EDTA from 29.31 to 114.18 µg/g ($F_{4,20}$ = 197.7, $p$ < 0.001). The interaction effects of Cd level and EDTA application on Cd concentration in shoot was also significant ($F_{4,20}$ = 75.7, $p$ < 0.001).

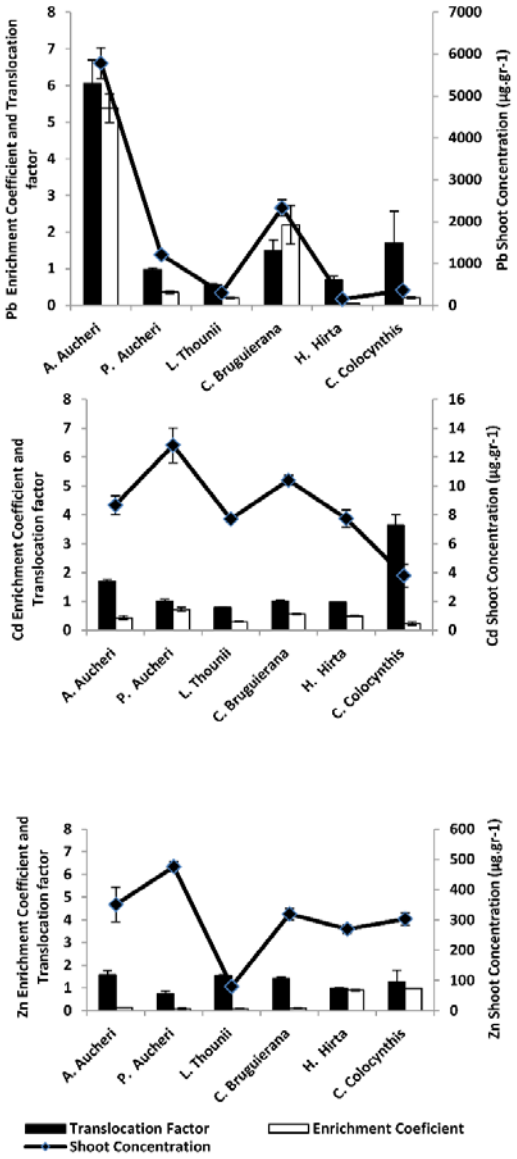


Fig 1. Pb, Zn and Cd translocation factors, enrichment coefficient and shoot concentration in different plant in field survey. Line show Pb, Zn and Cd shoot concentration in field study. E.C; Enrichment Coefficient. T.F; Translocation Factor.

**Table 2**. Effects on Elevated levels of Lead (Pb) and Cadmium (Cd) treatments on translocation factors, enrichment coefficient, and metal concentration in shoot and root in *ALcea aucheri* in greenhouse experiment. a, b means sharing the same superscript are not significantly different from each other.

| | | Applied metal (mg/kg) | Shoot Concentration (µg/g) | Root Concentration (µg/g) | Soil Concentration (mg/kg) * | T.F | E.C |
|---|---|---|---|---|---|---|---|
| **Pb** | No-EDTA | 0 | 5.00±3.3e | 7.53±2.7c | 27.67±8.4d | 0.65±0.25b | 0.18±0.09b |
| | | 300 | 43.61±5.0d | 34.80±3.5c | 143.5±20.9cd | 1.27±0.23ab | 0.31±0.07a |
| | | 600 | 94.22±2.8c | 67.18±6.7bc | 351.4±23.9c | 1.41±0.17ab | 0.27±0.02ab |
| | | 1200 | 221.1±21.4b | 123.5±34.1b | 793.7±67.4b | 1.93±0.76a | 0.28±0.02ab |
| | | 2400 | 375.3±14.4a | 208.2±61.4a | 1137.1±267.9a | 1.89±0.76a | 0.34±0.10a |
| | EDTA | 0 | 4.392±1.2d | 22.38±8.9e | 23.66±6.6e | 0.21±0.06c | 0.20±0.08c |
| | | 300 | 176.7±10.9c | 75.77±22.1d | 238.80±43.9d | 2.48±0.74a | 0.76±0.13ab |
| | | 600 | 239.3±4.0c | 296.2±4.6c | 344.7±31.9c | 0.81±0.03bc | 0.70±0.06b |
| | | 1200 | 579.6±54.6b | 469.8±13.8b | 881.9±105.3b | 1.23±0.12b | 0.66±0.02b |
| | | 2400 | 1785±153.6a | 1234±24.3a | 2002±45.4a | 1.45±0.11b | 0.89±0.08a |
| **Cd** | No-EDTA | 0 | 0.34±0.1c | 0.20±0.0e | 0.28±0.02e | 1.73±0.11a | 1.21±0.10a |
| | | 25 | 27.58±2.1b | 43.05±4.0d | 17.98±6.7d | 0.64±0.01b | 1.68±0.60a |
| | | 50 | 33.96±10.1b | 218.1±5.9c | 30.51±7.6c | 0.18±0.04c | 1.38±0.68a |
| | | 100 | 38.50±8.8ab | 336.8±12.2b | 87.29±2.0b | 0.10±0.03c | 0.39±0.09b |
| | | 150 | 46.19±4.1a | 463.9±7.2a | 164.6±8.2a | 0.10±0.01c | 0.28±0.02b |
| | EDTA | 0 | 0.56±0.1d | 0.28±0.1e | 0.60±0.1e | 2.10±0.42a | 0.94±0.16b |
| | | 25 | 41.10±3.8cd | 153.5±25.4d | 45.81±7.6d | 0.28±0.07b | 0.91±0.08b |
| | | 50 | 67.11±2.5c | 322.9±1.1c | 75.24±6.2c | 0.21±0.01b | 0.89±0.04b |
| | | 100 | 132.2±27.6b | 443.9±34.4b | 144.8±4.1b | 0.29±0.08b | 0.91±0.33b |
| | | 150 | 329.9±15.6a | 612.6±52.6a | 192.5±4.9a | 0.54±0.03b | 1.71±0.07a |

* Concentration of metal at the end of experiment.

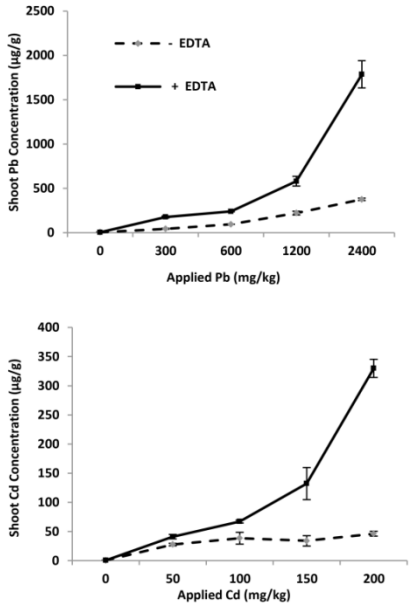


Fig 2. Pb and Cd shoot concentration (± 1SE) of Alcea aucheri after 48 days in greenhouse experiment at presence/absence of EDTA. Soils were spiked with different quantity of Pb (0, 300, 600, 1200 and 2400 mg/kg) and Cd (0, 50, 100, 150, and 200 mg/kg).

Concentration of Pb and Cd in shoot of hyperaccumolator plants correlated significantly with soil Pb and Cd, in line with previous study (Sun *et al.*, 2009). These uptake patterns confirm the field observation data and show that the *A. aucheri* could be a potential Pb-hyperaccumulator for phytoremediation process. The enrichment coefficient was less than 1 at greenhouse condition. Low availability of Pb in greenhouse calcareous soil may be possible explanation for low enrichment coefficient and translocation factor in greenhouse data. Enrichment coefficient increased significantly from 0.28 to 0.64 ($F_{1,28}$ = 11.0, $p$ < 0.001) when EDTA applied to soil as chelator of Pb treatment. This index increased with increase soil Pb concentration. This pattern also reported by others (Zhao *et al.*, 2010). The shoot concentration, enrichment coefficient and translocation factor of *A. aucheri* were increased with the increasing level of Pb in the soil indicating its ability to Pb adsorption and translocation in elevating Pb level in the soil. *A. aucheri* can accumulate more than 329 µg/g Cd in aerial parts in 48 days. It has noticeable enrichment coefficient, but relatively lower translocation factor compare to Pb.

Dry weight of *A. aucheri* was not affected by applied Pb (F4,20 = 1.1, p = 0.39) or Cd concentration (F4,20 = 1.9, p = 0.20), confirming the tolerance of this plant heavy metal contamination (Wei *et al.*, 2008). We did not observed visual symptoms of toxicity in any of the treatments. The mean dry weight of *A. Aucheri* was 5.3 g and 5.2 g at highest level of applied Pb and Cd after 48 days greenhouse experiment. The average amount of Pb and Cd concentration in aerial parts were 1081 µg/g and 196 µg/g in 2400 mg/kg Pb and 200 mg/kg Cd treatments. Considering a practicable sown density of 36 plants per square meter, we estimate that *A. aucheri* can reach a yield of 1.98 ton/ha after 48 days and remove 204.12 kg/ha Pb and 37.80 kg/ha Cd from soil in a 48 days period. Theoretically, *A. aucheri* would have the potential to remediate a 1000 mg/kg Pb contaminated area in 7 cultivation cycle, assuming 1400 tons surface soil per hectare. Total Cd uptake per hectare per year by *A. aucheri* is relatively higher than potential Cd uptake by the reference heavy metal accumulator *Thlaspi caerulescens* (Robinson *et al.*, 1998) (Bennett *et al.*, 1998).

Application of EDTA to soil Pb and Cd further increased Pb and Cd shoot concentrations in *A. aucheri* for Pb ($F_{1,20}$ = 458.2, $p$ < 0.001) and Cd ($F_{1,20}$ = 197.7, $p$ < 0.001) respectively (Fig. 2). This increase is in line with previous reports showing that EDTA can help increase uptake of Pb and Cd (Luo *et al.*, 2005; Lai & Chen, 2006; Shahid *et al.*, 2012).

**Heavy metal distribution in *Alcea aucheri***

In field conditions, concentration of Pb in *A. aucheri* exceeded 6800 µg/g in some old leaves, a value far above the root concentration of 400 µg/g in roots, a trend observed in phytoextractor plants. Concentration of metals in different organs of hyperaccumulator plants are in the order of: old leaves>young leaves>roots (Ebbs *et al.*, 1997). *Alcea aucheri* showed this trend for all three metals (Pb, Zn and Cd), and concentrations of Pb, Zn and Cd in its aerial parts were 5782.67 (SE=824), 350.44 (SE=93) and 8.67 (SE=1.5) µg/g compared to 971.53 (SE=210) , 320.06 (SE=54) and 5.085 (SE=1.5) µg/g in their roots, respectively. Among these elements, Pb and Cd is regarded as none or slightly mobile element in plants (Kumar *et al.*, 1995), meaning that Pb level should decrease from root to shoot, leaves, fruit and seeds (Sekara *et al.*, 2005). Low root to shoot translocation of Pb in plants has also been reported by others (Kumar *et al.*, 1995; Sekara *et al.*, 2005; Shahid *et al.*, 2012). The high Pb translocation factor (above 8) is remarkable in *A. aucheri* and suggests that *A. aucheri* harbours a specialized mechanism for absorption, accumulation and translocation of Pb. This high transfer of non-mobile elements in aerial parts is a huge advantage for

phytoremediation as it allows an easy harvesting of the biomass. This is clearly apparent in comparison with other candidate plants proposed for phytoremediations such as Indian mustard (*Brassica juncea*), which hyperaccumulates Pb but keeps it to more than 95%, complicating the extraction process (begonia, 1998).

High metal concentration in aerial parts and high biomass are two key factors for using plants in phytoremediation strategies (Ebbs *et al.*, 1997) and reduce the contaminant metals to an acceptable level (Kumar *et al.*, 1995). Even promising accumulators such as *Thlaspi rotundifolium* may not deliver sufficient results in field conditions due to their low biomass and growth rates (Huang & Cunningham, 1996). *Alcea aucheri* had higher biomass compared to the other Hyperaccumulators assessed in this study and is adapted to dry conditions, allowing its use to decontaminate semi-desertic area. We observed no visual symptom of chlorosis, necrosis and spot in the plants exposed to Pb and Cd even at highest level of heavy metal and also EDTA treatment. Furthermore the chlorophyll content in leaves did not show any significant difference at these levels of metal application (data not shown) confirming the ability of *A. aucheri* to accumulate Pb and Cd from highly contaminated soils without suffering from acute toxicity effects.

**Conclusion**

Wild plant species growing on heavy metal enriched soils bear a great potential for phytoremediation strategies. We propose *Alcea aucheri* as a candidate plant for large scale decontamination of heavy-metal contaminated drylands. It is especially efficient at taking up non-mobile elements such as Pb and to a lesser extent Cd. Its ability to remove high amounts of metals from the soil and translocating it to high concentrations in the aerial parts, coupled with a high biomass, allows removing high quantities of soil heavy metals in fewer growth cycles than alternative species. We propose that the biomass could be then burned and the ashes as ore for re-extracting the metals for further industrial use, making the decontamination process more attractive from an economical perspective.

**Acknowledgment**

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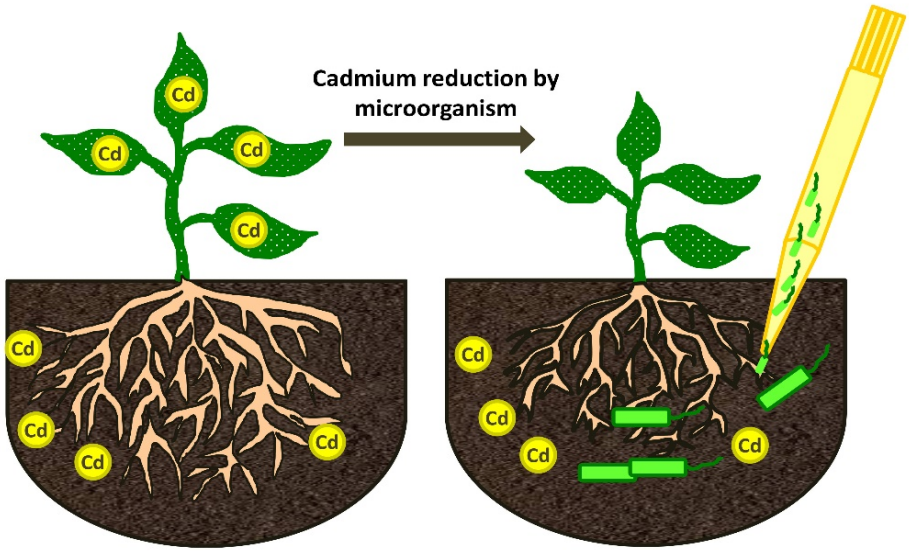
# 3

# Alteration of ethylene production by soil microorganisms prevents cadmium uptake and accumulation in plants

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Submitted

**Abstract**

Cadmium contamination in soil is a threat to global food safety. Large-scale soil decontamination is often impracticable, calling for strategies that block cadmium uptake by plants. Manipulation of plant physiology by root-associated microbiota may be an efficient strategy to block plant traits linked to heavy metal uptake.

We particularly address the reduction of plant ethylene levels by bacteria producing the enzyme ACC deaminase. Ethylene is a plant hormone essential for plant growth under stress conditions and we expect that its reduction may alter plant growth and cadmium uptake.

We grew three phylogenetically distinct plants *Rumex palustris*, *Alcea aucheri* and *Arabidopsis thaliana* on cadmium-spiked soil and inoculated them with the wildtype bacterium *Pseudomonas putida* UW4 or an isogenic ACC deaminase-deficient mutant as baseline. We followed ethylene hormone, plant growth and cadmium uptake.

ACC-deaminase producing bacteria consistently reduced shoot cadmium content by up to 40% across plant species. Ethylene concentration was negatively correlated to cadmium uptake, confirming the importance of this hormone.

***Synthesis and applications***: We show ethylene positively regulates cadmium uptake in plants. Ethylene reduction by root-associated microbes may thus offer new strategies to improve global food safety in a context of the widespread soil contamination.

**Key words**: ACC deaminase, Cadmium; Ethylene; Plant-growth promoting bacteria; Heavy metal, food safety.

## Introduction

Heavy metal contamination is an important food security concern worldwide. Around 100,000 ha of agricultural land in Europe and US alone are contaminated with heavy metals (Lewandowski *et al.*, 2006). Cadmium is a particularly problematic contaminant posing a health risk for humans and animals at concentrations that are far below phytotoxicity levels (Peijnenburg *et al.*, 2000) meaning that even healthy plants may contain dangerous levels of cadmium. Large-scale soil cadmium decontamination is impracticable and extremely expensive with currently available technologies (Tóth *et al.*, 2016). There is thus an urgent need for the development of new strategies to reduce heavy metal load in food and feed crops. Root-associated microbiota may offer promising tools to reach this goal by altering the plant physiological pathways linked to heavy metal uptake. In the present study we use

ethylene manipulation by plant-associated bacteria to demonstrate that microbial-induced changes in plant physiology determines plant cadmium uptake.

Plant ethylene is a central hormone playing a central role in stress resistance and may serve as effective target to alter heavy metal uptake. Ethylene regulates numerous plant traits involved in stress responses and nutrition (Sasidharan & Voesenek, 2015; Romera *et al.*, 2016) and is essential for plant survival in stressful conditions. However, the same stress response that is beneficial for the plant may make them unsafe for consumption: Ethylene regulates the expression of transporters for nutrients such as iron and zinc (Wong & Cobbett, 2009; Lee & An, 2009) that are potentially involved in cadmium uptake (Waters *et al.*, 2007). Ethylene further regulates several detoxification and transport processes (Keunen *et al.*, 2016), including metal phytochelation and transport to aerial parts (Bovet *et al.*, 2005; Hassan & Aarts, 2011). These mechanisms are crucial for heavy metal tolerance but may lead to a high heavy metal concentration in aerial parts, causing food safety issues. Ethylene signalling is deeply influenced by microorganisms that either degrade or increase the concentration of this hormone. Here we assess whether ethylene reduction by root-associated bacteria producing the enzyme ACC deaminase may help reduce cadmium concentration in plants. This enzyme is present in several soil microorganisms and reduces plant ethylene concentration by degrading its direct precursor ACC (1-amino cyclopropane-1-carboxylic acid; (Glick, 2014).

We inoculated three different plants growing in a cadmium-spiked soil with the model root-associated bacteria *Pseudomonas putida* UW4, producing ACC deaminase (hereafter referred to as "wildtype"), or an isogenic mutant lacking ACC deaminase but otherwise identical to the wildtype (hereafter referred to as "*acdS*$^-$ mutant"). We selected three model plants varying in phylogenetic affiliation and cadmium sensitivity from contrasting ecological zones: *Rumex palustris (Polygonaceae, high cadmium sensitive wetland plant), Arabidopsis thaliana* (*Brassicaceae*, moderate cadmium sensitivity), and *Alcea aucheri* (*Malvaceae*, cadmium tolerant from marginal drylands). *Rumex palustris* is adapted to high moisture conditions and is a model plant for studying ethylene-mediated stress responses (Voesenek *et al.*, 2003). *Arabidopsis thaliana* is the most intensively studied model organism in plant biology and genetics, and its inclusion allowed us to link our results to the wealth of physiological and molecular studies previously conducted with this species. *Alcea aucheri* is a newly reported lead- and cadmium hyperaccumulator plant that thrives in deserts and semi-dry conditions (Ravanbakhsh *et al.*, 2016). The use of the isogenic mutant of bacteria (*acdS*$^-$) allow us to distinguish the effects of ACC deaminase production from other effects of bacterial inoculation, providing a clean

background. We examined the effects of the bacteria on plant ethylene levels, and subsequently plant growth and shoot cadmium accumulation in the presence of moderate soil cadmium contamination. We hypothesized that (1) Ethylene positively regulates cadmium uptake and tolerance in plants, (2) ethylene reduction by ACC deaminase-producing bacteria reduces cadmium uptake in aerial parts.

**Method and Materials**

**Plant materials and experiment design**

Seeds of *Rumex palustris* Sm., were collected in 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 light, 25°C, 70 µmol $m^{-2}$ $s^{-1}$ photon flux density, and 12 h of dark, 10°C) for around 12 days. Seedlings were transferred to pots filled with a mixture (ratio 2:1) of potting soil (Jonkind, the Netherlands, pasteurized 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, saturated by 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). Plants were grown in a growth chamber (20°C; 70% relative humidity, 16 h of light; 200 µmol $m^{-2}$ $s^{-1}$ Photosynthetic Photon Flux Density (PPFD)) and inoculated with bacteria (see next section) 18 days after germination. After 24 days of growth, plants were selected based on uniformity of developmental stage and transferred to transparent rectangular glass boxes (25×35×5 cm, Rubox, Netherlands). Pots were drenched with 200 ml of 1 mM cadmium solution (as $Cd(NO_3)_2$ salt), resulting in 100 µM water-extractable cadmium in soil and incubated for 14 more days. The numbers of replicates were 6 plants per treatment.

Seeds of *Alcea aucheri* (Boiss.) Alef. were collected from a desert-like area in southern Iran (Ravanbakhsh et al, 2016) between UTM coordinates 640300 and 3158136 in 2014. Seeds were scarified in concentrated $H_2SO_4$ (90%v/v for 0.5 h), and germinated on polyethylene beads (Elf Atochem, Marseille, France) floating in a transparent container (12 h of light, 25°C, 70 µmol $m^{-2}$ $s^{-1}$ PPFD, and 12 h of dark, 10°C). Fourteen-day-old seedlings were transplanted into pots filled with a mixture (ratio 2:1) of potting soil (Jonkind, the Netherlands, pasteurized at 90 ° C) and sand (autoclaved at 130 ° C), 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 100 mL of nutrient solution as described above for *R. palustris*. Pots were placed in a walk-in

growth room (27°C; 70% relative humidity, 16 h of light; 250 µmol $m^{-2}$ $s^{-1}$ PPFD) and inoculated with bacterial strains (next section) 18 days after germination. After one week, plants were drenched with 200 ml of 1 mM cadmium solution (from $Cd(NO_3)_2$ salt), resulting in 110 µM water-extractable cadmium in soil, and placed back in the same growth chamber for an additional 14 days. The numbers of replicates were 7 plants per treatment.

*Arabidopsis thaliana* (L.) Heynh., Columbia (hereafter referred to as "Col-0") ecotype were grown from seeds stratified in the dark at 4°C for 4 days, sown on fertilized mixture of potting soil and perlite (both pasteurized at 90 ° C) in growth chambers (20°C; 70% relative humidity, 9-h photoperiod; 200 µmol $m^{-2}$ $s^{-1}$ PPFD), and grown for 14 days. On day 14, plants were inoculated with bacterial strains (next section). Forty-eight plants were selected based on uniformity of developmental stage on day 18, and pots were drenched with 200 ml of 1 mM cadmium solution (as $Cd(NO_3)_2$ salt) resulting in 105 µM water-extractable cadmium in soil. Plants were grown for an additional 14 days. Eight replicates were set up per treatment.

To validate the importance of ethylene signaling on cadmium concentration and accumulation, we grew of *Arabidopsis thaliana* Col-0 and its ethylene insensitive mutant *ein3eil1* (Alonso et al., 2003) in the same conditions as above, but without addition of bacteria, for total period of 45 days. The seeds of ethylene insensitive mutant, *ein3eil1,* were obtained from plant ecophysiology group, Utrecht University, the Netherlands, and were confirmed by PCR-based genotyping. Eight replicates were set up per treatment.

**Bacterial Strains**

*Pseudomonas putida UW4* wildtype strain (later "wildtype") and an isogenic ACC (1-aminocyclopropane-1-carboxylate) deaminase deficient mutant (later "*acdS-*") were obtained from Prof. Glick, Department of Biology, University of Waterloo, Canada. *Pseudomonas putida UW4* has been used as a model organism in several studies on the role of ACC deaminase production in plant-microbes interactions (Glick, 2014). The ACC deaminase deficient mutant (*acdS-*) was obtained by insertion of a tetracycline resistance gene in the ACC deaminase gene coding region (Li *et al.*, 2000). Bacterial strains were kept as frozen stocks at -80 °C. Prior to experiments, one single colony was grown overnight on TSB medium (for wild-type ACC deaminase containing bacteria) or TSB and 100 µg $ml^{-1}$ tetracycline (for mutant bacteria lacking ACC deaminase). Bacteria were harvested by centrifugation (6000 x *g*, 10 min), washed three times with 10 mM $MgSO_4$ and adjusted on $OD_{600}$ of 0.5 before inoculation.

**Enumeration of plant-associated bacteria**

We assessed the density of *Pseudomonas putida UW4* immediately after harvest. Roots were gently shaken to remove adhering soil, after which bacteria were recovered by shaking the roots in 10 mM $MgSO_4$ for 30 min at 200 $min^{-1}$, sonicating them for 20 seconds and vortexing for 30 seconds. Density of the wildtype strain was enumerated on DF agar (Dworkin & Foster, 1958) supplemented with ACC as sole nitrogen source (Penrose and Glick, 2003). The mutant strain (*acdS*$^-$) was enumerated on DF agar supplemented with $(NH_4)_2SO_4$ as nitrogen source and 100 μg $ml^{-1}$ tetracycline.

**Plant harvest, ethylene production rates and cadmium concentrations**

Shoots were carefully separated from the roots with a razor blade, and aerial parts placed in serum vials (30 ml). After 2 h, a 1 ml gas sample was taken from each vial and injected in a 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 to measure ethylene concentrations. Considering 2 h incubation time and measured shoot fresh weight, ethylene production rates were calculated (see Voesenek, et al, 2003). Plant materials (dry ashing procedure; Kalra et al, 1997) and soil samples (digestion with aqua regia; (McGrath & Cunliffe, 1985b) were digested by standard procedures. Concentrations of cadmium in plans and water extractable phase of soil (in soil paste) were measured by Inductively Coupled Plasma Mass Spectroscopy (Thermo Scientific ICP 6300 Fischer Scientific, Breda, Netherlands). Root to shoot translocation factors of cadmium were defined as the ratio of the element concentration in shoots and in roots.

**Statistical Analysis**

The effect of cadmium treatment on shoot dry weight and cadmium concentration in each plants was analyzed separately for cadmium and No-cadmium treatments using separate one-way ANOVAs. Then, we used two-way ANOVAs to evaluate the interactive effects of plant species and bacteria on shoot ethylene, dry weight, shoot cadmium concentration, translocation factor and accumulation in cadmium treated soils. We assessed the correlation between cadmium shoot concentration and translocation factor and ethylene production rate using separate one-way ANOVAs (in cadmium treated condition). The interactive effects of ecotypes (Col-0 and ein3eil1) and cadmium treatments (0 and 100 mM) on dry weight, cadmium concentration and accumulation were evaluated using two-way ANOVAs. When the F-test was significant, individual treatment means were compared by post

hoc Tukey's multiple range test (α= 0.05). All statistical analyses were performed in SPSS (V. 22).

## Results

### Cadmium treatment, toxicity symptom and plant growth

Shoot cadmium concentration and accumulation in the tree plant species significantly increased by growing on cadmium-spiked soil compared to the pristine one, confirming the ability of these plant species to take up soil cadmium and translocate it to the shoots (Table S1). Cadmium exposure reduced plant dry weight and each plant showed a different sensitivity. *Rumex palustris* was the most cadmium-sensitive plant, with cadmium exposure causing a 50% reduction in shoot dry weight, followed by *Arabidopsis thaliana* (19% reduction) and *Alcea aucheri* (6% reduction, Table S1).

### Effect of plant species and bacteria inoculation on cadmium shoot concentration, root-to-shoot translocation, and shoot cadmium accumulation in three plant species

The main effects of the plant species (*A. thaliana, A. aucheri,* and *R. palustris*), the bacterial treatment (non-bacterized control, wildtype and mutant (*acdS*$^-$)) and their interactive effects on shoot dry weight, cadmium concentration, accumulation, and shoot to root translocation factor was evaluated by Two-way ANOVAs (Table 1).

Table 1, ANOVA table summarizing the interactive effects of plant, bacterial treatment, and their interaction on cadmium concentration, accumulation, and plant dry weight in Arabidopsis thaliana, Rumex palustris, and Alcea aucheri. in Cd spiked soil. One linear model was fitted separately for each dependent variable.

| | | Cd concentration | | Cd accumulation | | Dry weight | | TF | |
|---|---|---|---|---|---|---|---|---|---|
| | *df* | *F* | *P* | *F* | *P* | *F* | *P* | *F* | *P* |
| Plant | 2 | 21.3 | <0.0001 | 88.26 | <0.0001 | 146.44 | <0.0001 | 22.3 | <0.0001 |
| Bacteria | 2 | 10.2 | <0.0001 | 11.50 | <0.0001 | 3.53 | 0.03 | 18.3 | <0.0001 |
| Plant× Bacteria | 4 | 2.39 | 0.06 | 5.41 | 0.001 | 1.40 | 0.245 | 2.15 | 0.086 |
| Error | 54 | | | | | | | | |

Cadmium shoot concentration and shoot accumulation varied significantly between the three plant species (main effect of plant species, Table 1). Bacterial

inoculations significantly alter these plant traits (main effect of bacteria, Table 1) and the effect depended on the presence of ACC deaminase gene (Fig. 1). Inoculation with ACC deaminase-producing bacteria (WT, *acdS*$^{+}$) significantly decreased shoot cadmium concentration and total content compared to the ACC deaminase-deficient mutant (*acdS*$^{-}$) and no-bacteria treatments (Fig. 1 A and B). This reduction was consistent between three plant species suggesting that the underlying mechanisms are conserved across a broad range of plant lineages.

Root-to-shoot Translocation of cadmium also significantly varied by plant species (main effect of plant species, Table 1) and bacterial inoculation (Table 1). *Alcea aucheri* showed higher translocation factor compared to *R. palustris* and *A. thaliana*, which showed the ability of this plant for cadmium transfer to plant aerial parts. Wildtype ACC deaminase-producing bacteria decreased the cadmium translocation factor in three plant species compared to mutant (*acdS*$^{-}$) and No-bacteria inoculation (Fig. 1C). This contributed to less shoot cadmium in three plant species.

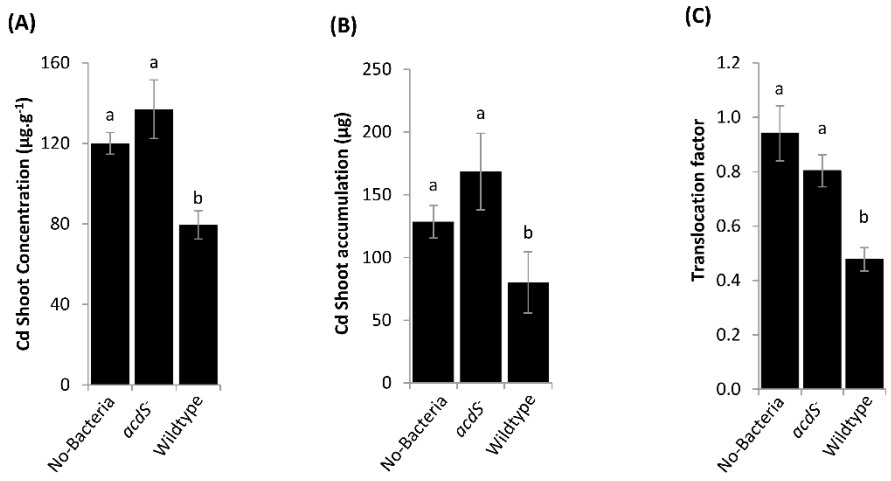


Figure 1. Effects of wildtype ACC (1-aminocyclopropane-1-carboxylate) deaminase-producing bacteria Pseudomonas putida UW4 (Wildtype) and its isogenic ACC deaminase deficient mutant (acdS-) and no bacteria control (No-Bacteria) on shoot cadmium concentration (A), accumulation (B), cadmium root-shoot translocation (C), and shoot dry weight (D) in Arabidopsis thaliana, Rumex palustris, and Alcea aucheri. The shown data represent the pooled data for the three plants. See fig. S2 for plant-

specific data. Different letters indicate significantly different treatments based on the Turkey's multiple range test ($p<0.05$). Error bars show ± SE.

**Effects of bacterial inoculation on ethylene production**

Ethylene production varied significantly between the three plant species ($F_{2,54}$ = 19.81, $p$ < 0.0001). Ethylene level in plants also varied in function of the bacterial treatment ($F_{2,54}$ = 13.68, $p$ < 0.0001). We found no significant interactive effects for plant and bacteria treatment in case of shoot ethylene production ($F_{4,54}$ = 1.40, $p$ = 0.25), indicating the effect of bacterial inoculation on ethylene production was consistent across the three species. In average, plant inoculation with the wildtype bacterial strain resulted in reducing ethylene concentration from 55.97 and 50.21 to 40.33 pmol $g^{-1}$ fresh weight $h^{-1}$ compared to the *acdS*$^{-}$ mutant bacterial inoculation and no-bacterial treatments in plants. In average, inoculation with wildtype *P. putida* UW4 consistently decreased shoot ethylene production rates across the three species examined by 20-34% compared to *acdS*$^{-}$ mutant bacterial treatment.

The central role of ethylene in uptake and transfer of cadmium is confirmed by the significantly positive correlation between shoot ethylene production and shoot cadmium concentration ($F_{1,61}$ = 31.25, $p$ < 0.0001, $R^2$ = 0.34), shoot to root translocation factor ($F_{1,61}$ = 19.95, $p$ < 0.0001, $R^2$ = 0.25) in the plant species (Fig. 2). We therefore conclude that the reduced uptake and transport of cadmium to aerial parts of plants in presence of ACC deaminase producing bacteria (Fig. S2) can be attributed to reduced ethylene production.

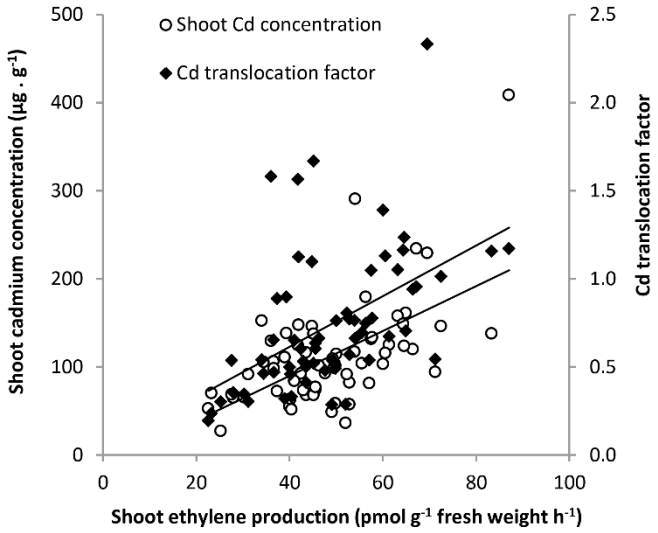


Figure 2. Correlation between ethylene production and root-to-shoot cadmium translocation (closed symbols, F1,61 = 19.95, p < 0.0001, R2 = 0.25) and shoot cadmium concentration (open symbols, F1,61 = 31.25, p < 0.0001, R2 = 0.34) is positively correlated with shoot ethylene production in Arabidopsis thaliana, Rumex palustris, and Alcea aucheri.

**Shoot dry weight**

Plant species varied in dry weight production in 1 mM cadmium-spiked soils (main effect of plant species, Table 1). Bacteria inoculations further significantly affect plant dry weight (main effect of bacterial treatment, Table 1). Inoculation with the *acdS*$^{-}$ mutant increased significantly shoot dry weight compared to wildtype (Table 1; Fig. 1C), indicating that plant growth promotion by the applied bacteria was not due to ACC deaminase activity. This is in strong contrast with the current paradigm that ACC deaminase producers are beneficial for plants under heavy metals (Albano & Macfie, 2016). The plant growth promoting effect of the *acdS*$^{-}$ mutant compared to wildtype was consistent between three plant species (Fig S2).

**Shoot cadmium concentration and accumulation in *ein3eil1* compared to Col-0 ecotype**

Shoot cadmium concentration was significantly ($F_{1,29}$ = 68.20, $p$ < 0.0001) lower in the ethylene insensitive mutant *ein3eil1* compared to Col0 *A. thaliana* (Fig S3, supporting materials). In line with this result, the total cadmium accumulation in

shoots was significantly lower in *ein3eil1* than in Col-0 ($F_{1,29}$ = 45.73, $p$ < 0.0001: Fig. S3). Shoot concentrations of Ca, Mg, Fe, and Cd and Shoot accumulation of almost all elements were significantly higher in Col0 ecotype compared to *ein3eil1* mutant (table S2).

**Bacterial colonization**

The density of recovered root-associated bacteria was varied between plant species ($F_{2,54}$ = 4.35, $p$ = 0.02). *Alcea aucheri* harbored more bacterial population in rhizoplane compared to *A. thaliana* and *R. palustris* plants. However, the densities of recovered wildtype ACC deaminase bacteria and ACC deaminase deficient mutant (*acdS*$^-$) were similar ($F_{2,54}$ = 1.19, $p$ = 0.31). The interactive effects of bacteria and plant was also not statistically different ($F_{4,54}$ = 1.98, $p$ = 0.11).

**Discussion**

Heavy metal contamination in agricultural land is a widespread problem for food safety. As soil decontamination remains difficult and costly, there is a growing demand for alternative strategies to deal with heavy metal contamination in agroecosystems. Steering plant physiology toward reduced uptake of heavy metals may allowing for the growth of safe food and feed crops on contaminated soils.

In the current study, we investigated the importance of ethylene as regulator of cadmium uptake. We then assessed whether plant-root associated bacteria can help restrict plant cadmium uptake in aerial plant parts by tweaking plant hormonal balance. We focused on ACC deaminase as a model bacterial function, as this enzyme has well-described effects on the plant hormone ethylene, itself a candidate plant hormone regulating nutrient uptake and transport in plants. By comparing a wildtype bacterium normally producing ACC deaminase and two separate controls (an isogenic *acdS*$^-$ mutant lacking this enzyme and a no-bacteria control), we were able to disentangle the specific effects of the ACC deaminase enzyme versus other bacterial effects on plant growth and heavy metal accumulation.

Across the three plant species examined, inoculation with the ACC deaminase-producing strain consistently decreased plant ethylene production rates by approximately 30%, as compared to the ACC deaminase deficient mutant (*acdS*$^-$) bacteria. Wildtype strain can consistently reduce cadmium uptake and transport across a range of plant species, differing in their heavy metal tolerance (Fig. 1B). On average, plants inoculated with ACC deaminase-producing bacteria showed approximately a 40% reduction in shoot cadmium accumulation compared to plants inoculated with the isogenic *acdS*$^-$ mutant lacking this enzyme.

In order to validate the importance of the ethylene pathway for cadmium uptake, we performed an additional experiment with an *ein3eil1* mutant of *A. thaliana* impaired in ethylene signaling. We compared the cadmium uptake of *A. thaliana* Col0 (normal ethylene signaling) and *ein3eil1* (ethylene insensitive). In agreement with the effect of ACC deaminase producers on cadmium uptake, we found that the concentration and uptake of cadmium in ethylene-insensitive mutant was significantly lower compared to wild type *A. thaliana* ecotype (Col0; Fig. S3), confirming the importance of ethylene for cadmium and nutrient uptake in cadmium contaminated soils. We could reproduce the effects of bacteria on plant phenotype with the *ein3eil1* mutant, which showed both a lower leaf cadmium concentration and accumulation in presence of cadmium, and validating the importance of ethylene signaling for the observed effects.

Ethylene is an important regulator of plant stress response (Abeles *et al.*, 2012). This hormone impacts plant nutrition via a range of effects including changes in root morphology and the expression of metal transporters (Lucena *et al.*, 2006; Waters *et al.*, 2007). These transporters are essential to take up elements such as Fe or Zn (Thomine *et al.*, 2000; Eren & Argüello, 2004) and also be involved in cadmium uptake, and transfer to aerial parts (Song *et al.*, 2003; Bovet *et al.*, 2005; Wong & Cobbett, 2009). Induced increased ethylene levels in plants may result in an overexpression of metal transporters, thereby resulting in increased heavy metal accumulation (Wong & Cobbett, 2009; Lee & An, 2009). We demonstrate that inoculation with bacteria degrading the precursor of ethylene results in a decline in ethylene levels in the plant, which in turn downregulates the expression of transporters responsible for cadmium uptake and keeps this metal from entering the plant.

We show that ACC deaminase producing bacteria alter plant physiology in a similar way to mutations impairing ethylene signalling. Lower ethylene will reduce heavy metal uptake, yet at the cost of a reduced plant growth. This is in line with the widely acknowledged importance of ethylene signaling for abiotic stress tolerance (Keunen et al., 2016). However, for some reason the reverse paradigm is used in plant-microbe interaction research, where ACC deaminase is typically implicitly assumed to enhance abiotic stress tolerance in plants (Safronova *et al.*, 2006; Zhang *et al.*, 2011). Possibly, the current paradigm results from the confounding effects of ethylene modulation and other microbial traits. By using a design explicitely disentangling these two effects, we show that ethylene is important for plant cadmium uptake and that reducing ethylene is deleterious from a plant perspective. Our results

call for a rethinking of this paradigm, which might benefit from being placed in the context of the latest advances on the ecophysiology of ethylene signalling.

**Conclusion**

Heavy metal contamination is an important issue for food safety worldwide. Taking ethylene as model cascade, we demonstrate that soil microbes modulating plant hormonal balance can help reduce heavy metal uptake in plant. Thanks to their ease of use, microbes may therefore provide an alternative to plant breeding and help obtain crop phenotypes that do not take up heavy metals, thereby remaining safe to eat despite of soil contamination.

**Authors' Contributions**

M.R. and A.J. conceived the ideas and designed methodology. M.R. was mainly responsible for establishment of the experiments, chemical analyses and collecting data, with substantial contributions from A.J. M.R. and A.J. drafted manuscript. All authors provided critical review and suggestion for the content. ALL authors read and approved the final manuscript.

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**Chapter 3, supporting materials**

**Supporting tables**

**Table S1.** The interactive effects of plant, cadmium treatment, and their interaction on shoot cadmium concentration, accumulation, and plant dry weight in *Arabidopsis thaliana, Rumex palustris,* and *Alcea aucheri* in Cd spiked soil compared to no-cadmium condition. One linear model was fitted separately for each dependent variable.

| | | Shoot dry weight | | Cd concentration | | Cd accumulation | |
|---|---|---|---|---|---|---|---|
| | *df* | *F* | *F* | *F* | *F* | *F* | *P* |
| Plant | 2 | 116.5 | <0.0001 | 10.84 | <0.0001 | 17.23 | <0.0001 |
| Cadmium | 1 | 11.49 | 0.002 | 367.96 | <0.0001 | 53.61 | <0.0001 |
| Plant× Cadmium | 2 | 1.18 | 0.318 | 10.28 | <0.0001 | 15.87 | <0.0001 |
| Error | 36 | | | | | | |

**Table S2**: ANOVA table summarizing the interactive effects of plant, cadmium treatment, and their interaction on cadmium concentration and accumulation in *Arabidopsis thaliana* Col0 and ein3eil1 genotype.

| | | Cd concentration | | Cd accumulation | |
|---|---|---|---|---|---|
| | *df* | *F* | *F* | *F* | *P* |
| Plant | 1 | 45.74 | <0.0001 | 68.2 | <0.0001 |
| Cadmium | 1 | 238.18 | <0.0001 | 208.18 | <0.0001 |
| Plant× Cadmium | 1 | 44.50 | <0.0001 | 66.65 | <0.0001 |
| Error | 28 | | | | |

**Supporting Figures**

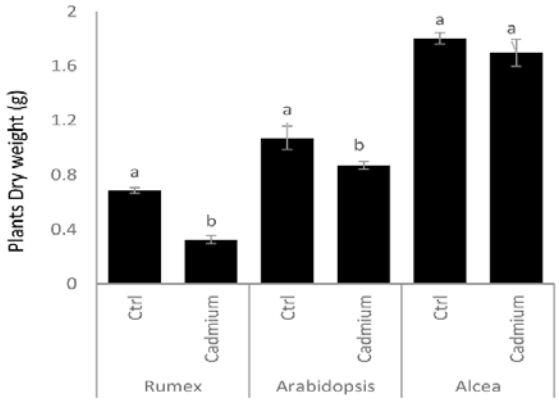
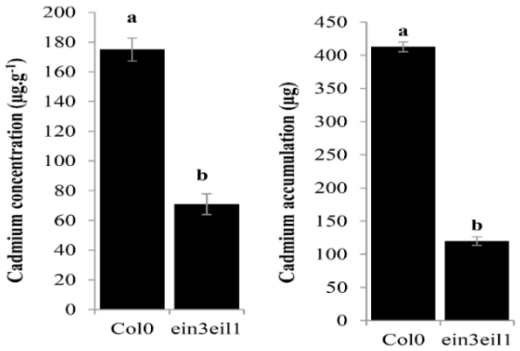



**Figure S1**. Effects of soil cadmium treatment (1 mM) on plant shoot dry weight in *Rumex palustris, Arabidopsis thaliana, and Alcea aucheri*. Differences between treatments were analyzed using two-way ANOVA ($p<0.05$) of plant species and bacterial inoculation (wildtype, mutant, and no-bacteria). Letters show significantly different effects based on Tukey s multiple range test. Error bars show ± SE.

**Figure S3**. Shoot cadmium concentration and shoot cadmium accumulation in Col0 and *ein3eil1* ecotype after applying 1 mM cadmium to soil. The differences between Col0 and *ein3eil1* ecotype were analyzed by the univariate analysis of plants at no-bacteria and 100 mM soil cadmium treatments. Error bars show ± SE.



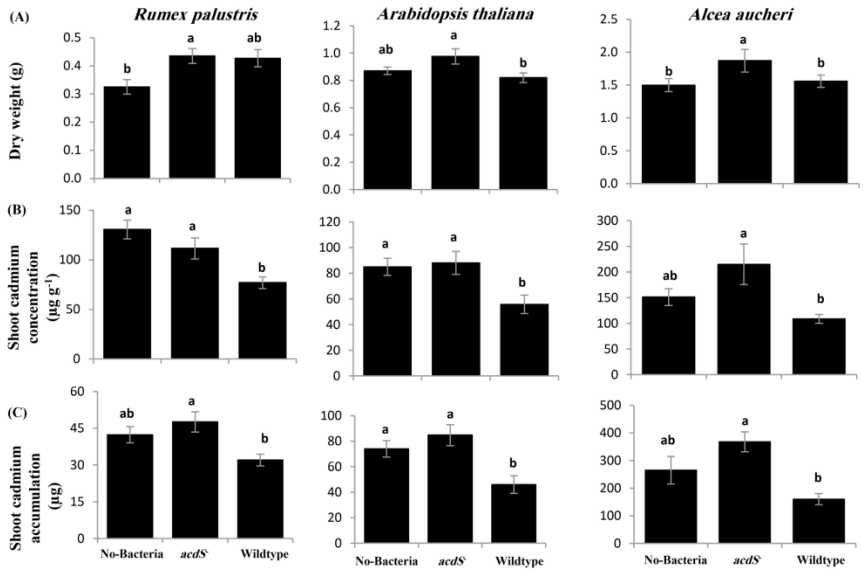
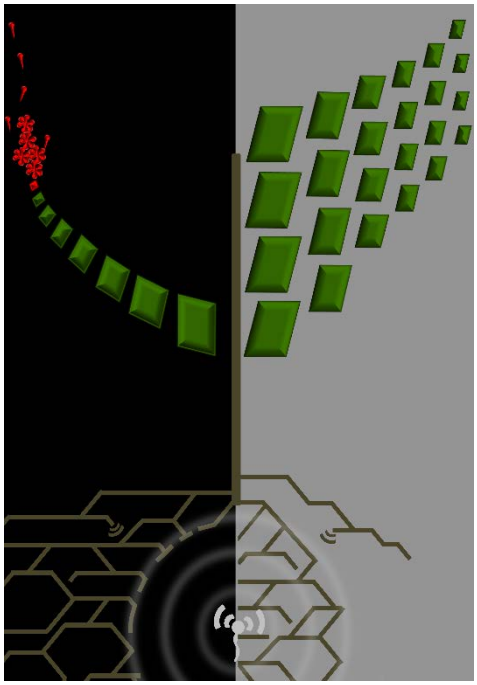



**Figure S2**. Shoot dry weight (A), shoot cadmium concentration (B), and shoot cadmium accumulation (C) of *Rumex palustris, Arabidopsis thaliana,* and *Alcea aucheri* in the three bacteria inoculant in 1 mM cadmium spiked soils. Treatments were: inoculation with a mock saline solution (No-Bacteria), wildtype *Pseudomonas putida UW4* (wildtype), ACC deaminase deficient mutant (*acdS*$^-$). The differences between No-Bacteria *vs* bacterized (Wildtype and *acdS*$^-$) treatments, as well as the differences between Wiltype *vs* Mutant Treatments, were analyzed with contrast analyses and are highlighted with horizontal lines. Significant effects are shown by asterisks ($p<0.05$ *, $P<0.01$ **). Error bars show ± SE.

# 4

## Friend or foe? Alterations of ethylene signalling by plant-associated microbiota cause maladaptation to stressful conditions



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Submitted

**Abstract**

Several plant hormonal pathways are co-regulated by plants and their associated microbiota. Although such interactions are often assumed to be beneficial, their effects on plants can be variable or even negative. This is particularly apparent in plant-microbe co-regulation of ethylene, a regulator of numerous interconnected plant traits such as those governing growth and stress responses. Ethylene reduction by microbiota has traditionally been viewed as beneficial for the plant, which is in direct contrast with studies in plant physiology and genetics that have pinpointed the essential role of ethylene signalling for stress tolerance. We propose that this discrepancy stems from a lack of insight into the complete life history consequences of hormonal manipulation. We assess how inhibition of ethylene signalling by microbiota or chemical treatment affects plant growth along a heavy metal stress gradient. We show that reducing ethylene levels in plant increases plant biomass in absence of stress, but also causes hypersensitivity to stress. We finally confirm the results with an ethylene insensitive plant mutant, which exhibited the same patterns. We conclude that alteration of ethylene signalling by microbiota shifts plant phenotypes along existing growth-stress resistance tradeoffs, similar to the influence of natural genetic variation. Purportedly beneficial plant-microbe interactions can cause plant maladaptation across a range of environmental conditions, calling for a reassessment of the plant growth promotion concept in the light of plant life history strategies.

**Key words**: ACC deaminase, Cadmium; Ethylene; Plant-growth promoting bacteria; Heavy metal, tolerance.

**Introduction**

Plants are faced with multiple stressors during their life cycle, and balancing resource allocation between biomass or seed production and stress resistance mechanisms is essential for fitness (Huot *et al.*, 2014). In plants, resource investment into stress resistance is to a large extent coordinated by ethylene signalling (Roux *et al.*, 2006). Ethylene is a central plant hormone regulating several aspects of plant development, as well as stress responses. Plants react to stress by upregulating ethylene, which triggers a range of physiological adaptations (Sasidharan & Voesenek, 2015; Thao *et al.*, 2015). These adaptations can help the plant cope with stressors, but they are also costly for plants. Optimal ethylene homeostasis is therefore important to reach an optimal phenotype maximizing fitness under the given environmental constrains.

Plant-microbe interactions play a central role in the regulation of a plant's hormonal balance. In addition to the plant's own enzymatic machinery, ethylene levels are to a large extent co-regulated by plant-associated microbiota(chapter 7). In particular, microbial production of the enzyme ACC (1-aminocyclopropane-1 carboxylic acid) deaminase, which degrades the direct precursor of ethylene, has received a sustained attention during the last decades. ACC deaminase has been typically been considered as a plant-growth promoting trait, helping plants shift resources from stress resistance to growth. However, this concept is at odds with recent developments in plant ecophysiology that highlight the core role of ethylene signalling and priming for stress resistance in plants(Sasidharan & Voesenek, 2015; Thao *et al.*, 2015; Keunen *et al.*, 2016): While microbiologists have pointed to ACC degradation by microbiota, and the resulting reduction in ethylene concentration, as a potential way to alleviate stress in plants, plant physiologists have developed the opposite claim, namely that high ethylene levels are essential for plant stress resistance and that priming them with ACC increases stress tolerance (Cox *et al.*, 2004).

We propose that this discrepancy stems from widespread use of single-trait approaches, in which researchers examine the effect of a treatment on one or a few trait of interest under a given set of conditions. While this approach is useful and informative, it neglects that most plant traits are connected and that altering one trait may have a deep impact on other life history traits (Adler *et al.*, 2014). Here, we propose to assess the role of ethylene manipulation by microbiota in the context of a growth-stress resistance tradeoff, an inherent evolutionary constraint on all organisms from bacteria to plants and animals (Gudelj *et al.*, 2010; Huot *et al.*, 2014). We start from the premises that growth and stress tolerance are negatively correlated (Araújo *et al.*, 2011) and that both are under the regulation of ethylene (Thao *et al.*, 2015).

We examined the effect of microbial reduction of ethylene on plant growth along a gradient of cadmium stress, a widespread heavy metal with a high toxicity to plants (Mendelssohn *et al.*, 2001). We inoculated *Arabidopsis thaliana* with *Pseudomonas putida* UW4, a model bacterium able to degrade the ethylene precursor ACC, or an *acdS*$^{-}$ isogenic mutant lacking this ability. We then followed plant growth in the absence or presence of stress and used the growth difference between the two bacterial treatments as a proxy for the impact of ethylene reduction on growth. We validated the importance of ethylene by growing an *A. thaliana* ethylene-insensitive mutant (*ein3eil1*) as well as by treating plants with the ethylene biosynthesis inhibitor α-aminoisobutyric acid (AIB).

We hypothesized that microbial decrease of ethylene improves growth in the absence of stress by steering resource allocation into growth, but that this comes the cost of hypersensitivity under stressful conditions. This context dependent effect of microbial association may lead to maladaptive plant responses if not matched correctly with designed plant phenotype.

## Method and Materials

### Plant materials

*Arabidopsis thaliana* (L.) Heynh., ecotype Columbia (hereafter referred to as "Col-0") was obtained from Plant Ecophysiology group of Utrecht University, the Netherlands. To validate the importance of ethylene signalling, we grew an ethylene insensitive mutant *ein3eil1* (Alonso *et al.*, 2003) in the same conditions as *Arabidopsis thaliana* Col-0 for both experimental designs (pot experiment and solidified agar; see below). Seeds of the ethylene insensitive mutant *ein3eil1* were also obtained from plant ecophysiology group, Utrecht University, the Netherlands, and were confirmed by PCR-based genotyping.

### Bacterial Strains

*Pseudomonas putida UW4* wild-type strain (later "wild-type") and an isogenic ACC (1-aminocyclopropane-1-carboxylate) deaminase deficient mutant (later "*acdS*$^-$") were obtained from Prof. Glick, Department of Biology, University of Waterloo, Canada. *Pseudomonas putida UW4* has been used as a model organism to assess the role of ACC deaminase production for plant-microbe interactions (Glick, 2014). The ACC deaminase deficient mutant (*acdS*$^-$) was obtained by insertion of a tetracycline resistance gene into the ACC deaminase gene coding region (Li *et al.*, 2000). Bacterial strains were kept as frozen stocks at -80 °C. Prior to experiments, one single colony was grown overnight on TSB medium (for wild-type ACC deaminase containing bacteria) or TSB and 100 μg ml$^{-1}$ tetracycline (for mutant bacteria lacking ACC deaminase). Bacteria were harvested by centrifugation (6000 x *g*, 10 min), washed three times with 10 mM $MgSO_4$ and adjusted to an $OD_{600}$ of 0.5 before inoculation. Bacteria suspension was inoculated to the base of each plant after seedling transfer.

### Experimental design

### Pot experiment

After sterilization and stratification (in the dark at 4°C, for 4 d), plant seeds were sown on 12 cm square petri dishes containing agar-solidified Murashige and Skoog (MS) medium supplemented with 0.5 % sucrose. Petri dishes were transferred

and positioned vertically in a growth chamber (20°C; 70% relative humidity, 9-h photoperiod; 200 µmol $m^{-2}$ $s^{-1}$ PPFD). For the pot experiment, 10-day old seedlings were transferred to pots containing a mixture of fine sand and perlite (both pasteurized for 45 min at 90 ° C), saturated by a modified Hoagland nutrient solution (Smeets *et al.*, 2008) and placed in the walk-in growth chamber (20°C; 70% relative humidity, 9-h photoperiod; 200 µmol $m^{-2}$ $s^{-1}$ PPFD). Ninety seedlings were selected based on uniformity of developmental stage on day 24 and inoculated with bacterial strains (see previous section). The pots were drenched with 50 ml of deionized water or cadmium solution (from 1 mM $CdSO_4$ salt stock solution) resulting in 0, 10, 50, 100, 250 µM water-extractable cadmium in solution. Plants were grown for an additional 14 days. A full factorial experiment was designed using six replicates per treatment, and the pots were randomized every three days. The plants then harvest and bacterial colonization, plant biomass and ethylene production were measured.

We assessed the density of *Pseudomonas putida UW4* immediately after harvest. Roots were gently shaken to remove adhering soil, after which bacteria were recovered by shaking the roots in 10 mM $MgSO_4$ for 30 min at 200 $min^{-1}$, sonicating them for 20 seconds and vortexing for 30 seconds prior to serial dilution on appropriate culture medium: The density of the wild-type strain was enumerated on DF agar (Table S1, composition of medium) (Dworkin & Foster, 1958) supplemented with ACC as sole nitrogen source (Penrose and Glick, 2003). The *acdS*$^-$ mutant strain was enumerated on DF agar supplemented with $(NH_4)_2SO_4$ as nitrogen source and 100 µg $ml^{-1}$ tetracycline.

On the day 38, shoots were carefully separated from the roots with a razor blade and aerial parts placed in serum vials (50 ml). After 2 h, a 1 ml gas sample was taken from each vial and injected into a 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 to measure ethylene concentration. Ethylene production rates were calculated based upon the 2 h incubation time and expressed in nmol g (fresh weight)$^{-1}$ $h^{-1}$ (see Voesenek, et al, 2003). Plant materials (dry ashing procedure; Kalra et al, 1997) and water extractable of soil samples were measured by Inductively Coupled Plasma Mass Spectroscopy (Thermo Scientific ICP 6300 Fischer Scientific, Breda, Netherlands).

**Growth and stress resistance of ethylene-insensitive plant mutants**

To validate the importance of ethylene signalling in the pot experiment, we grew *Arabidopsis thaliana* Col-0 and the ethylene-insensitive mutant *ein3eil1* (Alonso *et al.*, 2003) in the same conditions as above, but without the addition of bacteria, using 9 replicate plants per treatment. The Col-0 and *ein3eil1* plants pots were

drenched with 50 µM cadmium solution at a similar growing stage (four full leaves; day 25). Plants were harvested after two weeks, and plant dry weight was measured.

***In vitro* assay using Col-0, *ein3eil1*, and chemical inhibitor of ethylene oxidase**

To evaluate the effects of ethylene production and signalling on plant biomass and root architecture, we grew *Arabidopsis thaliana* Col-0 and *ein3eil1* genotype on cadmium-treated solidified agar without addition of bacteria. We used an ethylene biosynthesis inhibitor (10 µM α-aminoisobutyric acid, AIB) to evaluate the effects of ethylene oxidase inhibition on plant development in the cadmium-treated medium. Uniform 10-day-old seedling (prepare as before mentioned) of plants were transferred to new agar-solidified Hoagland medium (Hoagland & Arnon, 1938) containing either 0 or 10 µM cadmium. Eight plants were grown on each square plate and at least three replicate plates were assessed for each treatment. Plants were positioned vertically in a walk-in growth chamber (as before mentioned) for 10 more days, after which roots were scanned (EPSON Perfection 4900) to examine root architecture (WinRHIZO Arabidopsis Pro V 2015 software, Quebec, Canada) and plant biomass was measured.

**Statistical Analysis**

We used two-way ANOVAs to evaluate the interactive effects of cadmium concentration and plant treatment by bacteria (three levels: non-bacterized control, WT and *acdS*$^-$) on ethylene production, shoot and root dry weight for different experimental set-ups. For the experiment on the plant genetic background, we evaluated the interactive effects of plant genotype (two levels: Col-0 and *ein3eil1*) and cadmium treatments (0 and 50 µM) on shoot dry weight. Similar statistical analyses were also performed for the *in vitro* plants assays. We assessed the correlation between root dry weight and ethylene production rate using separate one-way ANOVAs (separate in control and cadmium treated condition). In order to delve deeper into the effect of ethylene reduction, we calculated for each stress level the weight difference between plants treated with wild-type and *acdS*$^-$ bacteria (which we used as a proxy for the net effects of ACC deaminase enzyme). All analyses were performed in SPSS (V. 22).

**Results**

**Effects of bacterial inoculation and cadmium level on ethylene production**

Both bacterial treatment ($F_{2,75}$ = 52.65, $p$ < 0.0001) and cadmium concentration ($F_{4,75}$ = 88.80, $p$ < 0.0001) significantly affected ethylene levels in

*Arabidopsis thaliana*. Ethylene concentration increased with increasing cadmium level in soils. However, it decreased at the highest level of cadmium treatment (250 μM) (Fig. S1). Plant ethylene levels in *A. thaliana* also varied as a function of the bacterial strain inoculated (interactive effects of bacterial treatment and cadmium levels; $F_{8,75}$ = 12.96, $p < 0.0001$). Inoculation with the wild-type bacterial strain reduced shoot ethylene significantly from 0.401 to 0.351 nmol $g^{-1}$ fresh weight $h^{-1}$ compared to the no-bacteria control. However, inoculation with *acdS*$^-$ mutant bacteria increased ethylene concentration resulting in 0.524 nmol $g^{-1}$ fresh weight $h^{-1}$ in this treatment.

**Shoot and root fresh weigh and dry weigh in gradient assay**

Fresh weight and dry weight of *A. thaliana* significantly decreased with increasing cadmium (Table. 1, main effects of cadmium levels). At high cadmium concentration (250 μM), the plant showed serious symptoms of toxicity, even in the presence of bacteria. The effects of bacterial inoculation on shoot and root dry weight were significant (Table. 1, main effects of bacteria inoculation). The effects of bacteria on plant growth was further modulated by the cadmium concentration (Cadmium x Bacteria interaction, Table 1). In order to appreciate better the impact of ACC deaminase, within the background of other bacterial traits that might affect plant growth responses, we performed further analyses in which we compared the weight difference between plants inoculated with WT and *acdS*$^-$ bacteria as a measure of the effect of ACC deaminase. In absence of stress, ACC deaminase-producing bacteria increased plant weight compared to the *acdS*$^-$ mutant (Fig. 1). However, this effect switched to being negative under cadmium stress (Fig. 1). This indicates that ACC deaminase-producing bacteria may increase plant growth in the absence of stress, but causes a hypersensitivity to stress. In other words, plant is shifted along a growth-stress tolerance tradeoff.

Table 1. Two-way ANOVA table summarizing the interactive effects of cadmium treatment levels (0, 10, 50, 100, and 250 mM), bacterial treatment (wild-type ACC deaminase bacteria, its isogenic ACC deaminase deficient mutant, and no-bacterial control), and their interaction on plant fresh, dry weight, and shoot ethylene concentration in *A. thaliana.* One linear model was fitted separately for each dependent variable.

| | | | Fresh weight | | Dry weight | | Ethylene Conc. | |
|---|---|---|---|---|---|---|---|---|
| | | *Df* | *F* | *P* | *F* | *P* | *F* | *P* |
| **Shoot** | Cadmium (Cd) | 4 | 244.14 | **<0.0001** | 34.17 | **<0.0001** | 90.07 | **<0.0001** |
| | Bacteria | 2 | 0.104 | 0.901 | 6.98 | **0.002** | 53.97 | **<0.0001** |
| | Cd × Bacteria | 8 | 4.11 | **<0.0001** | 0.41 | 0.91 | 15.52 | **<0.0001** |
| | Error | 75 | | | | | | |
| **Root** | Cadmium (Cd) | 4 | 121.2 | **<0.0001** | 169.53 | **<0.0001** | | |
| | Bacteria | 2 | 2.92 | **0.06** | 5.54 | **0.006** | | |
| | Cd × Bacteria | 8 | 2.75 | **0.014** | 7.01 | **<0.0001** | | |
| | Error | 75 | | | | | | |

The central role of ethylene was confirmed by the significantly negative correlation between shoot ethylene production and root dry weight ($F_{1,17}$ = 5.42, $p$ = 0.03, $R^2$ = 0.15) and total plant weight (marginally significant, $F_{1,17}$ = 3.36, $p$ = 0.08, $R^2$ = 0.21) in the no-cadmium treatment (Fig. 2A). However, under cadmium stress, ethylene production in *A. thaliana* showed a positive correlation with root dry weight ($F_{1,71}$ = 10.26, $p$ = 0.002, $R^2$ = 0.28) and total plant dry weight ($F_{1,71}$ = 8.25, $p$ = 0.002, $R^2$ = 0.42), suggesting that this hormone coordinated physiological adaptations to increase stress tolerance (Fig. 2A). In addition, ethylene reduction by ACC deaminase bacteria yields contrasting impacts on root dry weight in no-cadmium versus cadmium-amended soils (Fig. 2B).

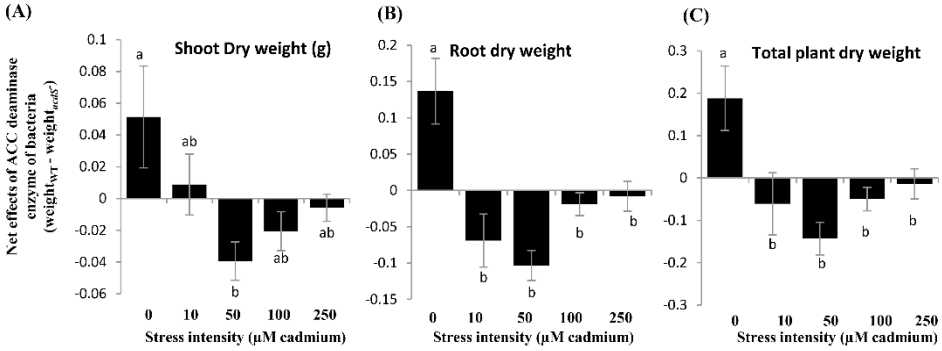


**Fig. 1.** Net effects of ethylene reduction by root-associated microbiota on plant growth along a stress gradient. The net effect was defined as the difference in shoot (A), root (B) and total dry weight (C) between *Arabidopsis thaliana* Col-0 plants inoculated with WT *Pseudomonas putida* UW4 or an isogenic ACC deaminase-deficient mutant. A positive net effect indicates that ACC deaminase-mediated reduction of plant ethylene increases plant biomass; a negative effect indicates an inhibition. Differences between treatments were analyzed using one-way ANOVAs of difference (wild bacteria

subtracted mutant). Asterisks and line above each bar indicate statistically significant differences based on Tukey test (p<0.01 *, P<0.0001 **). Error bars show ± SE.

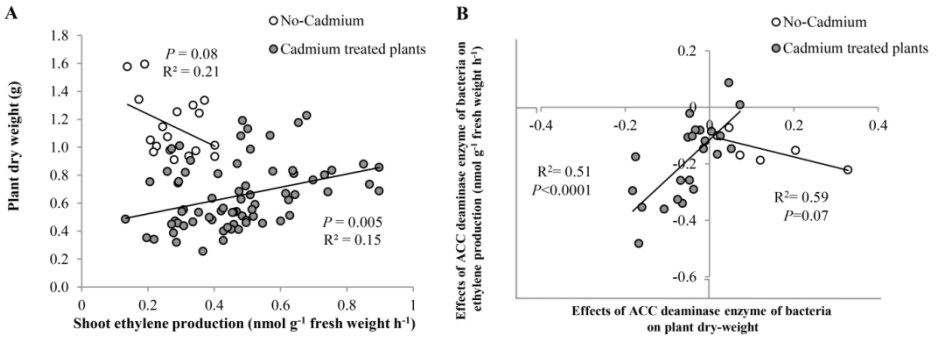


**Fig. 2**. Ethylene reduction by rhizosphere microbes is deleterious for plants under stress conditions. Panel A: High ethylene production is positively correlated with biomass production in *A. thaliana* under cadmium stress, but not in stress-free control, highlighting the importance of this hormone for stress tolerance. Panel B: Reduction of ethylene level by ACC deaminase producing rhizosphere bacteria is linked to a reduction of plant biomass under cadmium stress. The net effects of the ACC deaminase enzyme is defined as the difference of weight effects of wild-type ACC deaminase bacteria and its isogenic mutant.

**Plant growth and stress tolerance of *Arabidopsis thaliana* Col-0 and *ein3eil1***

In order to confirm the importance of ethylene reduction as a mechanism linking ACC deaminase to plant growth and hypersensitivity to stress, we assessed the biomass production of sterile *A. thaliana* Col-0 (WT, normal ethylene response) and *ein3eil1* (ethylene insensitive) growing at 0 (no stress) and 50 µM water-soluble cadmium (stress). In the absence of stress, *ein3eil1* and Col-0 showed similar levels of growth. However, *ein3eil1* showed a higher sensitivity to stress, with cadmium causing a biomass reduction of 38% relative to the no-stress treatment, in comparison with an 11% reduction for Col-0 (Fig. 3 A; Table S2). Together, these results indicate that ethylene signalling is essential for the plant's stress response, and deactivation of this response comes at a cost of hypersensitivity in the presence of a stressor.

Similar effects where obtained by the AIB treatment Col-0 and *ein3eil1* grown on cadmium-treated solidified agar plates. In the absence of cadmium, plant biomass, lateral root number, and lateral root length were significantly higher in the ethylene impaired mutant, *ein3eil1*, and in the treatment with the ethylene inhibitor AIB, as compared to Col-0 (Fig. S2). In contrast, *ein3eil1* and the AIB treatment yielded

deleterious effects on plant biomass and root architecture under cadmium stress (Fig. S2). These results are in line with the results observed for the reduction of ethylene via microbial activity, again showing that the impact of ethylene reduction on plant growth and phenotype depends on the stress status.

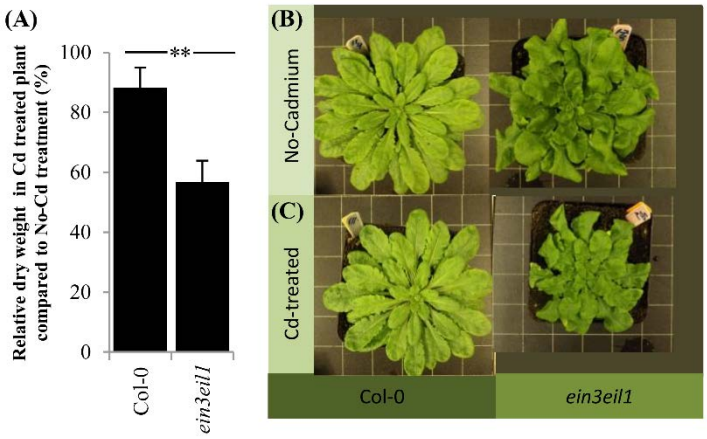


**Fig. 3**. panel (A): Sensitivity to cadmium stress in Col-0 and *ein3eil1* genotype growing in 50 µM cadmium (C) compared to a no-cadmium treatment (B). *ein3eil1* genotype showed 43% reduction in dry weight compared to 11% reduction in Col-0. The Differences between Col-0 and *ein3eil1* genotype were analyzed by two-way ANOVA of plants and cadmium treatment (0 and 50 µM). Significant effects are shown by asterisks ($p<0.05$ *, $P<0.01$ **). Error bars show ± SE.

**Bacterial colonization**

The density of recovered root-associated bacteria per g root fresh weight decreased slightly along with cadmium gradient ($F_{4,75} = 2.17$, $p = 0.08$). The densities of recovered wild-type ACC deaminase bacteria and ACC deaminase deficient mutant (*acdS*$^-$) were similar ($F_{2,75} = 1.59$, $p = 0.21$).

**Discussion**

Plants face various stressors during their life cycle, and growth-stress resistance tradeoffs are an integral determinant of plant fitness (Huot *et al.*, 2014). In plants, this balance is to a large extent determined by ethylene signalling. There is an increasing awareness that ethylene signalling is co-regulated by plants and their

associated microbiota (refer to chapter 7). In this study, we examined how the expression of ACC deaminase enzyme, which reduces ethylene levels in the plant by degrading its direct precursor, affects plant growth and stress resistance. We thereby aimed to resolve an important paradox related to current paradigms in plant-microbe interactions and plant physiology: Bacteria harboring ACC deaminase have widely been proposed as beneficial to plants (Safronova *et al.*, 2006; Zhang *et al.*, 2011; Glick, 2014), yet plant physiology studies consistently highlight that ethylene is essential for the plant's response to stress (Thao *et al.*, 2015; Keunen *et al.*, 2016).

In this study, we show that this apparent contradiction can be harmonized if one takes plant life history tradeoffs into account, specifically the tradeoff between growth and stress tolerance. Tradeoffs emerge when investment in one potentially adaptive trait reduces the ability to develop alternative potentially adaptive traits.. It has been well established that investment into growth diverts resources from defense mechanisms and vice-versa (Huot *et al.*, 2014). In this context, any change in hormonal balance will not be *per se* beneficial or deleterious, but will instead cause a shift along existing tradeoffs.

In the present study, we show that ethylene reduction by mutation, chemical inhibitors or microbiota increases plant growth under stress-free conditions, but that this comes at a cost of making them hypersensitive to stress. This effect was directly proportional to the stress level imposed. In absence of stress, ethylene concentrations negatively correlate with plant biomass production, suggesting that plants with a higher ethylene concentration invest less into vegetative growth. Accordingly, reduction of ethylene leads to an increase in biomass. In the presence of a moderate stress, however, the situations becomes reversed. In this case, ethylene levels positively correlate with biomass, highlighting the importance of this hormone as a coordinator of physiological adaptations that allow the plant to cope with stressors. In line with this observation, reduction of ethylene levels will cause a decrease in plant performance. Finally, as stress intensity increases and goes beyond the physiological adaptation potential of plants, ethylene ceases to be relevant. Plants are inhibited in all cases, and the alteration of a now ineffectual signalling scheme no longer impacts plant growth.

A key finding of this study is that microbiota harboring ACC deaminase impact plant stress responses to a similar degree as complete shutdown of ethylene signalling by mutations causing ethylene insensitivity (*ein1eil3*) or by chemical inhibition of ethylene synthesis (by AIB).

Our results are in line with studies in plant physiology that highlight the key role of ethylene signalling for abiotic stress tolerance (Sasidharan & Voesenek, 2015;

Thao *et al.*, 2015). They, however, contrast with the general perception that ACC deaminase activity is predominantly a plant-beneficial trait, especially under stressful conditions (Safronova *et al.*, 2006; Zhang *et al.*, 2011). We propose that this discrepancy can be resolved by assessing ethylene signalling as a coordinator of full life history strategies as opposed to focusing on single plant traits. Further, using functional mutants was demonstrated as a highly useful approach to disentangle the effects of ACC deaminase from other bacterial traits that may affect plant growth.

We conclude that microbial manipulation of plant ethylene signalling (and potentially of other hormones) is not a plant growth-promoting trait *per se,* and we call for a reconsideration of the standard plant-microbe interactions concept. Instead of being "beneficial" or "deleterious", we propose to approach microbiota as modulators of plant phenotype, helping plants explore the potential phenotypic space offered by their genetic background. The effect of microbiota is thus similar to breeding, which allows for the creation of new phenotypes by reshuffling existing traits. Plants remain restricted by existing life-history tradeoffs and ultimately, the best phenotype will be that best matches the actual challenges that are expericed. Reducing ethylene may for instance be beneficial as long very little stress is present, but can cause plant maladaptation under more stressful conditions.

**Acknowledgment**

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# 5

# ACC deaminase-producing rhizosphere bacteria modulate plant responses to flooding

Photographer: Nathan Van Ewijk

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**Abstract**

Flooding events are predicted to increase over the coming decades, calling for a better understanding of plant responses to submergence. Specific root-associated microbes can interfere with the plant hormonal signals that steer plant flood responses, yet plant responses to such stressors have traditionally been assessed in isolation. We hypothesized that the presence of such microbes may affect plant responses to submergence.

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.

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.

**Synthesis**. We demonstrate that root-associated bacteria can alter plant plastic response under environmental stress by interfering with hormonal balance. Plant-microbes 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.

**Keywords:** Ethylene; ACC deaminase; PGP-bacteria; *Rumex palustris*; plastic response; submergence**;** Ecophysiology

## 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 signaling, 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 (Glick, 2014). Until now, these bacteria have mainly been studies in the context of crop plants in terrestrial ecosystems, where they can alleviate peaks of stress by reducing deleteriously high ethylene levels (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 signaling is required. ACC deaminase-producing bacteria are very common in the rhizosphere. 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 several 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") and an isogenic ACC deaminase-deficient mutant (later referred to as "mutant"), and subjected plants to short- and long-term submergence. We then recorded ethylene production rates, plant elongation and biomass production after 3 and 17 days of full submergence. We expected that (1) plant ethylene levels would increase under submergence, triggering elongation of the aerial parts and that (2) 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 (autoclaved at 90 ° C) and sand (autoclaved at 130 ° C), 0.14 mg of MgOCaO 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 and 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 and 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.

**Experimental design, growth conditions, and measurements**

Plants were inoculated with one of three bacterial treatments (control, WT, mutant) seven days after the transfer of the seedlings to the pots. After two weeks, plants with 4 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 $m^{-2}$ $sec^{-1}$ (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 d, 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.*, 2003). Root-associated bacterial population densities were measured (next section), 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 seconds and vortexing for 30 seconds. Re-suspended bacteria were enumerated on DF salt minimal medium (Dworkin & Foster, 1958) supplemented with ACC as sole nitrogen source (Penrose & Glick, 2003b). 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 submergence and bacterial treatments on petiole size, leaf size, dry weight, fresh weight, plant height, post-submergence ethylene production rates were evaluated using separate two-way ANOVAs. If the F-test of variance ratio was significant, 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 versus control), ACC deaminase enzyme (WT versus mutant) and effect due to the genetic background of the bacteria independently of ACC deaminase activity (mutant versus 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 ($F_{1,47}$ = 107.87; $p$ < 0.001) in submerged *R. palustris* compared to controls (non-submerged) from 5.56 pmol $g^{-1}$ Fresh Weight (FW) $h^{-1}$ (control) to 293.12 pmol $g^{-1}$ FW $h^{-1}$ (submerged) (Fig. 1a). Inoculation of *R. palustris* with ACC (1-aminocyclopropane-1-carboxylate) deaminase-producing bacteria (WT) decreased significantly ($F_{2,23}$ = 5.25; $p$ = 0.02) 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).

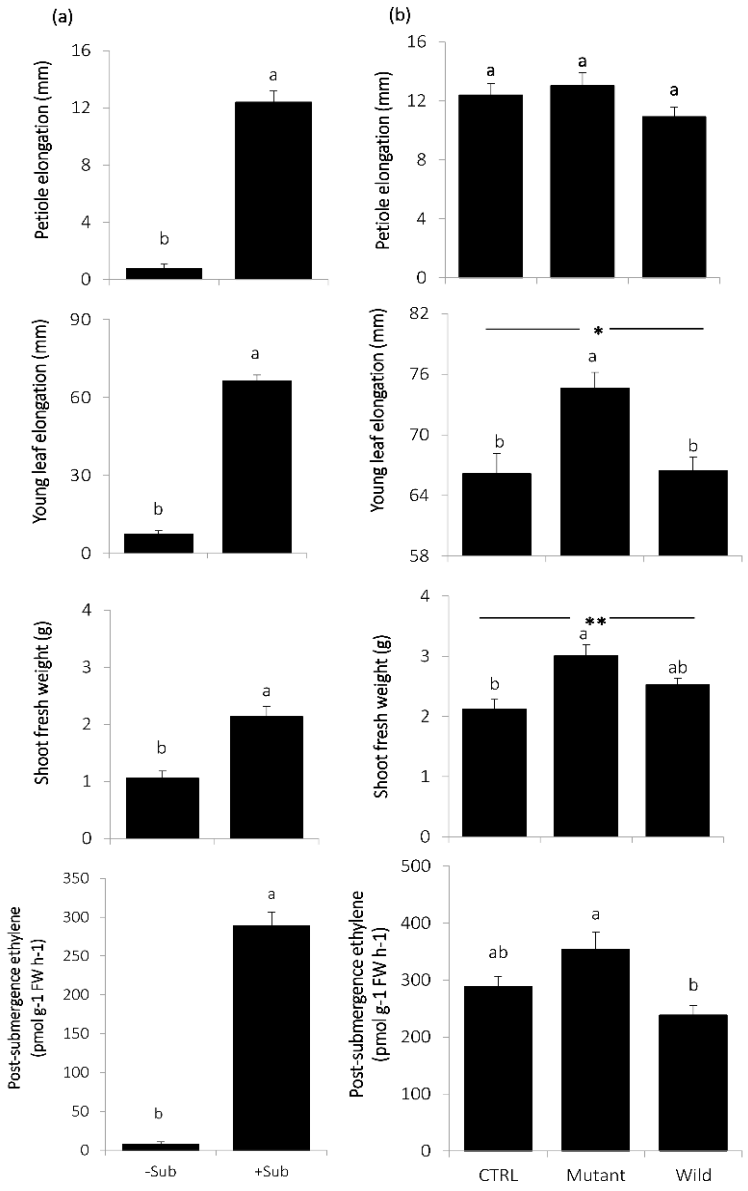


**Fig. 1.** a, Effects of 72 h of complete submergence on post-submergence ethylene production and morphology of *Rumex palustris*. –Sub; no submergence (air-control), +Sub; short-term (72 h) submergence. (b): Effects of ACC (1-aminocyclopropane-1-carboxylate) deaminase-producing bacteria *Pseudomonas putida UW4* (WT), its isogenic ACC deaminase deficient mutant (mutant), and non-bacterial (CTRL) on

*Rumex palustris* ethylene-mediated responses (young leaf, petiole elongation and fresh weight) to short-term submergence. In part b, the differences between treatments was analyzed by the polynomial linear contrast analysis using three contrasts: CTRL vs bacterized plants (WT and Mutant), WT vs Mutant, and Mutant vs CTRL. Significant effects based on contrast analysis are showed by asterisk ($p<0.05$ *, $P<0.01$ **). Error bars show ± SD.

Plant inoculation with the ACC deaminase negative mutant bacterial strain increased post-submergence ethylene production ($t(24) = 2.01$; $p = 0.06$) in *R. palustris* compared to non-bacterial control treatment, suggesting positive effects of other bacterial traits on this factor in submergence condition (Fig. 1b).

There was a strong negative correlation ($R^2 = -0.58$) between the WT population density on the rhizoplane and post-submergence ethylene production in *R. palustris* associated with WT bacteria ($F_{1,8} = 11.54$; $p = 0.01$), 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 6.74 mm to 70.53 mm after 72 h submergence ($F_{1,47} = 1512.1$; $p < 0.001$; Fig. 1a). Submergence also resulted in significantly larger older petioles: leaf 3 ($F_{1,47} = 212.83$; $p < 0.001$) and leaf 2 ($F_{1,47} = 322.82$; $p < 0.001$) from 0.77 to 12.33 mm and 0.52 mm to 12.12 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. Wild type (WT) bacterial inoculation decreased elongation of the youngest leaf ($F_{2,23} = 8.75$; $p = 0.001$) and of the older third leaf petiole ($F_{2,23} = 2.84$; $p = 0.08$) compared to ACC deaminase negative mutant bacterial inoculation after 72 h submergence (Fig. 1b).

Total shoot fresh weight of *R. palustris* increased significantly ($F_{1,47} = 85.00$; $p = 0.001$) after 72 h submergence from 1.23 g to 2.76 g (Fig. 1a). Wild type (WT) bacterial inoculation decreased *R. palustris* shoot fresh weight significantly compared to the ACC deaminase deficient mutant bacterium ($F_{2,23} = 7.91$; $p = 0.002$) from 3.01 g to 2.52 g, suggesting negative effects of ACC deaminase activity on ethylene-mediated shoot fresh weight and 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 ($t(24) = 2.49$; $p = 0.02$) compared to the no bacteria treatment (Fig. 1b). This factor also increased significantly ($t(24) = 3.57$; $p = 0.002$) 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 ($t(24) = 3.33$; $p = 0.003$) and mutant inoculation ($t(24) = 3.97$; $p = 0.001$) 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 no-bacterial inoculation plants did not change after 72 h of submergence implying that no extra biomass was produced while the plant was submerged ($F_{1,47} = 1.41$ ; $p = 0.24$). However, bacterial inoculation increased ($F_{2,23} = 6.06$; $p = 0.01$) dry weight significantly compared to the no bacterial treatment (Fig. 3). There was no significant difference between wild type (WT) and ACC deaminase negative mutant inoculation with respect to this factor ($t(24) = 0.41$; $p = 0.68$). Similarly, the root fresh weight ($F_{1,47} = 1.31$; $p = 0.27$) and dry weight ($F_{1,42} = 0.81$; $p = 0.38$) of *R. palustris* did not change significantly during 72 h of submergence (see table S1 in Supporting Information). Bacterial inoculation did increase both root fresh weight ($t(24) = 3.14$; $p = 0.004$) and root dry weight ($t(24) = 2.81$; $p = 0.01$) significantly compared to the no bacteria treatment during the short-term submergence (Fig. 3).

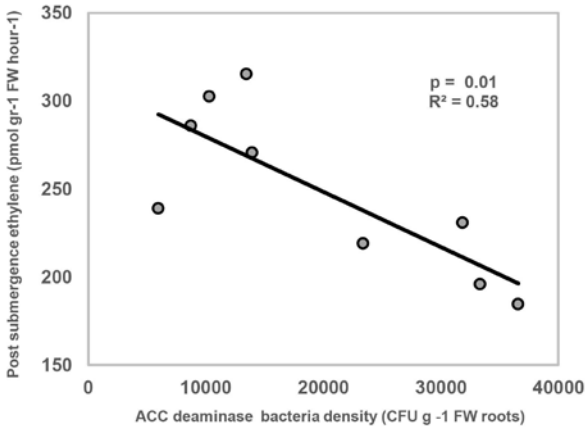

**Fig. 2.** Relationship between the density of ACC (1-aminocyclopropane-1-carboxylate) deaminase-producing bacteria on the roots of *Rumex palustris* and post-submergence ethylene production (pmol $g^{-1}$ FW (Fresh Weight) $h^{-1}$).

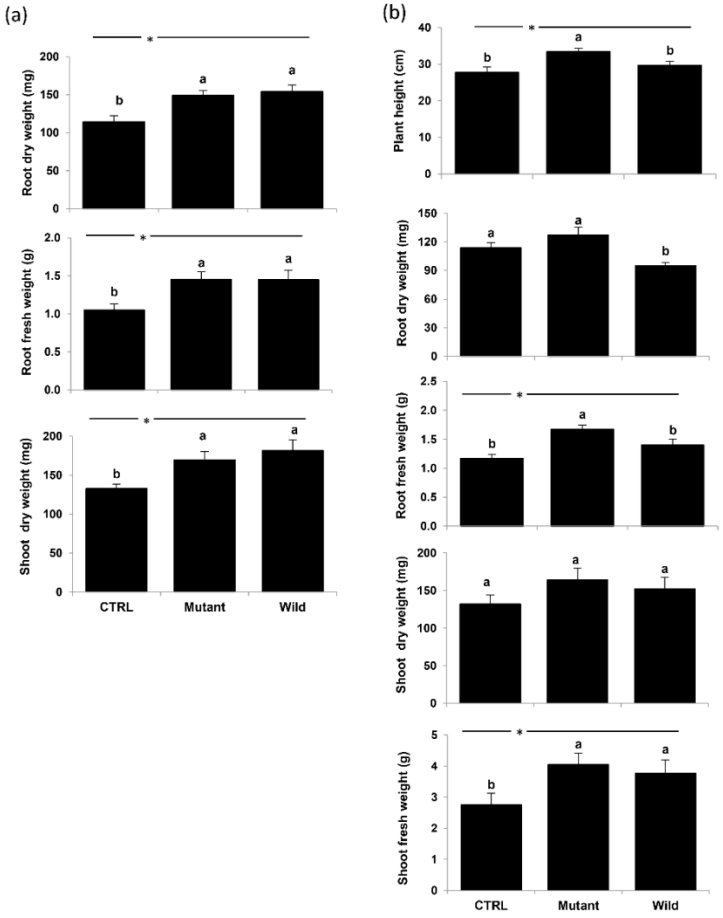


**Fig. 3.** a) Shoot fresh weight, shoot dry weight, root fresh weight, and root dry weight of *Rumex palustris* in the three experimental treatments after 72 h of complete submergence. Treatments were without bacteria treatment (CTRL), *Pseudomonas putida UW4* (WT), ACC deaminase deficient mutant (Mutant). b) 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. Treatments were without bacteria treatment (CTRL), *Pseudomonas putida UW4* (WT), ACC deaminase deficient mutant (Mutant). Differences between treatments were analyzed by the polynomial linear contrast analysis using three

contrasts: CTRL vs bacterized plants (WT and Mutant), WT vs Mutant, and Mutant vs CTRL Significant effects based on contrast analysis are showed by asterisk ($p<0.05$ *, $P<0.01$ **). Error bars show ± SD.

**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 ($F_{2,22}$ = 5.49; $p$ = 0.03), shoot dry weight ($F_{2,22}$ = 2.44; $p$ = 0.14), root fresh weight ($F_{2,22}$ = 8.56; $p$ = 0.002) and final plant height ($F_{2,22}$ = 4.95; $p$ = 0.04) compared to the no bacteria treatment (Fig. 3b). Furthermore, inoculation with the WT bacterial strain significantly decreased root fresh weight (t (22) = 2.45; $p$ = 0.02), root dry weight (t (22) = 3.74; $p$ = 0.001) and final plant height (t (22) = 2.83; $p$ = 0.01) compared to plants inoculated with the ACC deaminase negative mutant (Fig. 3b). 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. Plant stress responses are often quantified in plants grown under more-or-less sterile conditions. Here we show that common root-associated microbes can alter adaptive responses by interfering with plant hormonal balance.

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.

We assessed the impact of ACC-deaminase producing bacteria, which can reduce plant ethylene levels, on submergence responses of the riparian plant *Rumex 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 *Pseudomonas putida UW4* strain (WT) and an isogenic ACC deaminase deficient mutant (mutant). This

mutant is otherwise identical to the wild type, 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.*, 2003). 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.

*Rumex palustris* responds to submergence by elongation of its aerial parts, resulting in a higher shoot fresh weight (but not dry weight), indicating that productivity remained unchanged) and an ability to reach the water surface (Banga *et al.*, 1996; Voesenek *et al.*, 2003).

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 signaling pathway results in strongly reduced submergence responses (Voesenek *et al.*, 2003; 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. Our study shows that this reduction in ethylene impeded the ability of *R. palustris* to express its normal morphological adaptation to flooding. Furthermore, plant flood responses can be improved by addition of ACC (Voesenek *et al.*, 2003; Cox *et al.*, 2004). Thus, reducing plant ethylene levels may have contrasting impacts on plant survival depending on the ecological context.

In addition to the effect of ACC deaminase activity, 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 versus

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. The model bacterial strain used in this study also has other traits that may stimulate growth, such as production of the phytohormone auxin (Duan *et al.*, 2013). We would like to emphasize that comparisons can be drawn only between the WT and ACC deaminase negative mutant treatment, as the control, non-bacterial treatment is an artificial and unnatural treatment where plants grow in absence of mutualistic microbes. 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 to field situations. Given the high abundance of ACC deaminase-producing bacteria around plant roots (Duan *et al.*, 2008), they may have 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.*, 2003). 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 *et al.*, 2009). Hence, this elongation is particularly useful in shallow and prolonged floods (Pierik *et al.*, 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 *et al.*, 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 *et al.*, 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.

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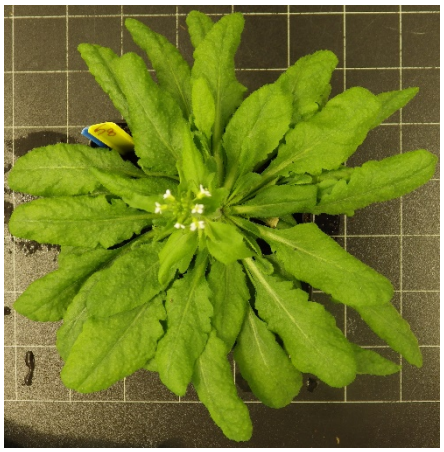
**Author Contributions**

All authors contributed to designing the research and writing of the manuscript. A.J. and M.R. contributed to data analysis. M.R. was mainly responsible for establishment of the experiment, chemical analyses and plant harvest, with substantial contributions from A.J. All authors contributed critically to the drafts and gave final approval for publication.

# 6

## Fitness effects of ethylene modulation by microbiota depends on bacterial genetic background



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**Abstract**

Ethylene is a central hormone regulating plant growth and stress resistance. Ethylene production is co-regulated by the plant and its associated microbiota. Ethylene reduction by root-associated microbes has been intensively investigated as a way to alleviate plant stress. However, microbiota can affect plant physiology via a range of traits beyond ethylene signaling. We therefore hypothesized that plant responses to ethylene-reducing bacteria is not only a function of changes in plant ethylene levels but also other microbial traits.

We inoculated *Arabidopsis thaliana* with four ACC deaminase-producing bacteria (degrading the ethylene precursor ACC) or their respective isogenic mutants lacking this ability. We assessed plant growth and resistance to cadmium and salt stress. Using this experimental design, we could examine both the direct impact of ACC deaminase activity, versus other properties associated with the bacterial genetic background, on plant growth and stress responses.

We found strong interactive effects between the presence of the ACC deaminase enzyme, bacterial strain identity and stress, with effects ranging from deleterious to positive.

We conclude that procession of the ACC deaminase enzyme is not *per se* a beneficial or deleterious trait, but rather that its effect on plant growth will essentially depend on the genetic background of bacteria as well as the environmental conditions. Understanding which plant and bacterial traits determine the fitness effects of ethylene reduction by plant-associated microbiota will help develop more reliable strategies to enhance plant growth under stressful conditions.

**Keywords**: ACC deaminase enzyme, bacterial genetic background, plant stress, salinity, cadmium

**Introduction:**

Ethylene is a central plant hormone regulating several aspects of plant growth, development, and stress responses (Sasidharan & Voesenek, 2015; Thao *et al.*, 2015). Ethylene concentrations increase under stress, which triggers adaptive responses (Pieterse *et al.*, 2009). Ethylene cascade is conserved across most plant species. However, stress perception, transduction, and the final response can vary greatly between plants. As ethylene is a multifunctional plant regulator, increased ethylene levels will induce a range of pleiotropic effects in addition to the target response (Roux *et al.*, 2006; Scarpeci *et al.*, 2017). Different plant species and even genotypes can greatly vary in stress perception and response (Li *et al.*, 2013; Veen *et*

*al.*, 2013; Voesenek *et al.*, 2015). Furthermore, ethylene levels within the plant are not only determined by plant physiology, but also co-regulated by plant-associated microbiota.

Plant-associated microbiota producing the enzyme ACC deaminase can decrease plant ethylene levels and have long been considered as beneficial for plant growth. This enzyme degrades ACC, the direct precursor of ethylene, potentially lowering ethylene in plants. However, numerous plant traits can impact plant physiology and signaling. We therefore propose that the impact of ACC deaminase-producing bacteria will not only depend on this trait, but will represent the net result of numerous bacterial activities as dictated by the overall genetic background of bacteria in question.

To disentangle the specific role of ACC deaminase activity from other bacterial activities on plant responses, we conducted two experiments to quantify the importance of the bacterial genomic background on the effects of ACC deaminase activity on plant growth. We examined the impacts of selected four pairs of ACC deaminase-producing bacteria and their respective isogenic ACC deaminase deficient mutants. We inoculated each of these trains onto *Arabidopsis thaliana* growing under cadmium or salt stress. We then evaluated the effect of ACC deaminase on root architecture and plant growth. We hypothesized that both bacterial strain identity and the presence of ACC deaminase will affect plant growth. We further expected a strong interactive effect between strain identity and ACC deaminase on plant growth.

**Method and materials**

**Plant materials**

*Arabidopsis thaliana* (L.) Heynh., Columbia (hereafter referred to as "Col-0") ecotype seed was obtained from plant ecophysiology group, Utrecht University, the Netherlands. After sterilization and stratification (in the dark at 4°C, for 4 d), seeds were sown on square petri dishes containing agar-solidified Murashige and Skoog (MS) medium supplemented with 0.25 % sucrose. Petri dishes were transferred and positioned vertically in a growth chamber (20°C; 70% relative humidity, 9-h photoperiod; 200 μmol $m^{-2}$ $s^{-1}$ PPFD). For the *in vitro* assay, uniform 10-day old seedlings of Col0 were transferred to new agar-solidified Hoagland medium (Hoagland and Arnon, 1938) without NaCL or amended with 20 μM NaCL. Each treatment consisted of eight plants on each 12 cm square plate and at least four replicates. Plants were inoculated with one of eight bacterial strains (see next section), and positioned vertically in the same growth chamber for 10 more days at which time root and shoot biomass, root length and lateral root number were analysed. In

addition, a “no bacteria” control was performed in which the plants did not receive any bacterial inoculum.

For the pot experiment, 10-day-old seedlings (prepare as described above) were transferred to pots containing a mixture of fine sand and perlite (both pasteurized at 90 ° C), saturated by a modified Hoagland nutrient solution (Smeets et al, 2008), and positioned in the same growth chambers. Forty-five plants were selected based on uniformity of developmental stage on day 21 and inoculated with bacterial strains (see next section). The pots were drenched with 50 ml of deionized water or cadmium solution (from $CdSO_4$ salt), resulting in 20 µM water-extractable cadmium in solution. Five plants were examined per treatment. Plants were grown for an additional 14 days at which shoot and root dry weights were determined.

**Bacterial strains**

The bacterial strains used in this study are listed in Table 1. The ACC deaminase deficient mutant ($acdS^-$) of these bacteria were obtained by insertion of an antibiotic resistance gene in the ACC deaminase gene coding region (Li *et al.*, 2000; Ali *et al.*, 2014; Vacheron *et al.*, 2016). Bacterial strains were kept as frozen stocks at -80 °C. Prior to experiments, one single colony was grown overnight on TSB medium (for wild-type ACC deaminase containing bacteria) or TSB and 100 µg $ml^{-1}$ tetracycline (to isolate mutant bacteria lacking ACC deaminase). Bacteria were harvested by centrifugation (6000 *g*, 10 min), washed three times with 10 mM $MgSO_4$ and adjusted to an $OD_{600}$ of 0.5 before inoculation.

**Table 1.** The bacterial strains used in this study are listed in Table 1.

| Bacteria Identity | Reference |
|---|---|
| *Pseudomonas putida UW4* (Wild type) | |
| *Pseudomonas putida UW4* (ACC deaminase deficient mutant) | (Li et al., 2000) |
| *Pseudomonas fluorescens F113* | |
| *Pseudomonas fluorescens F113* (ACC deaminase deficient mutant) | (Vacheron et al., 2016) |
| *Pseudomonas migulae 8R6* | |
| *Pseudomonas migulae 8R6* (ACC deaminase deficient mutant) | (Ali et al., 2014) |
| *Pseudomonas fluorescens YsS6* | |
| *Pseudomonas fluorescens YsS6* (ACC deaminase deficient mutant) | (Ali et al., 2014) |

**Data analysis**

The general effects of bacterial strain inoculation, the ACC deaminase enzyme, and their interactive effects on plant biomass and root architecture in control, salt and

cadmium stress experiments were evaluated by two-way ANOVAs. The effect of ACC deaminase enzyme was evaluated as the difference of the measured plant variables between plants inoculated with the wild type or their respective isogenic mutant, and expressed as a function of bacterial ACC deaminase enzyme. Four replicate for first (salt stress condition) and five replicates for second (cadmium stress condition) experiment were set up for every treatment. All analyses were performed in SPSS (V. 22).

**Results**

**Effects of bacteria strains and the ACC deaminase enzyme on plant growth in control and salt stress condition**

Bacterial inoculation significantly increased shoot fresh weight, root fresh weight, and the number of lateral root in *A. thaliana* grown under both control and salt stress conditions (Table. S1, contrast analysis of bacterized versus no-bacterized plants, supporting material). These effects depended both on bacterial strain identity (main effects of bacterial strains, Table 2A), the presence of ACC deaminase enzyme (main effect of ACC deaminase enzyme, Table 2A). Bacterial ACC deaminase increased plant biomass and promoted lateral root number compared to ACC deaminase deficient mutant in *A. thaliana* Col0. However, the effects of the ACC deaminase enzyme on plant biomass and root architecture was dependent on the specific bacterial strain that was inoculated (Significant interactive effects of Bacteria strain x ACC deaminase enzyme) (fig. 1).

Bacterial strains also showed significant effects on plant biomass and root architecture under the control growth conditions (Main effects of bacterial strains, Table 2B). The presence of ACC deaminase did not show significant effects on plant biomass and root architecture in the no-salt condition. However, the interactive effects of bacterial strains and possession of the ACC deaminase enzyme on plant biomass and the number of lateral roots were significant (Significant interactive effects of Bacteria strain x ACC deaminase enzyme, Table 2B). The effects of ACC deaminase bacteria on plant biomass and root architecture was depends on specific bacterial strains (Fig S1).

Table 2. The effects of bacteria strains, the ACC deaminase enzyme and their interaction on shoot fresh weight, root fresh weight, root length, and lateral root number of *Arabidopsis thaliana* under salt stress and control conditions.

| | **Factors** | ***df*** | ***F*** | ***P- value*** |
|---|---|---|---|---|
| **A) Salt treated medium** | | | | |
| Shoot fresh weight | Bacteria strains | 3 | 5.92 | **0.003** |
| | ACC deaminase | 1 | 6.79 | **0.01** |
| | Bacteria × ACC deaminase | 3 | 8.91 | **P<0.0001** |
| Root fresh weight | Bacteria strains | 3 | 7.99 | **0.001** |
| | ACC deaminase | 1 | 8.64 | **0.007** |
| | Bacteria × ACC deaminase | 3 | 13.75 | **P<0.0001** |
| Root length | Bacteria strains | 3 | 10.249 | **P<0.0001** |
| | ACC deaminase | 1 | 3.55 | **0.07** |
| | Bacteria × ACC deaminase | 3 | 2.24 | *0.10* |
| Root lateral number | Bacteria strains | 3 | 8.27 | **P<0.0001** |
| | ACC deaminase | 1 | 1.89 | **0.18** |
| | Bacteria × ACC deaminase | 3 | 7.51 | **0.001** |
| | Error | 28 | | |
| **B) Control medium** | | | | |
| Shoot fresh weight | Bacteria strains | 3 | 8.232 | **P<0.0001** |
| | ACC deaminase | 1 | 0.018 | 0.895 |
| | Bacteria × ACC deaminase | 3 | 2.722 | *0.061* |
| Root fresh weight | Bacteria strains | 3 | 15.553 | **P<0.0001** |
| | ACC deaminase | 1 | 3.325 | *0.078* |
| | Bacteria × ACC deaminase | 3 | 13.657 | **P<0.0001** |
| Root length | Bacteria strains | 3 | 32.065 | **P<0.0001** |
| | ACC deaminase | 1 | 0.335 | 0.567 |
| | Bacteria × ACC deaminase | 3 | 0.263 | 0.852 |
| Root lateral number | Bacteria strains | 3 | 11.896 | **P<0.0001** |
| | ACC deaminase | 1 | 0.739 | 0.396 |
| | Bacteria × ACC deaminase | 3 | 9.186 | **P<0.0001** |
| | Error | 28 | | |

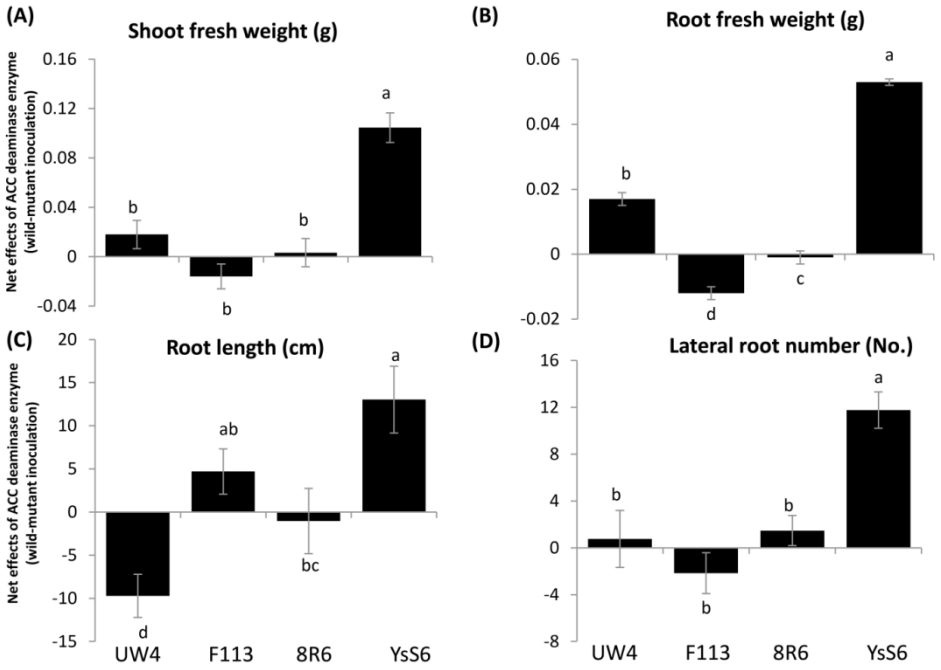


**Fig. 1.** Net effects of ACC deaminase production on shoot fresh weight (A), root fresh weight (B), root length (C), and lateral root number (D) of *A. thaliana* under 25 µM NaCl-treated solidified Hoagland medium agar. Net effect was defined as the difference between plants inoculated with wild type bacteria and their respective *acdS*$^-$ mutant. The differences between treatments was analyzed by two way NAOVAs complemented with Tukey's range test. Error bars show ± SE.

**Effect of bacterial strain identity and the ACC deaminase enzyme on plant growth under cadmium stress**

Bacterial inoculation significantly increased root and shoot biomass of *A. thaliana* plants in cadmium treated soils (Table. 3), with an interactive effect between the bacterial strain and the presence of the ACC deaminase enzyme.

**Table 3**. The effects of bacteria strains, ACC deaminase enzyme and their interaction on shoot fresh weight, root fresh weight, root dry weight and shoot dry weight of *A. thaliana* under control and cadmium stress condition. Differences between treatments were analyzed using two way ANOVAs.

| | Factors | *df* | *F* | *P- value* |
|---|---|---|---|---|
| **Cadmium treated soil** | | | | |
| Shoot Fresh weight | Bacteria strains | 3 | 3.2 | **0.03** |
| | ACC deaminase | 1 | 7.538 | **0.01** |
| | Bacteria × ACC deaminase | 3 | 3.766 | **0.02** |
| Shoot dry weight | Bacteria strains | 3 | 2.991 | **0.04** |
| | ACC deaminase | 1 | 1.69 | 0.20 |
| | Bacteria × ACC deaminase | 3 | 3.714 | **0.02** |
| Root Fresh weight | Bacteria strains | 3 | 8.499 | **P<0.0001** |
| | ACC deaminase | 1 | 4.609 | **0.03** |
| | Bacteria × ACC deaminase | 3 | 2.539 | *0.07* |
| Root dry weight | Bacteria strains | 3 | 4.625 | **0.008** |
| | ACC deaminase | 1 | 9.098 | **0.005** |
| | Bacteria × ACC deaminase | 3 | 7.839 | **P<0.0001** |
| | Error | 32 | | |
| **Control soil** | | | | |
| Shoot Fresh weight | Bacteria strains | 3 | 2.161 | 0.11 |
| | ACC deaminase | 1 | 0.752 | 0.39 |
| | Bacteria × ACC deaminase | 3 | 2.138 | 0.12 |
| Shoot dry weight | Bacteria strains | 3 | 1.276 | 0.30 |
| | ACC deaminase | 1 | 0.092 | 0.76 |
| | Bacteria × ACC deaminase | 3 | 1.535 | 0.23 |
| Root Fresh weight | Bacteria strains | 3 | 3.121 | **0.04** |
| | ACC deaminase | 1 | 0.857 | 0.36 |
| | Bacteria × ACC deaminase | 3 | 2.525 | 0.08 |
| Root dry weight | Bacteria strains | 3 | 2.982 | **0.05** |
| | ACC deaminase | 1 | 0.001 | 0.98 |
| | Bacteria × ACC deaminase | 3 | 1.418 | 0.26 |
| | Error | 24 | | |

Bacterial strains showed significant effects on plant fresh and dry weight of *A. thaliana* under cadmium stress (main effects of bacterial strains, table 3). The ACC deaminase enzyme of bacteria showed significant effects on shoot fresh weight, root fresh weight, and root dry weight of *A. thaliana* (main effects of ACC deaminase enzyme of bacteria, table 3). The presence of ACC deaminase was associated with significantly decreased root fresh and dry biomass compared to ACC deaminase deficient mutant under cadmium stress conditions. However, the effect of bacterial ACC deaminase on plant biomass dependent on the specific bacterial strain in question (Significant interactive effects of ACC deaminase enzyme x Bacterial strain), demonstrating that the effects of ACC deaminase is a function of the bacterial genetic background (Fig. 2).

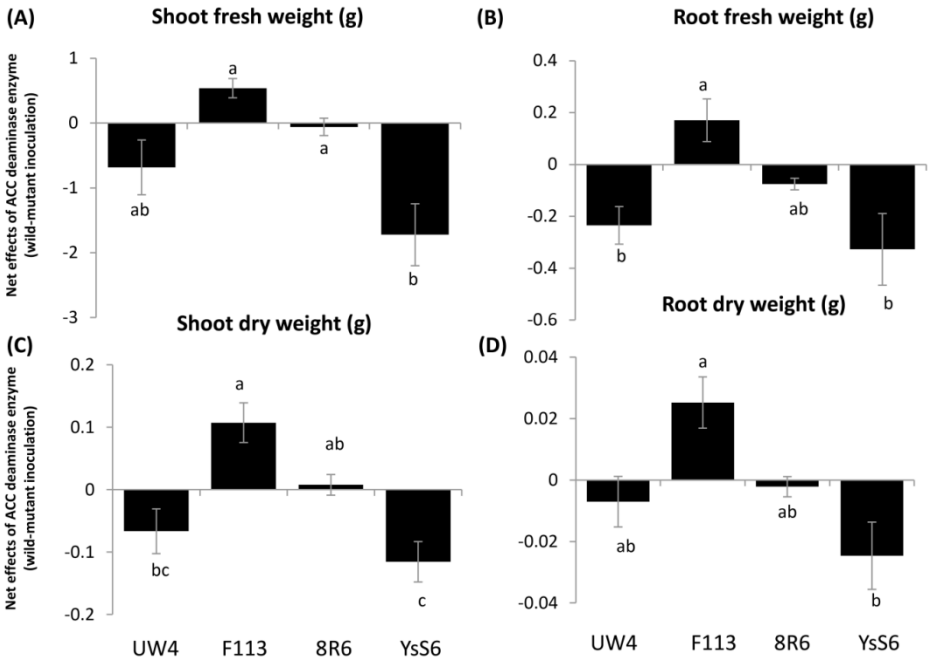


**Fig. 2.** The net effects of ACC deaminase producing bacteria (difference of wild type and mutant) on shoot fresh weight (A), root fresh weight (B), shoot dry weight (C), and root dry weight (D) of *A. thaliana* under cadmium stress. The differences between treatments was analyzed by two way NAOVAs complemented with Tukey's range test. Error bars show ± SE.

**Discussion**

Ethylene is a central regulator of the balance between plant growth and stress tolerance and as such an interesting target to study plant phenotypes and life history tradeoffs. As ethylene is co-regulated by the host plant and its associated microbes, it is further a promising model to adress how plant-microbe interactions shift plant phenotype. In particular, ACC deaminase producing microbes, which degrade the precursor of ethylene, have long been scrutinized in relation to their ability to promote plant growth under stress. However, bacteria are not only altering ethylene signaling. Several other traits can influence plant physiology and potentially interact with ethylene signalling. In the present study we assessed whether the effect of one specific bacterial trait (ACC deaminase) on plant phenotype is conserved or in contrast varies as a function of the genetic background of the bacteria.

We demonstrate that the effects of the ACC deaminase production in root-associated microbes on plant growth are context dependent and range from stimulation to inhibition as a function of the applied stress and the genetic background of the bacteria. By comparing pairs of bacteria harboring or lacking ACC deaminase but otherwise genetically identical, we could tease apart the effect of this trait from the bulk of strain-specific bacterial traits that may also affect plant growth.

We observe a general trend for a stimulation of plant growth by inoculation with bacterial strains. ACC deaminase producing enzyme altered plant growth in the stress treatment, but not in the control treatment. Under heavy metal stress the main effect of ACC deaminase was negative, but in the salt treatment, ACC deaminase enzyme promote plant growth. No or little effects of ACC deaminase enzyme were observed in the control treatment. This is in line with the importance of ethylene modulation by ACC deaminase enzyme and its effects on plant stress response (Cheng *et al.*, 2007; Vacheron *et al.*, 2016).

In addition, the effect of ACC deaminase production on plant growth was dependent on the specific bacterial strain and stress condition. Comparing different pairs of wildtype and mutants revealed that knocking out the acdS gene had vary idiosyncratic effects on plant development. For instance, under salt stress, ACC deaminase production in *Pseudomonas putida UW4* led for instance to a stunted root length, in addition to an increase in shoot biomass. In contrast, in *Pseudomonas fluorescens YsS6*, ACC deaminase production led to an increase in both shoot and root growth as well as a more ramified root system. Both these strains decrease plant growth at the presence of cadmium stress condition.

Part of the differences in plant morphology may stem from differences in ACC deaminase production among bacterial (Vacheron *et al.*, 2016). However, such an

effect does not account for the change in the sign of the interaction depending on the strain. The effect of carrying ACC deaminase on plant growth varied from strongly negative to strongly positive depending on the bacterial identity. We propose therefore that other bacterial traits may interact with ethylene reduction.

Ethylene has been shown to be an important modulator of plant stress tolerance (Abeles *et al.*, 2012). Diverse effects of ethylene on plant stress tolerance depend on both the plants and microbial agents examined (Ma et al 2014; Yang et al., 2015). Microbiota may alter plant ethylene levels either by directly reducing ethylene precursor levels (*e.g.* via the action of ACC deaminase) or altering plant signaling and transcription that triggers ethylene production. They can also modulate ethylene response by producing plant hormones that are interacting with ethylene signaling (Chanclud & Morel, 2016). Bacteria also can modulate other hormones and pathways that interact with ethylene, such as auxin, cytokinin, gibberellin or salicylic acid (Weiss & Ori, 2007; Arteca & Arteca, 2008; Guan *et al.*, 2015).

Comparing the effects of ACC deaminase mutant bacteria compared to wild type is a rapid and rigorous approach to isolate the effects of the ACC deaminase enzyme (Contesto *et al.*, 2008) (Contesto et al, 2008). Using this approach, in which we compared four pairs of ACC deaminase or deficient strains and their impact on plant growth under salt and cadmium stress conditions, we could demonstrate the potential complexity of plant-microbe interactions in remodeling plant stress responses.

The genetic background effects of bacterial strains have previously been highlighted in some studies (Remold & Lenski, 2004; Cao *et al.*, 2007). With respect to the effects of ACC deaminase, it has been suggested that it might be important to consider the genetic background effects of bacterial strains in order to disentangle the complex and diverse results observed when relating this function to plant growth (Hall *et al.*, 1996; Vacheron *et al.*, 2016; Ravanbakhsh *et al.*, 2017). Given that there are dozens of candidate bacterial genes that may impact plant growth, identifying the genes interacting with ACC deaminase goes beyond the scope of the current study. Identification of the most important factors for determining the cumulative effect of bacterial ethylene of ethylene represents an interesting and line for future research, and such work might include the use of multiple mutants across a range of genetic backgrounds. A more complete understanding of this joint co-regulation of plant phenotype may prove to be crucial to the development of microbial inoculants that reliably promote plant growth.

**Conclusion**

We show here that harboring the ACC deaminase gene is not a good predictor of plant growth promotion by a given bacterial species. Adding or removing this gene can improve or negatively affect plant growth depending on the stress type, and the bacterial genetic background. Given this contingent effect of ACC deaminase, we call for a reinterpretation of the current paradigm that treats this trait as a premium plant growth-promoting function, often without regard for other critical bacterial properties. These results may explain the wide range of effects observed in bioaugmentation studies aiming at increasing the abundance of ACC deaminase gene in the rhizosphere. We propose that accounting for the functional genes already present in the introduced and native microbiota may help provide a better way to improve plant growth under stress conditions.

**Supporting materials**

**Table S1.** The effects of bacteria strains (bacterial inoculation versus no-bacterial inoculation) on shoot fresh weight, root fresh weight, root length, and lateral root number of *A. thaliana* under control and salt stress conditions. The results is based on contrast analysis of no-bacterial versus bacterized (wild type and mutant) treated plants.

| | *t* | *df* | *P value* |
|---|---|---|---|
| **Control medium** | | | |
| Root FW | 3.214 | 34 | 0.003 |
| Shoot FW | 3.903 | 34 | P<0.0001 |
| Length of root | -11.407 | 34 | P<0.0001 |
| Lateral root No. | 7.19 | 34 | P<0.0001 |
| **Salt treated medium** | | | |
| Root FW | 4.343 | 36 | P<0.0001 |
| Shoot FW | 5.382 | 36 | P<0.0001 |
| Length of root | -7.812 | 36 | P<0.0001 |
| Lateral root No. | 6.072 | 36 | P<0.0001 |

**Table S2.** The effects of bacteria strains (bacterial inoculation versus no-bacterial inoculation) on shoot fresh weight, shoot dry weight, root fresh weight, and root dry weight of *A. thaliana* under control and cadmium stress conditions. The results is based on contrast analysis of no-bacterial versus bacterized (wild type and mutant) treated plants. Bacterial treatment increased significantly fresh and dry roots of *A. thaliana* in control and cadmium spiked soils.

| | *t* | *df* | *P value* |
|---|---|---|---|
| **Control soil** | | | |
| Shoot FW | 0.641 | 1 | 0.526 |
| Shoot DW | 0.829 | 1 | 0.414 |
| Root FW | 2.786 | 1 | P<0.0001 |
| Root DW | 2.513 | 1 | P<0.0001 |
| | | 31 | |
| **Cadmium treated soil** | | | |
| Shoot FW | 0.904 | 1 | 0.372 |
| Shoot DW | 1.553 | 1 | 0.128 |
| Root FW | 2.639 | 1 | 0.012 |
| Root DW | 2.15 | 1 | 0.038 |
| Error | | 40 | |

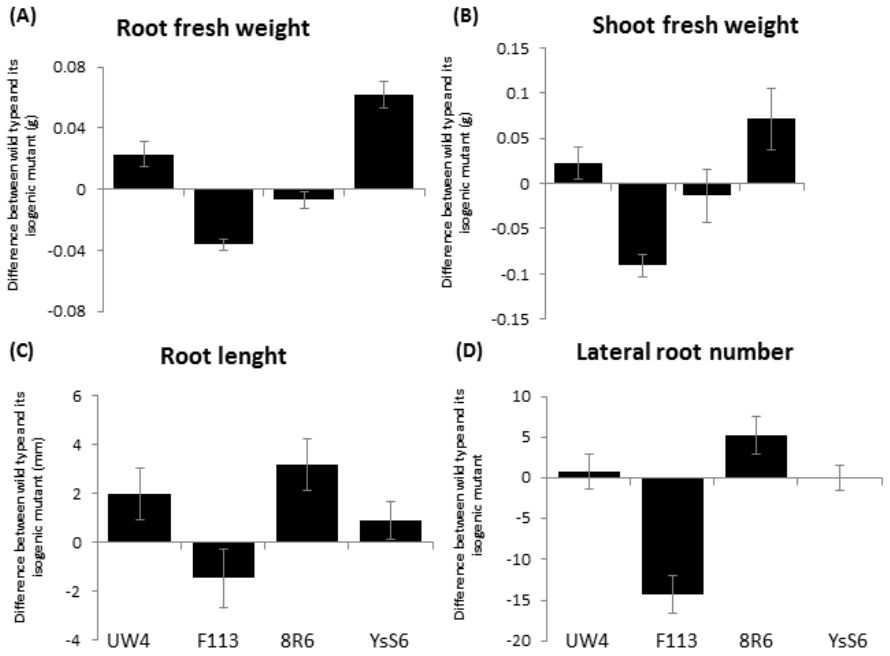

**Fig. S1.** The net effects of ACC deaminase producing bacteria (difference of wild type and mutant) on shoot fresh weight (A), root fresh weight (B), root length (C), and lateral root number (D) of *A. thaliana* on solidified Hoagland medium agar. Error bars show ± SE.

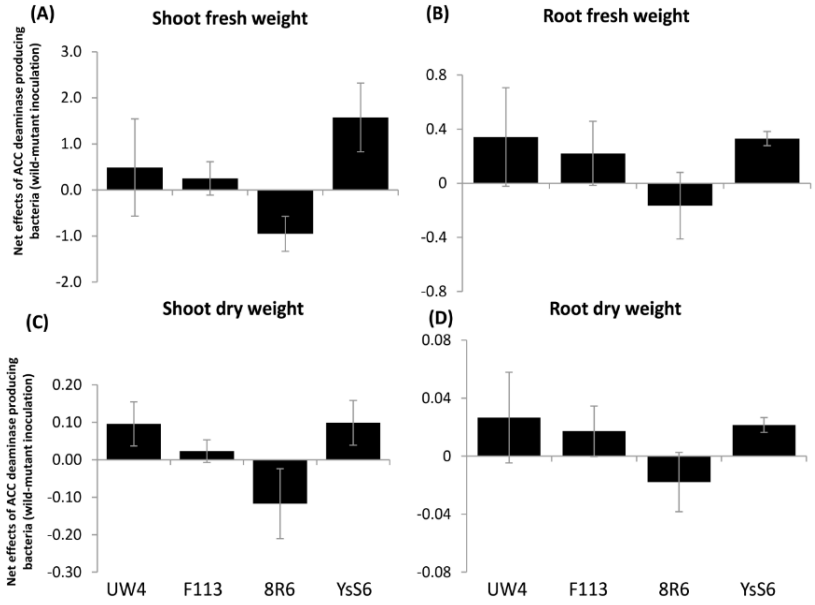


**Fig. S2.** The net effects of ACC deaminase producing bacteria (difference of wild type and mutant) on shoot fresh weight (A), root fresh weight (B), shoot dry weight (C), and root dry weight (D) of *A. thaliana* under control (no-cadmium) conditions. Error bars show ± SE.

# 7

# Microbial modulation of plant ethylene signalling: ecological and evolutionary consequences

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**Abstract**

The plant hormone ethylene is one of the central regulators of plant development and stress resistance. Optimal ethylene signalling is essential for plant fitness and is under strong selection pressure. Plants upregulate ethylene production in response to stress, and this hormone triggers defense mechanisms. Due to the pleiotropic effects of ethylene, adjusting stress responses to maximize resistance, while minimizing costs, is a central determinant of plant fitness. Ethylene signalling is influenced by the plant-associated microbiome. We therefore argue that the regulation, physiology and evolution of the ethylene signalling can best be viewed as the interactive result of plant genotype and associated microbiota. In this article, we summarize the current knowledge on ethylene signalling and recapitulate the multiple ways microorganisms interfere with it. We present ethylene signalling as a model system for holobiont-level evolution of plant phenotype: this cascade is tractable, extremely well studied from both a plant and a microbial perspective, and regulates fundamental components of plant life history. We finally discuss the potential impacts of microbiota on plant ecology and evolution. We assert that ethylene signalling cannot be fully appreciated without considering microbiota as integral regulatory actors, and we more generally suggest that plant ecophysiology and evolution can only be fully understood in the light of plant-microbiome interactions.

**Keywords:** evolution**,** holobiont, plant, phenotype, physiology, ethylene, microbiota, microbiome, ACC deaminase.

## Background

### Environmental stress and plant fitness

Plants are constantly facing a range of different environmental stressors linked for instance to temperature, water availability, presence of toxic minerals or pathogens. Stress can be permanent, for instance when a plant lives outside its ecological optimum, or acute during climatic extremes such as drought and flooding waves. Environmental stress has an important effects on plant fitness and elicits specific adaptations (Bohnert *et al.*, 1995). Plants have evolved a range of physiological and morphological responses to stressors, allowing them to cope with the prevailing environmental conditions. Although these responses vary widely, they all share one characteristic: They all come at a cost to the plant, diverting resources from growth and reproduction, and causing negative side effects that may have consequences on other traits that can result in indirect fitness costs. Optimizing the

relative investment into stress response and other life history traits is thus essential to maximize fitness (Huot *et al.*, 2014). Due to the variability of stressors and their interactive effects with plant genotype, regulating stress response is a complex task with several possible optima. Further, adaptation to one specific set of environmental conditions may negatively affect plant fitness under other conditions (Denison, 2015). Plant transpiration illustrates this dilemma well: stomatal closure, a typical plant response to drought, reduces water loss, but comes at a cost of lower photosynthesis, gas exchange and sap flow. Given that stomata are also an entry point for several pathogens (Melotto *et al.*, 2006), the optimal aperture will be a function of several parameters including water availability, plant sensitivity to desiccation, and presence of pathogens (Araújo *et al.*, 2011).

To optimize fitness, stress responses must be therefore carefully adjusted. This implies that plants need to perceive different stressors, process the signals and trigger an optimal stress response, thereby maximizing resistance while minimizing costs and side effects. Signal integration in plants is generally achieved by alterations in hormonal balance. Hormones such as ethylene, auxin or jasmonic acid interactively shape the relative investment of plants into growth, reproduction and stress defense (Leon-Reyes *et al.*, 2009). Hormone concentrations are dictated by the combine action of the plant's own regulatory pathways as well as the activities of its associated microbiota. Hormonal regulation therefore offers an excellent model to approach plant physiology, ecology and evolution from a holobiont perspective in which plants and microbes form a coherent unit of selection (Vandenkoornhuyse *et al.*, 2015).

In this review, we approach ethylene, a central plant hormone regulating the balance between growth and stress tolerance, from a holobiont perspective. We first briefly, summarize the importance of ethylene for stress tolerance and other life history traits. We then go on to provide an overview of how plants and their associated microbiota jointly shape hormonal balances, thereby shifting plant response toward or away from adaptation to specific situations.

## Regulation of stress response by plants

### Role of ethylene

Ethylene is a central plant hormone regulating several aspects of plant growth and development, throughout the whole plant life cycle, from germination to senescence (Bleecker & Kende, 2003). In addition, this hormone is essential to regulate stress responses and confer stress tolerance (Sasidharan & Voesenek, 2015; Thao *et al.*, 2015). Stress results in increased levels of ethylene in plants. Stress

derived ethylene is a signal triggering adaptive responses and influences other hormonal signalling pathways (Pieterse *et al.*, 2009). Due to the multiple effects of ethylene on plant phenotype, increased ethylene levels will induce a range of pleiotropic effects, such as growth inhibition and late flowering (Scarpeci *et al.*, 2017), in addition to the target response (Roux *et al.*, 2006). Precisely controlling cellular ethylene levels is thus a key aspect of plant physiology (Roux *et al.*, 2006).

**Ethylene production, signal transduction, and response**

An important step in ethylene production is the synthesis of its precursor ACC (1-aminocyclopropane-1 carboxylic acid) by ACC synthase (ACS) enzyme (Fig. 1). Upon stress detection, ACS mediates the synthesis of the ethylene precursor ACC, which is transformed to ethylene by the enzyme ACC oxidase (ACO; Fig. 1). Both *ACS* and *ACO* form large multigene families in plants and different members can be regulated by different internal and external stimuli (Barry *et al.*, 1996; Johnson & Ecker, 1998; Schellingen *et al.*, 2014). Ethylene triggers the expression of response genes. Ethylene binds to the negative regulator CTR1 (Constitutive Triple Response), frees the positive regulator EIN2 (Ethylene Insensitive 2) (Alonso *et al.*, 1999), which triggers the transcription factors EIN3 (Ethylene Insensitive 3) and EIL1 (Ethylene Insensitive 3-like 1) (An *et al.*, 2010). These transcription factors will increase the expression of ethylene responsive transcription factors (ERFs) (Groen *et al.*, 2013), resulting in ethylene-mediated stress responses in plants (Kacperska, 1997). ERF-regulated traits include activation of plant immunity (Loon *et al.*, 2006; Wees *et al.*, 2013), metabolic and morphological adaptations to flooding (Morgan & Drew, 1997; Sasidharan & Voesenek, 2015), expression of systems for scavenging reactive oxygen species and modification of enzymatic activity under heavy metal and salinity stress conditions (Peng *et al.*, 2014; Tao *et al.*, 2015; Keunen *et al.*, 2016). The type of ethylene-mediated response is highly variable, as discussed below in "ethylene variation in plants".

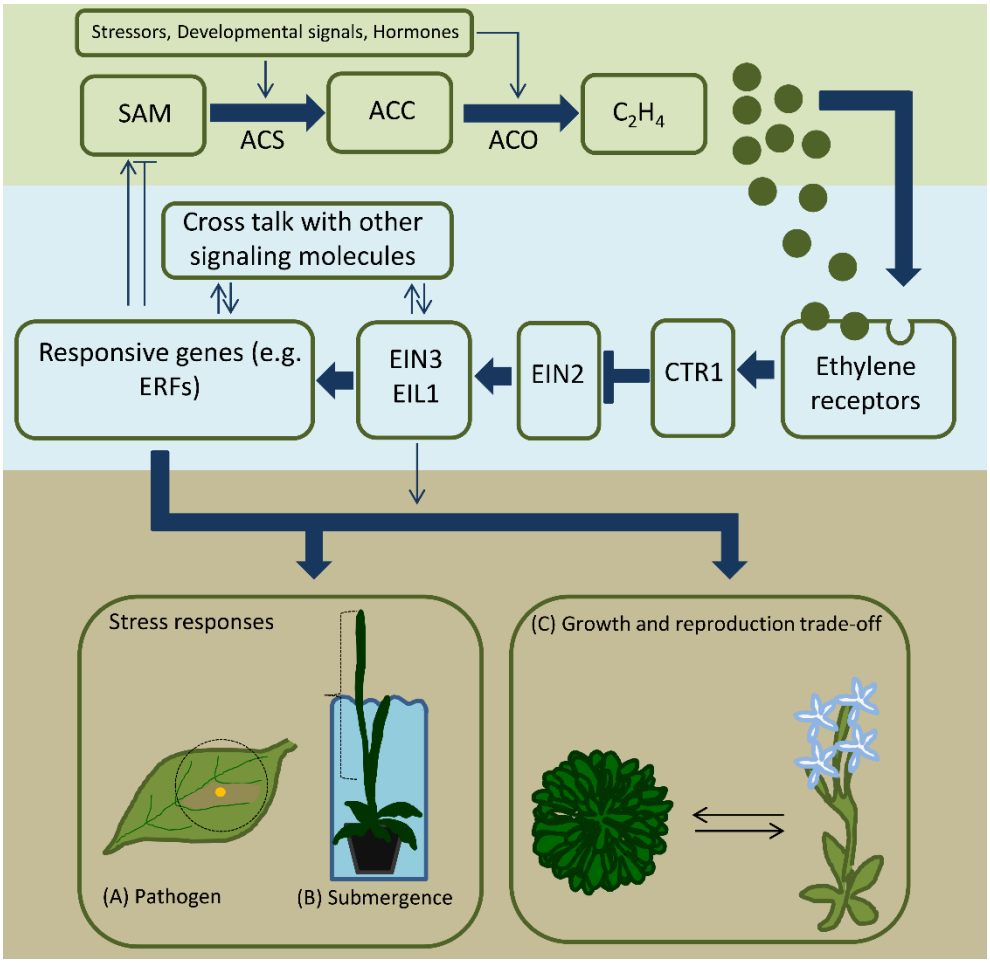


**Fig. 1**. Overview of the pathways linked to ethylene production (top panel), signal transduction (central panel) and response (bottom panel). Ethylene concentration determines plant resource allocation into growth, reproduction and stress response (Roux *et al.*, 2006). The thick arrows show the main ethylene cascade, the thin ones point to possible interaction with external and internal stimuli. We illustrate plant response with three well-investigated ethylene-dependent phenotypic adaptations (A) Ethylene coordinates plant response against pathogens, such as hypersensitive response, preventing pathogen spread (Kacperska, 1997). (B) Ethylene accumulation triggers escape strategy involving accelerated shoot growth in submerged plants, allowing them to regain atmospheric contact (Veen *et al.*, 2013). (C): Growth-reproduction tradeoffs: higher ethylene causes plants to invest more resources into

seed production under harsh conditions that may compromise vegetative stage survival. SAM: S-adenosylmethionine, ACC: 1-aminocyclopropane-1-carboxylic acid, ACS: ACC synthase, ACO: ACC oxidase, C2H4: plant hormone ethylene, CTR1: Constitutive triple response 1, EIN2: Ethylene-insensitive protein 2, EIN3: Ethylene-insensitive protein 3, EIL1: ethylene insensitive 3-like 1 protein, ERFs: Ethylene Response Factors.

**Ethylene varies as a function of stress type and intensity**

Ethylene production depends on the intensity and duration of stress periods. For instance, different levels of heavy metal (Vassilev *et al.*, 2004; Keunen *et al.*, 2016) or different dehydration rates (Berumen & Lownds, 1996) differentially modulate ethylene biosynthesis and signalling. In another example, low-levels of stress stimulate ethylene production, while high levels may decrease it (Berumen & Lownds, 1996; Remans *et al.*, 2012), either as part of a targeted stress response or as the result of impaired plant metabolism.

**Ethylene response from ecological and agricultural perspective**

Plants are continually confronted with variable environmental conditions, to which they must respond and adapt. Ethylene has a central role in plant survival and adaptation in dynamic environments. Ethylene-dependent stress response enhances survival in stress conditions such as heavy metal (Keunen *et al.*, 2016), salinity (Tao *et al.*, 2015), and drought (Wilkinson & Davies, 2010). However, high stress tolerance may cause pleiotropic effects on plant phenotype under such stress conditions (Keunen *et al.*, 2016). Ethylene triggers for instance early flowering, helping plants complete their life cycle before resources become depleted (Wilkinson & Davies, 2010). This comes, however, at the cost of a reduced biomass (Roux *et al.*, 2006). Therefore, considering multiple components of plant fitness may be essential to provide a full view of ethylene-related impacts. Instead of purely looking at single traits such as short-term biomass production, as has often been done in plant-microbe research, we propose approaching ethylene as coordinator balancing different life history traits to reach the best possible phenotype for survival in prevailing ambient conditions, thereby maximizing reproductive fitness. Variation in ethylene levels might be due to selection on the plant genetic material or, as we will discuss in the next section, on the microbiota that co-regulate ethylene (Fig. 2).

## Ethylene variation in plants

### Variability in ethylene-based stress responses across plant species

The core ethylene transduction cascade is highly conserved in plants, and a wide range of plants use ethylene as a regulator of their stress responses. However, plants vary greatly in how ethylene impacts stress perception, transduction, and the final response. Plants evolved in a certain environment have adapted ethylene signalling to the stresses typical for that environment or even dropped it, if not useful. For instance, plants living in flood-prone or riparian areas, including rice and *Rumex palustris*, use flooding-induced accumulation of ethylene to trigger important adaptive responses (Bailey-Serres & Voesenek, 2008). In contrast, some aquatic plants adapted to a permanently submerged life style, lost many genes involved in ethylene signalling (Voesenek *et al.*, 2015; Street & Schaller, 2016). Contrasting ethylene-mediated responses are even seen in closely related plant species. For instance, different species of *Rumex* sp. (van Veen et al, 2013) or different varieties of rice all use ethylene as a flooding signal to trigger adaptive responses, yet the responses itself are highly variable, ranging from compensatory growth to complete quiescence (Bailey-Serres & Voesenek, 2008).

### Variability in stress perception and ethylene signal transduction

Both *ACS* and *ACO* ethylene biosynthetic genes are encoded by large multigene families. These genes are organ-specific and are differentially regulated by different environmental signals (Barry *et al.*, 1996; Lasserre *et al.*, 1996; Peng *et al.*, 2005). The size of these multigene families can vary between plant species and could link to the variation in ethylene-mediated stress perception. For instance, apple harbors 19 *ACS* genes as compared to 12 *ACS* genes in *Arabidopsis thaliana* and 9 in tomato (Vogel *et al.*, 1998; Peng *et al.*, 2005; Li *et al.*, 2013). Deletion of one single gene, *ACS6*, resulted for instance in a 85–90% reduction in ethylene production in maize (Young *et al.*, 2004). Variation in the ethylene response can also occur at the level of ethylene perception across different plant genotypes linked to changes in receptor affinity, expression pattern and/or turn-over (Dal Cin *et al.*, 2006). Furthermore, knocking out *EIN3* showed opposite effects on salinity tolerance in *Arabidopsis* and rice (Yang *et al.*, 2015). Ethylene signalling and response are highly dependent on plant genotype (Pierik *et al.*, 2007), organ, growth stage (Dugardeyn & Van Der Straeten, 2008), and associated microbiota.

## Microbiota

### Importance of microbiota as co-regulators of stress response

Plants are associated with a complex microbiome, including bacteria, fungi, and protists that have impact on diverse aspects of plant growth, health and evolution (Zilber-Rosenberg & Rosenberg, 2008). Plant-associated microbiota can be either vertically transmitted, as is the case for endophytes that live within plant tissues, or horizontally for instance by recruiting microbiota to the rhizosphere from the surrounding soil species pool. Microbiota form an integral part of a plant's immune system, metabolism, and hormonal balance (Berendsen *et al.*, 2012). They directly alleviate stress, for instance by producing protective compounds that enhance drought resistance (Fan & Liu, 2011; Ruíz-Sánchez *et al.*, 2011), degrading organic pollutants (Horvath, 1972), or chelating heavy metals (Lloyd & Lovley, 2001). Plant-associated microbes can also fine-tune hormonal balance and physiology by modulated plant hormone levels and the pathways they steer. In the case of ethylene, several possible mechanisms have been described by which microbiota can affect plant hormonal levels. Below, we examine the mechanisms by which the plant-microbe dialog determines ethylene-mediated plant responses as the basis for a more general model on holobiont-level regulation of plant hormonal balance.

### Ethylene modulation as a holobiome process

Ethylene signalling forms a perfect example of a holobiont-level physiological cascade. From the holobiont perspective, plant physiology is controlled by a combination of traits encoded in the host genome as well as its associated microbes, which collectively form the holobiont (Vandenkoornhuyse *et al.*, 2015). This association offers a broader genetic pool than the plant alone: ethylene-modulating microbes could increase the reservoir of genetic information linked to ethylene signalling, enabling a greater plant phenotypic plasticity in response to stressors. Microbiota can 1) impact plant-perceived stress 2) co-regulate ethylene which affects plant fitness, and 3) perceive ethylene, potentially responding to it.

### Plant and ethylene-modulating microbes as unit of selection

Ethylene levels are a strong determinant of fitness in dynamic environments. Given that plants and microbes work in concert to modulate ethylene-mediated responses, the holobiont level of selection is the most appropriate: the ethylene cascade provides an important link between the host and its associated microbes, and forms an integrated biological entity (Zilber-Rosenberg & Rosenberg, 2008). This

interaction even has the potential to be evolutionarily stable: Plants rely on microbes to optimize their fitness and microbes directly benefit from a more vigorous host that may provide more nutrients and energy. As plants can select associated microbes on the basis of the functions they perform, mutualistic interactions may persist across generations.

**Ethylene modulation by microbiota and their evolutionary impact on plants**

**a) Reduction of stress perception by microbiota**

Microbiota may contribute to plant stress tolerance in an ethylene-independent way by providing protection mechanisms expressed outside of the host plant. For instance, plant-associated microbiota may reduce the intensity of stress experienced by the plant by detoxifying chemicals or providing protective substances against desiccation (Horvath, 1972; Lloyd & Lovley, 2001; Ruíz-Sánchez *et al.*, 2011). From an evolutionary perspective, a plant's reliance on the microbiome to reduce stressors may lead to a reduced ability of the plant to respond to the acute stressors (Fig. 3B), a task delegated to the associated microbiota.

**b) Altered ethylene level by microbiota**

Microbes can potentially influence all regulatory steps of the ethylene pathway (Fig. 2). The most direct way of acting on ethylene signalling is to either directly produce or degrade ethylene. Several plant-associated microbes can increase plant ethylene levels by directly synthesizing ethylene or inducing plant ACS activity (Freebairn & Buddenhagen, 1964; Fukuda *et al.*, 1993; Suganuma *et al.*, 1995; Weingart & Volksch, 1997). Ethylene production by microbes was first reported in the pathogen *Ralstonia solanacearum,* which, among other symptoms, induces banana premature ripening (Freebairn & Buddenhagen, 1964). Microbial ethylene production was later mainly investigated in relation to pathogenic bacteria (Fukuda *et al.*, 1993; Weingart & Volksch, 1997). However, biosynthetic pathway studies (Nagahama *et al.*, 1992) and the examination of available bacterial genomes has revealed that the relevant genes and pathways can be found across a wide range of microorganisms (Sato *et al.*, 1997; Eckert *et al.*, 2014). For instance, more than one third of all cultivable soil bacteria can produce ethylene via different pathways (Nagahama *et al.*, 1992). Phylogenetic studies of ethylene-forming enzymes show that multiple ethylene-producing pathways have evolved independently and later spread between bacterial phyla by horizontal gene transfer (Nagahama *et al.*, 1992; Eckert *et al.*,

2014). Ethylene production by microbes may have deep effects on plant physiology and life history, as demonstrated by the accelerated fruit ripening in plants inoculated with *Escherichia coli* engineered to produce ACC oxidase (Digiacomo *et al.*, 2014). Rhizosphere microbes can further increase ethylene indirectly by secreting auxin (Patten & Glick, 2002; Veselov *et al.*, 2003) and cytokinin (Vogel *et al.*, 1998; Veselov *et al.*, 2003), two hormones that upregulate the expression of ACS-coding genes (Yeong-Biau & Shang Fa; Lorteau *et al.*, 2001).

Microbiota can also decrease ethylene levels, for instance by producing ACC deaminase. This enzyme degrades ACC, ultimately leading to lower plant ethylene concentrations. ACC deaminase can be found in both commensal (Glick, 2014) and pathogenic microbes (Singh *et al.*, 2015). ACC deaminase genes are widespread in bacteria, fungi and members of stramenopiles (Nascimento *et al.*, 2014). ACC deaminase genes of bacteria and fungi shared high sequence identity (Nascimento *et al.*, 2014), pointing to a single evolutionary origin and frequent horizontal transfer of this gene in bacteria and fungi (Hontzeas *et al.*, 2005; Blaha *et al.*, 2006; Nascimento *et al.*, 2014). The reduction of ethylene levels caused by ACC-deaminase producing microbes is of the same magnitude as the one resulting from knocking out *ACS* genes (Young *et al.*, 2004; Ravanbakhsh *et al.*, 2017). In contrast to commonly held assumptions, ACC deaminase-producing microbes are not necessarily good and the effects of ACC deaminase on plant physiology and plant growth greatly depend on the interactive effects of plant genotype (Hall *et al.*, 1996; Vacheron *et al.*, 2016) and the environnement (Desbrosses *et al.*, 2009; Ravanbakhsh *et al.*, 2017). For instance, root growth reduction by ethylene is a common adaptation to avoid salt and pollutants (Koevoets *et al.*, 2016). Alleviating this inhibition may bring a short-term increase in root growth, but may ultimately be deleterious for the plant.

Co-evolution of plants with bacteria that increase or inhibit ethylene levels may have various consequences: Co-evolution of plants with microbes increasing ethylene levels may cause the plant to reduce ethylene production in order to maintain homeostasis. This could result in a lower ability to produce ethylene, a higher sensitivity to stressors and a dependency on microbial ethylene production (Fig. 3D). In contrast, plants associated with ethylene-reducing microorganisms such as ACC deaminase producers may need to produce more ACC to compensate for microbial degradation (Fig. 3C). Thus, plants may evolve a higher expression of *ACS* genes, which can allow a wide range of responses, but may also lead to overreactions to stress without modulation by the associated microbiome.

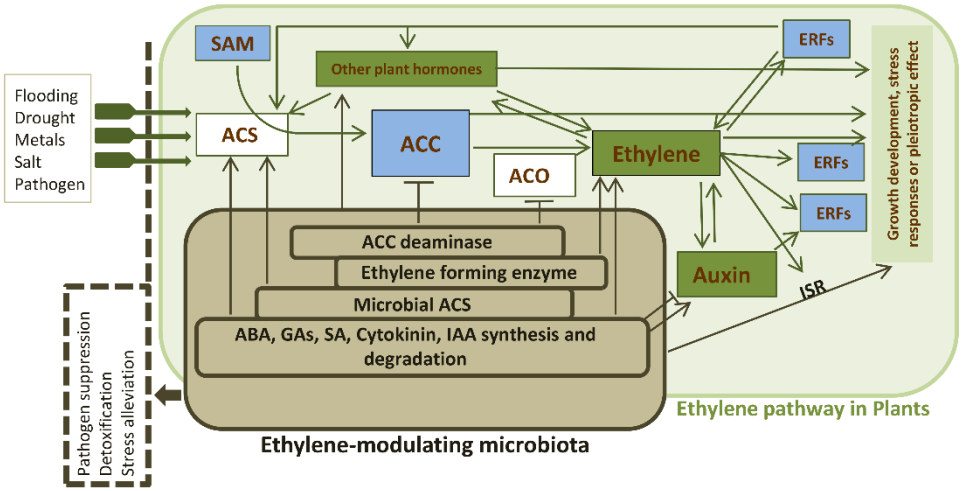


**Fig. 2**. A holobiont-level regulation of ethylene signaling and plant stress response**.** Ethylene pathway in plants (green area). ACC (1-Aminocyclopropane-1-carboxylic acid) is synthesized from SAM (S-adenosylmethionine) by action of ACC synthase enzyme (ACS). ACC is then converted to ethylene by the enzyme ACC oxidase (ACO), triggering different ethylene response factors (ERFs). Plant-associated microbiota can alter virtually all steps of ethylene signalling. Microbiota can increase ethylene production by ACC oxidase (microbial ethylene forming enzyme), by inducing ACC synthase directly, or by affecting other plant hormones indirectly. They can also modulate ethylene response by producing plant hormones that interact with ethylene signaling (Lorteau *et al.*, 2001; Arteca & Arteca, 2008; Guan *et al.*, 2015). Microbiota can also decrease ethylene production by cleaving its precursor ACC. White boxes show ethylene biosynthetic enzymes, green boxes show plant hormones and signals, and blue boxes show the molecules involved in the ethylene pathway. ABA; Abscisic acid, GA: Gibberellic acid, SA: Salicylic acid.

**Microbial alteration of plant ethylene response and intertwined signalling**

In addition to direct manipulation of ethylene levels, some microbiota are an integral component of stress perception and response. For instance, some microbial species can perceive environmental stressors relevant to the plant (Carlos *et al.*, 2016) as well as sense and respond to plant ethylene (Street & Schaller, 2016). This suggests that they may potentially be part of the holobiont-level ethylene-regulated traits, communicating stress perception to plants and monitoring plant stress status. As ethylene sensitivity in plant-associated microbiota is widespread, we propose that ethylene signaling may be part of a hologenome-level stress response in which genetic traits carried by both microbiota and the plant are activated in response to stressors. Ethylene modulation is under this perspective the result of the co-evolution of both plant and microbial traits. Bacteria could receive signals from environment or plants and trigger plant ethylene response factors (Fig. 3E) or even express their own ethylene response factors in response to plant ethylene or environmental cues (Fig. 3F).

Such intertwined signaling between plants and microbes might contribute to complete (Golicz *et al.*, 2015) or a partial (Osborne *et al.*, 1996) loss of plant ethylene pathways over the course of evolution, as observed in the loss of the ACC biosynthetic route in several gymnosperms (Groen & Whiteman, 2014) or the production of ethylene via an ACC independent pathway in several plants (Osborne *et al.*, 1996). From a co-evolutionary perspective, such plants will become more dependent on microbiota for ethylene pathway modulation.

**BOX 1**

**What makes co-evolution possible?**

a) Plants live in close association with a wide range of microbes. Roots select and feed a specific microbiome (Campbell & Greaves, 1990). A wide range of the rhizosphere-enriched microbiota have the ability to modulate plant ethylene signalling. For instance, genes linked to ethylene production or reduction can be found in a broad range of bacteria and fungi (Nagahama *et al.*, 1992; Duan *et al.*, 2009; Nascimento *et al.*, 2014). The constant contact with ethylene signaling-altering microbiota may cause the evolution of a modified pathway optimizing plant response in the presence of external perturbations.

b) Positive feedback loops: under stressful conditions, plants produce more ACC (Schellingen *et al.*, 2014). This confers an advantage to microbiota producing ACC deaminase that are able to use ACC as a nitrogen and carbon source. This

may result in an increased density of ACC-degrading microbes, whose effect can be counteracted by the plant by producing more ACC. The outcome might be beneficial only for microbes (parasitism of plant nitrogen), or mutually beneficial (symbiosis via shared ethylene signalling).

c) Plant adaptation to fluctuating environments requires a rapid rewiring of stress response pathways such as ethylene signaling. However, this adaptation may be too slow in plants, requiring several generations to acquire and spread the needed mutations. Emergence of genetic variation in the microbiome is many orders of magnitude faster than in plants (Rosenberg & Zilber-Rosenberg, 2016). Modulation of plant hormone levels via the microbiome may thus provide a new mechanism to match plant phenotype to environmental conditions.

d) Modulation of plant hormone levels via the microbiome may thus provide a new mechanism to match plant phenotype to environmental conditions.

e) Ethylene-modulating microbiota can be transmitted vertically, from one generation to the next generation, thus allowing co-evolution of microbes and the host as a cohesive unit of selection (Rosenberg & Zilber-Rosenberg, 2016). Vertical co-evolution may allow more gene transfer to the next generation, and the establishment of relatively stable associations. Nonetheless, vertical transmission is probably essential for the last of our proposed coevolutionary dynamics (Intertwined signalling; Fig 3E and F). The ethylene modulation genes could transfer between ethylene modulating bacteria by horizontal transfer (Hontzeas *et al.*, 2005; Blaha *et al.*, 2006), or through symbiotic island exchange (Nascimento *et al.*, 2012).

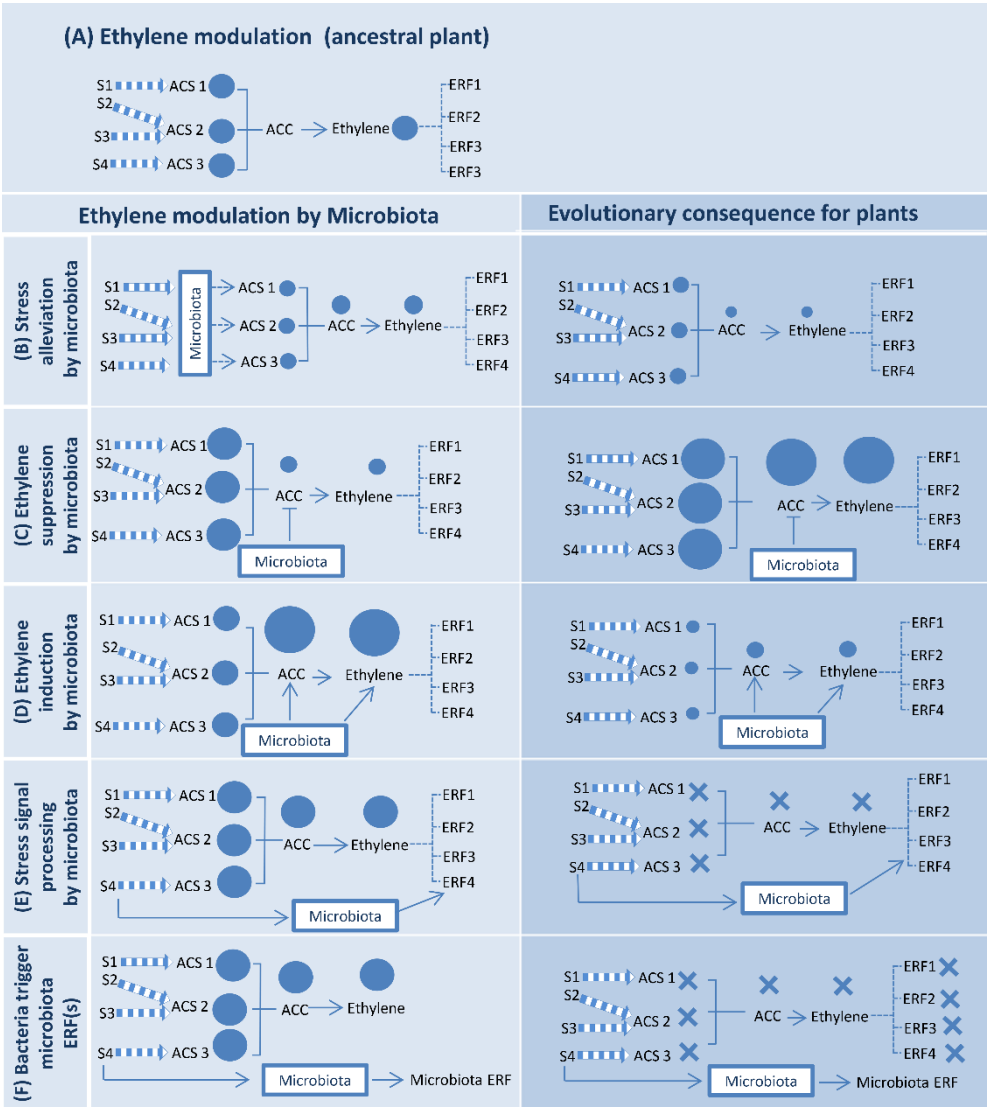


**Fig. 3**. Potential consequences of evolution of an intertwined ethylene signalling involving both plant and microbiota (A): in ancestral plant phenotype, ACC (1-Aminocyclopropane-1-carboxylic acid) is produced by the action of ACC synthase enzymes (ACS). ACC is then converted to ethylene by the enzyme ACC oxidase, triggering different ethylene response factors (ERFs) (B): bacteria reduce the intensity of stress experienced by the plant. Plant reliance on the microbiome to reduce stressors may lead to a reduced ability of the plant to respond to the acute stressors. (C, and D): bacteria alter ACC and ethylene in plants, leading to over- or under-

expression of ethylene pathway genes in plants, (E and F): bacteria integrate plant signals and trigger plant ethylene response factors (ERFs) or express their own ERFs, contributing to partial or complete loss of ethylene pathway in the plant. The dashed lines (for instance between stressors and ACS and ethylene and ERFs) showed indirect connections. The size of each circle indicates relative levels of ACC synthase (ACS) activity, ACC, and ethylene production in response to stressors (S1-S4).

**Evolutionary implications of ethylene modulating bacteria and plants**

Based on existing scientific evidence, ethylene signaling most likely evolved within the context of long term co-evolution processes between plants and their associated microbes. We propose that the joint regulation between microbes and plants can lead to several implications:

**a) Alterations of ethylene signaling may offer new functions and shift the niche of the holobiont**

Altering ethylene levels might allow plants to exploit new niche space, where other trait combinations are optimal. Co-evolution leading to ethylene overproduction (Fig. 3C) and insensitivity (Fig. 3D) might also shift plant niches, as well as restrict the chances for a plant to re-inhabit its ancestral range.

**b) Change in plant-encoded ethylene signalling genes**

During co-evolution, some microbiota are potentially part of holobiont-level ethylene-regulated traits (Fig. 3 E and F). This association might reduce some parts of the plant genome working in parallel with microbiota, saving the cost of gene expression and maintenance of redundant genes. In addition to losing some part of the ethylene signalling pathway, based on the amount of plant dependency on associated microbes, dispersal of seeds to new environments with completely different microbial communities might cause them to die before they are able to adapt to the new conditions and pass the traits down to their offspring.

**c) Uncouple plant phenotype from mutations in the plant genome**

Mutations can alter plant evolution by affecting different pathways including the ethylene pathway. Mutations in the plant ethylene biosynthesis and signalling pathway (for instance, the ability to overproduce ethylene) could cause new morphological traits or functions that promote plant fitness in a new environment, and therefore increase the chances for natural selection. Associated microbiota influence this selection, by making the

ancestral microbe associated plants more successful in competition, thereby decreasing the advantage of mutations, as microbes might override the plant-bacteria co-evolution by altering different parts of the ethylene pathway.

**Conclusion**

The plant hormone ethylene mediates many aspects of plant life history. At the holobiont level, ethylene signalling is a regulatory cascade composed of both plant- and microbiota associated traits, which together provide a dynamic and fine-tuned response to environmental conditions and stressors. The holobiont perspective in plant hormone regulation also has large evolutionary implications in which plants have become dependent on their microbiome for fully adaptive ethylene-mediated responses. From an agricultural perspective, the plant holobiont may facilitate appropriate or maladapted stress responses depending on the match or mismatch of plant and microbiome traits. Many aspects of plant health related to the microbiome and ethylene signalling may represent a useful model case to further our general understanding of plant holobiont ecology and evolution.

# 8

## Summarizing discussion

**Mohammadhossein Ravanbakhsh**

**Importance of ethylene for plant growth and stress response**

Phytohormones play crucial roles in numerous aspects of plant growth and development. The gaseous plant hormone ethylene is a key coordinator of growth, development and senescence of all plant organs, including roots, leaves, flowers, and fruits during the entire plant life cycle (Bleecker & Kende, 2003). In addition, ethylene is the central coordinating hormone of the plant stress response. The ethylene-dependent stress response enhances survival under stress conditions such as during exposure to heavy metals (Keunen *et al.*, 2016), high salinity (Tao *et al.*, 2015) and drought (Wilkinson & Davies, 2010). High ethylene signalling may also cause pleiotropic effects on the plant phenotype under stress condition (Keunen *et al.*, 2016). However, the effect of ethylene signalling on plant fitness is highly variable and depends, for instance, on the specific plant species as well as the environmental conditions encountered.

**Discrepancy between plant ecophysiology and plant-microbe interaction research**

Ethylene can either induce plant growth stimulation or inhibit growth depending on hormonal cross-talk in the plant as well as the conditions of the surrounding environment (Pierik *et al.*, 2007). Evidence has also recently been accumulating with respect to the role of ethylene as an important regulator of plant stress response (Tao *et al.*, 2015; Thao *et al.*, 2015). Given the high pleiotropic costs associated with stress tolerance, accurate ethylene regulation is important for phenotypic adjustment to different environment. Plant ethylene levels are a product of plant metabolism combined with the activities of associated microbiota. However, the subtle role of ethylene in plant growth and stress responses contrasts sharply with research targeting plant-microbe interactions, where ethylene is mostly seen as a negative hormone that is deleterious for the plant. The crucial role of ethylene production, signalling and perception for a plant's response to stress (Chen et al., 2005; Hall et al., 2007; Tao et al., 2015) is at odds with the widely held notion that ethylene reduction via the activity of microbiota, such as bacteria possessing the enzyme ACC deaminase, is beneficial to the plant (Reviewed by Glick, 2014). In this thesis, I have strived to reconcile these two fields of investigation and place the effect of bacterial ACC deaminase activity in the context of recent advances in plant ecophysiology. My central premise is that ethylene signaling is an essential component of the plant stress response. As such, its alteration by ACC deaminase-producing microorganisms, which degrade the direct precursor of ethylene, may

prevent the plant from building up proper defense mechanisms, thereby leading to predominantly negative effects on fitness when plants are subjected to stress.

**Context-dependent effects of ACC deaminase-producing bacteria**

The consequences of ethylene reduction by microbiota may depend on several factors, including plant genotype, microbial genome, and the environmental conditions encountered. I show in chapter 4 that the effects of microbial ethylene reduction on the plant depend on the stress intensity: Ethylene reduction by ACC deaminase-producing microbiota has contrasting effects in the presence or absence of stressor. Ethylene reduction by microbiota promotes plant growth under non-stressful conditions, but, contrary to the commonly held paradigm, may reduce plant growth under various abiotic stressors. I propose that ethylene reduction by microbes can increase plant growth by shifting the investment of plant resources from stress tolerance toward biomass production. Such an effect may increase productivity as long as no stress is present. However, may also interfere with a plant's potential to adapt properly to stress, resulting in an impaired growth as soon as plants are faced with an abiotic stress. The effect of microbial ethylene reduction on plant growth is thus not a positive or negative plant-microbe interaction *per se*, but instead depends on the ecological setting. In chapter 3, I further showed that the effects of ethylene reduction differ between plant species. Inoculation of three different plants with the same bacterium under heavy metal stress revealed that the effect of bacteria depends on the ability of the host plant to cope with the present stressor. For instance, *Alcea aucheri* (cadmium hyperaccumulator) was less affected by cadmium stress as compared to *A. thaliana* and *R. palustris*. Interestingly, reduction of ethylene by associated bacteria showed more impact on this plant both in terms of cadmium reduction and growth inhibition. Subsequently, in chapter 6, I show that the effects of bacterial ACC deaminase are dependent on the bacterial genetic background. In total, this thesis therefore challenges the generally accepted paradigm that bacterial ethylene reduction is a purely positive plant-microbe interaction by demonstrating that this activity may actually be deleterious for the plant across a broad range of conditions. This calls for a rethinking of the concept of what constitutes a beneficial plant-microbe interaction. To achieve this, I have developed a new concept that places microbial modulation of ethylene signalling into a co-evolutionary framework, using the latest developments of the holobiont concept (Chapter 7).

**Ethylene modulation by bacteria from the holobiont perspective**

Plants harbor a wide range of microbiota on and within their tissues. From the holobiont perspective, it is a combination of the host genome and the associated microbes that controls plant physiology and function (Vandenkoornhuyse *et al.*, 2015). Plant responses to stressors are mediated by phytohormones and regulators, including ethylene. Several microbes can modulate plant hormonal balance, potentially up- or down-regulating ethylene levels. To date, plant stress responses have often been studied under sterile conditions. This has enabled an extensive understanding of the genetic basis of plant physiology, but such approaches to not account for the co-evolutionary history of plants and associated microbes. Plant traits can be regulated by specific microbiota (Berendsen *et al.*, 2012), and the microbiome is selected along with the plant (Lau *et al.*, 2017). I propose that ethylene-mediated stress response is the product of a co-regulation by plants and microbiota, providing a perfect example of a holobiont-level approach to plant ecophysiology. In chapter 7, I develop a holobiont perspective to ethylene signalling and present evidence that the ethylene-mediated balance between plant growth and defense is the result of a co-evolutionary process between plants and microbes. Ethylene signaling forms a complex regulatory network allowing plants to respond to multiple stressors, a situation that is typical throughout a plant's lifetime. Due to pleiotropic effects, purely plant evolution of ethylene signaling represents a rather rigid mechanism to respond to specific environmental conditions, as responses may also impact non-target ethylene responsive factors. Fine-tuning of ethylene by microbes may provide a means of fine tuning the plant's ethylene levels to respond rapidly to environmental stress. A proper match between microbes and the host plant may help the plant realize an optimal adaptive response to stress in a fluctuating environment. However, a mismatch will result in ethylene levels that are either too high or too low, resulting in a suboptimal phenotype.

**Evolutionary consequences of bacteria application**

From an evolutionary perspective, ethylene signalling is a co-product of plant- and microbe-associated traits, which together provide a dynamic and fine-tuned response to environmental change. The holobiont perspective in plant hormone regulation also has large evolutionary implications in which plants have become dependent on their microbiome to achieve fully adaptive ethylene-mediated responses. The final outcome might make plants vulnerable in the absence of

appropriate associated microbes, yielding maladaptive plant responses when ethylene levels are not kept in check by the activities of the plant's microbial partners.

**Applications and perspectives**

The results of this thesis call for a reinterpretation of ethylene modulation by plant-associated microbiota. As such, the work presented opens up new avenues for the improvement of crops and cultivation techniques. In addition, this work will hopefully set the base for the development of a more comprehensive framework aiming at a more balanced vision of plant-microbe interactions. Below, I will therefore discuss the perspectives and open questions raised by the work reported in this thesis.

**a) Manipulation of plant phenotype by bacterial inoculation**

In chapter 3, I demonstrated that the phenotypic changes induced by ethylene reduction by ACC deaminase-producing bacteria is comparable with the effect of drastic manipulations of ethylene signalling in the plant genotype. I could, for instance, reproduce the effects of ACC deaminase-producing bacteria on the plant phenotype with an ethylene insensitive mutant in which all known genes responsible for ethylene signalling had been deactivated. Plant treated with ACC deaminase-producing bacteria as well as those that were genetically altered both showed lower leaf cadmium concentrations (supporting material P1) and a hypersensitivity to cadmium. This raises the prospect that bacteria could potentially function as an efficient replacement for tedious and costly genetic manipulation of plants. Using the proper associated bacteria might contribute to the desired phenotype and physiology response in the wild type plant (Fig. 1). However, a poor or uninformed choice of bacterial inoculant might lead to undesired or unexpected plant phenotypes, stressing the importance of the holobiont approach to understanding plant performance. Further investigation is thus needed to better understand the contingency of plant-microbe interactions.

**Fig. 1**. Ethylene-modulating bacteria Inoculation offer new plant phenotypes. Twelve different wild type cadmium resistance ACC deaminase producing bacteria were inoculated to *Arabidopsis thaliana* Col0 at 20 µM water extractable cadmium in soil solution.

**b) Application of bacteria to decrease heavy metals in food and feed**

Heavy metal contamination is an important issue for food safety worldwide. A wide range of agriculture soils is contaminated with low or moderate levels of heavy metals worldwide. As large-scale decontamination of heavy metal-polluted areas is often impracticable, manipulation of plant physiology by root-associated microbiota may offer an efficient strategy to reduce or block plant traits linked to heavy metal uptake. Taking ethylene as model cascade, we demonstrate that soil microbes modulating plant hormonal balance can help reduce heavy metal uptake in the plant and might help in the future to fine tune the plant phenotype and physiology to enhance food safety.

**c) From plant growth-promoting traits in bacteria to phenotype-modifying microbiota**

Plant-associated bacteria can fine-tune plant a plant's hormonal balance and physiology by modulating the hormone network. Such interactions have classically been expected to improve plant tolerance to stressors by improving plant stress tolerance and growth. However, the ideal phenotype in the presence of stressors is not an absolute concept and may differ based upon the ecological setting and plant

species. For instance, shoot biomass reduction under drought conditions helps to reduce water desiccation and is therefore an excellent adaptation. Reducing ethylene by microbiota may block such an adaptation to drought. It may enhance plant growth on the short term but at the cost of a waste of water that may kill the plant in later stages. I showed in chapters 4 and 5 that the ACC deaminase bacteria could have deleterious effects on the plant when ethylene is necessary for plant stress tolerance. Microbial auxin has also been shown to either promote or inhibit plant growth depending on the concentration (Barazani & Friedman, 1999). There are also other examples of potentially adaptive plant responses that are instead maladaptive under specific environmental conditions. For instance, triggering the plant's immune response in the absence of a pathogen can reduce growth and yield (Tian et al, 2003). Similarly, investing carbon in mycorrhizal fungi under conditions of sufficient phosphorus can come at a growth and fitness cost (Fitter, 1991). The work presented here and such examples show that the impact of presumably beneficial microbiota might actually be detrimental if the ecological context does not require the given activities. Hence, the final outcome of microbiota harbouring a specific trait is not *per se* "plant growth promotion". The associated microbiota allow for a modulation of the plant phenotype as dictated by a variety of microbial traits, and the final outcome might be beneficial or deleterious based on the plant's environmental conditions (Goh *et al.*, 2013; Ravanbakhsh *et al.*, 2017). I therefore assert that it might be more appropriate to speak of, "plant phenotype-modifying bacteria" as oppose to plant growth-promoting bacteria, and this might be extended to specific traits such as ACC deaminase activity. Microbiota effect phenotype. If the resulting phenotype is matched well to the environmental conditions (stress, nutrients, natural enemies), it will result in growth promotion, but the opposite may be true if the microbial modification of the plant's phenotype is inappropriate.

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**Samenvatting**

Stressfactoren zorgen ervoor dat planten zich continu aanpassen door het veranderen van fysiologie en fenotype. In een veranderend milieu, zorgen hormonen ervoor dat de plant zich kan aanpassen door grondstoffen te verdelen tussen groei en stresstolerantie. De aanpassingen van planten aan stressfactoren worden meestal bestudeerd zonder rekening te houden met het microbioom. Echter, planten leven samen met een grote hoeveelheid micro-organismen die invloed hebben op alle stadia van de ontwikkeling van een plant.

In dit proefschrift laten we zien dat het microbioom een belangrijke factor is die bepaalt hoe planten reageren op stressfactoren. Micro-organismen kunnen planten beschermen tegen stress of de fysiologie van de plant en de hormoonbalans mede bepalen. De stof ethyleen is een van belangrijkste regelaars voor plantengroei, -ontwikkeling en de reactie op stressfactoren. De productie van ethyleen door de plant wordt bepaald door planten, maar ook door micro-organismen. Micro-organismen hebben eigenschappen om de productie van ethyleen zowel te verhogen als te verlagen, en bepalen daarmee mogelijk mede de reactie van planten op stress. Recent onderzoek naar de fysiologie van planten heeft de belangrijke rol van ethyleen bij stress-tolerantie vastgesteld. In onderzoek naar interacties tussen planten en microben, echter, wordt de vermindering van ethyleen door micro-organismen normaliter gezien als een eigenschap die zorgt voor een toename in plantengroei, met name tijdens stress-condities. Dit proefschrift combineert recente ontwikkelingen op het gebied van plant fysiologie met onderzoek op het gebied van interacties tussen planten en microben.

Planten nemen zware metalen op uit de bodem. Voedselproductie op vervuilde bodems kan daarom gevaarlijk zijn voor de volksgezondheid. Grootschalige sanering van deze vervuilde bodems is vaak onpraktisch. Ethyleen speelt een belangrijke rol bij de opname van nutriënten en zware metalen door planten. We laten zien dat verlaging van de ethyleen productie van planten door bacteriën zorgt voor een verlaging van de opname van het zware metaal cadmium. Dat betekent dat bacteriën gebruikt kunnen worden als biotechnologie om de opname van zware metalen in gewassen te reduceren.

Verder laten we zien dat het verlagen van de ethyleen productie bij planten door micro-organismen invloed kan hebben op de stress-tolerantie van planten. Ten eerste kan het verlagen van de ethyleen productie door het ACC deaminase enzym van bacteriën positieve effecten hebben voor planten onder stressvrije omstandigheden. Tegelijkertijd is het mogelijk dat er ook negatieve effecten zijn op de normale aanpassingen van planten op stressfactoren zoals de aanwezigheid van zware metalen.

Ethyleen is voor planten ook belangrijk voor het aanpassen aan overstromingen. We laten zien dat de reductie van ethyleen productie door bacteriën belemmerd de aanpassing van planten op overstroming. Dit is in tegenspraak met de gangbare opvattingen in het onderzoeksveld van interacties tussen planten en microben. In deze opvattingen zorgt het ACC deaminase enzym juist voor een toename van plantengroei tijdens stress. Ons onderzoek toont echter aan dat het ACC deaminase enzym vooral negatieve effecten heeft op plantengroei tijdens stressvolle omstandigheden.

Verder laten we zien dat de sterkte van de afname van ethyleen productie door het toedoen van bacteriën vooral afhangt van de genetische achtergrond van de bacteriën. Dit betekent dat ACC deaminase enzymen in bacteriën met een verschillende genetische achtergrond resulteren in verschillende effecten op plantengroei.

Als laatste concluderen we dat ethyleen signalering bij planten alleen volledig begrepen kan worden door rekening te houden met de invloed van het hele microbioom van de plant. De verandering van de ethyleen productie van de plant als reactie op stressfactoren wordt bepaald door zowel de plant als het bijbehorende microbioom die samen één holobiont (superorganisme) vormen. Dit samenwerkingsverband kan grote implicaties hebben voor de evolutie van deze soorten: Planten zijn voor aanpassing aan stress afhankelijk van hun microbioom.

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**List of papers from this book:**

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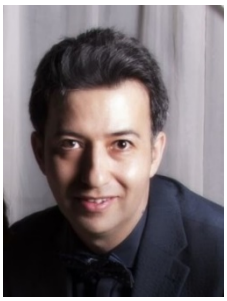
**Curriculum Vitae**



Mohammad Ravanbakhsh was born on 16 September 1981 in Shiraz, Iran. He graduated in 2004 with a BSc in “Agriculture, Soil Science” from Shiraz University, Iran, following with an MSc in the same major from Ferdowsi University of Mashhad, Iran in 2006. Afterwards, he worked for Fars Research Center for Agriculture and Natural Resources, Iran (2006-2013) as a junior researcher. As an international collaboration, he coordinated the Iran case study projects of “Sustainable Management of Marginal Drylands (SUMAMAD, 2009-2013), and “Ground Water and Human Security (GWAHS, 2008-2010) with UNESCO, UNUINWEH, and UNU in the area of nature conservation and land rehabilitation. In parallel, he worked as the executive manager of “Water Management Journal” between 2009-2012. Returning to study in late 2013, he began his PhD research at the Soil Science Department of Shiraz University focusing on “phytoremediation of heavy metal contaminated area using hyperaccumulator plants”. In May 2015, he was invited to join the ecology and biodiversity group at Utrecht University and work with Prof. G.A. Kowalchuk and Dr. A.L.C. Jousset where he spent 2015-2017 performing a large portion of the experimental work presented in this thesis. This work was done in close collaboration with prof. L.A.C.J. Voesenek, and Dr. Rashmi Sasidharan form plant ecophysiology group. Mohammad continues to work at Utrecht University, now as a postdoctoral researcher, working on the evolution of plant pathogens.