

A.1 APPLICANT

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B.1 BASIC DETAILS

B.1.1 Title

“The Soil-Borne Legacy and drought stress”

B.1.2 Abstract

Drought negatively affects crop health and yield and is expected to increase in abundance and duration in the future. Over the last decades, however, countless beneficial microbes have been described that can mitigate the effect of drought stress on plants, providing the plant with an increased tolerance. The assemblage of these beneficial microbes is largely unknown. The aim of this proposed research is to investigate the drought-induced promotion of microbes mitigating drought stress in the *Arabidopsis thaliana* rhizosphere and endosphere in natural soils. First, new experimental methods will be optimized for examining plant-induced microbiome composition changes under drought stress, bypassing the direct effects of drought on the soil community. Then, the change in microbiome composition is visualized and metabolite fingerprinting is performed in search of the molecular signal promoting specific microbes. Finally, the long-term effect covering several generations called the soil-borne legacy, is examined. The newly gathered knowledge will help future research and crop cultivation in terms of drought tolerance and beneficial microbe selection.

B.1.3 Layman’s summary

Droogte is een van vele abiotische stressen die planten te verduren kunnen krijgen. Het beïnvloedt de groei, gezondheid en opbrengst van een plant en kan uiteindelijk leiden tot sterfte. Al enkele decennia geleden werd ontdekt dat microben kunnen helpen tegen de effecten van droogte. Sindsdien zijn er talloze bacteriën, schimmels en virussen ontdekt die de plant helpen te overleven tijdens perioden met weinig beschikbaar water. Deze microben leven in of rond de wortels van de plant, waar ze leven van nutriënten uit de bodem en stoffen die worden uitgescheiden door de plant zelf. Over de precieze manier waarop de plant en deze micro-organismen samenwerken is echter nog maar weinig bekend. In dit voorstel gaan we onderzoeken of de plant *Arabidopsis thaliana* bepaalde microben, die kunnen helpen bij het overleven van droogte, actief kan aantrekken en deze kan stimuleren ten opzichte van andere microben. Dit doen we door eerst te kijken naar welke microben worden gestimuleerd in de grond rond de wortels (de rhizosfeer) en in de wortels (de endosfeer). Daarna gaan we op zoek naar het signaal dat de planten geven in en rond de wortels om deze microben aan te trekken en te stimuleren. Vervolgens kijken we of er een lange termijn effect bestaat wat over meerdere generaties opgebouwd wordt, om zo steeds de nieuwste generatie beter voor te bereiden op mogelijke periodes van droogte.

B.1.4 Keywords

Soil-Borne Legacy - Drought tolerance - Beneficial microbes - Microbiome composition - Rhizosphere & endosphere

B.2 SCIENTIFIC PROPOSAL

B.2.1 Research topic

Objectives

Our main objective is to discover **whether drought stress induces a shift in microbial composition** in the rhizosphere and endosphere, **does this shift promote drought tolerance** in *Arabidopsis thaliana* and **how this shift is controlled** from within the plant.

Key objectives:

- 1) Isolate and identify promoted microbes in the rhizosphere and endosphere of *A. thaliana* exposed to drought conditions
- 2) Analyze the changes in the *A. thaliana* root transcriptome and root exudates under drought stress
- 3) Test for the presence of the Soil-Borne Legacy induced by drought stress in the *A. thaliana* root system

Scientific background

Drought stress and beneficial microbes

Drought is one of many abiotic stresses impacting plant growth and health. Many environmental factors can cause drought conditions, i.e., high temperatures, high light intensity and dry winds (Dia, 2011). All these factors increase evaporation of water from both the soil and plants directly, having a double negative effect on the water availability for crucial plant processes. Plants experience an inaccessibility of water due to a low soil moisture or an imbalance in water uptake and water loss, occurring when the transpiration rate from leaf surfaces is higher than the water uptake by the roots (Salehi-Lisar & Bakhshayeshan-Agdam, 2016).

As drought is a multidimensional stress for plants, it can severely affect all kinds of processes. First and foremost, it influences growth and development by affecting processes including cell division, elongation and differentiation (Fig. 1). Leaf anatomy is altered, resulting in smaller leaves with less stomata and cutinization (Fig. 2). Together with an increase in premature leaf senescence, these alterations result in a reduction of net photosynthesis in plants exposed to drought stress (Shao *et al.*, 2008). The lowered photosynthesis rate, partly due to a decrease of chlorophyll content, results in a size reduction of the whole plant. In contrast with the size reduction of above-ground parts, root systems typically increase in size under drought stress in order to create a higher capacity for water uptake (Salehi-Lisar & Bakhshayeshan-Agdam, 2016). The fastest effect is the reduction of relative water content (RWC), which leads to closing of the stomata and consequently to a decrease in transpiration rate. Although less transpiration seems like a positive factor during drought stress, it results in an increase in leaf temperature. This can cause denaturation of proteins and changes in membrane flexibility (Bhargava & Sawant, 2013). Additionally, decreasing water content results in a decrease in

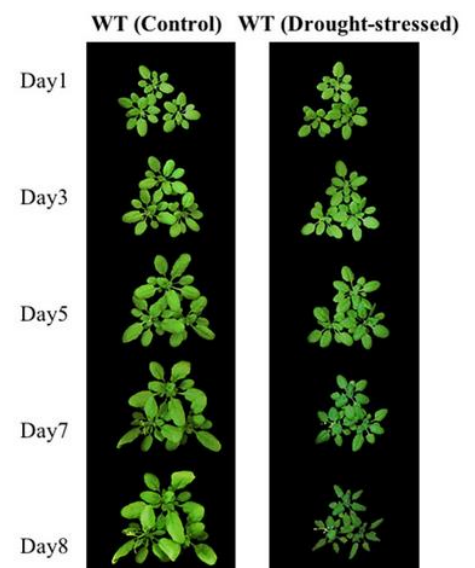


Figure 1: Effect of drought on plant growth. Edited from Yao *et al.* (2018).

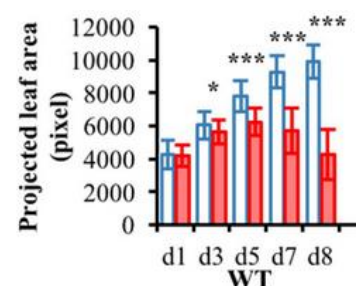


Figure 2: Effect of drought on projected leaf area. Edited from Yao *et al.* (2018).

total nutrient availability in soils, lowers the uptake of nutrients by roots and consequently reduces concentrations in tissues (Farooq *et al.*, 2012). Further, decreased water availability affects the hormonal balance, synthesis of a wide scale of proteins and amino acids and leads to the accumulation of reactive oxygen species, alongside of a multitude of other impacts (Salehi-Lisar & Bakhshayeshan-Agdam, 2016).

Over decades of research on the effects of drought on plant health and yield, many plant responses have been uncovered and thoroughly described. Besides direct effects from and within the plant, microbes were found to play an important part in mitigating drought stress. Microorganisms in all sorts and sizes colonize the plant's rhizosphere and endosphere (Fig. 3), interacting with other microbes and the host plant. Interactions vary from pathogenic to beneficial, creating a complex pool of multifaceted life. Beneficial microbes can support the plant in its fight against both abiotic and biotic stresses in a wide variety of ways.

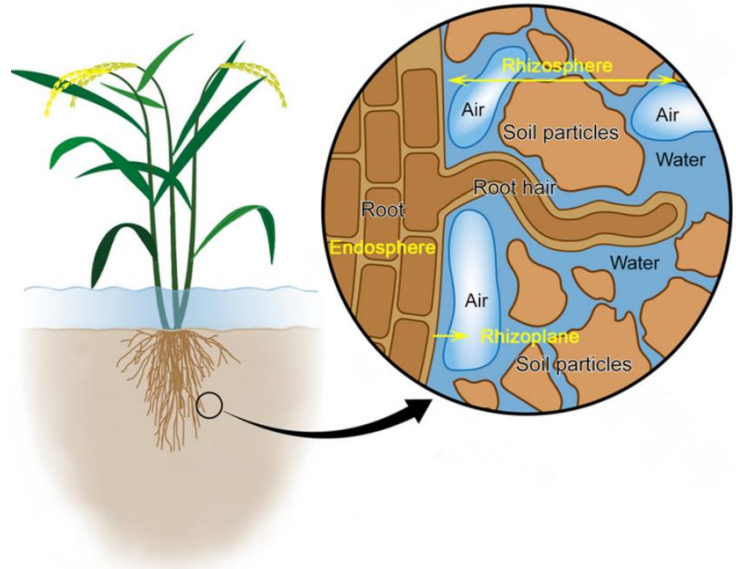


Figure 3: Endosphere and rhizosphere. Edited from Ding *et al.* (2019).

Concerning drought stress, beneficial factors which can be traced back to microbes include the production of several plant-growth promoting substances and biocontrol agents, increased nutrient availability, changed soil structure, pH and oxygen availability (Finkel *et al.*, 2017; Ullah *et al.*, 2018). The ability to mitigate drought stress in plants is not limited to a specific selection of microbes, as numerous members of a wide variety of fungal and bacterial families (and even some viruses) were reported to display this effect (Kim *et al.*, 2012).

The Soil-Borne Legacy

Recent studies showed changes in the composition of the rhizosphere microbiome of *Arabidopsis thaliana* (hereafter *Arabidopsis*) upon infection of leaves with a foliar pathogen. When leaves were infected with the biotrophic pathogen *Hyaloperonospora arabidopsidis*, three bacterial isolates were actively promoted in the rhizosphere. Upon further investigation, these bacterial strains were reported to act synergistically in biofilm formation and had beneficial effects on the plant, including plant growth and resistance against the pathogen which induced the response in the first place (Fig. 4; Berendsen *et al.*, 2018; Yuan *et al.*, 2018). Moreover, research showed that the rhizosphere community changes favored not only the first, but even next generations. This phenomenon was named the "soil-borne legacy" (Bakker *et al.*, 2018).

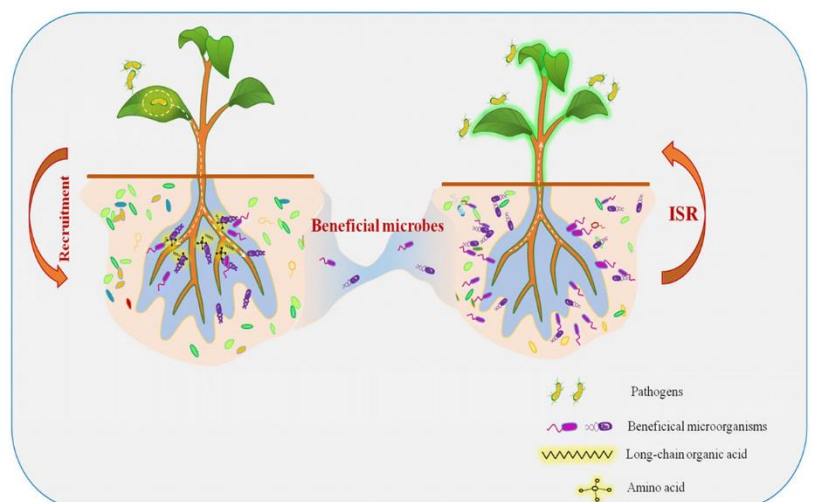


Figure 4: Mechanistic model of soil-borne legacies by foliar pathogens. Recruitment induced by pathogen infection causes increased resistance through induced systemic resistance (ISR). Edited from Yuan *et al.* (2018).

Exciting future?

Combining the knowledge on both the microbes mitigating drought stress and the soil-borne legacy, the theory of a similar phenomenon induced by drought stress rises. Does drought stress induce a soil-borne legacy in the rhizosphere and endosphere of *Arabidopsis* mitigating the negative effects of drought on plant fitness? The proposed research contains experiments in search of an active change in rhizosphere and endosphere microbiome composition induced by drought stress, favoring both the first and/or later generations in terms of drought tolerance. Several studies have shown a (long-lasting) effect of drought on the rhizosphere and endosphere community composition either directly or via plant alterations. Both Fitzpatrick *et al.* (2018) and Santos-Medellín *et al.* (2021) described an enrichment of specific microbial families i.e., *Streptomyces* (linked to growth promotion) in rice plants and angiosperms in general, respectively. However, the exact interaction remains largely unknown. With the duration and abundance of drought periods expected to increase in the coming decades due to climate change (Field & Barros, 2014), with the impact on agriculture increasing in a similar fashion, uncovering such a phenomenon would be ground-breaking, providing us knowledge on “drought-stress suppressive soils” and opening up new applications of beneficial plant-microbe interactions in the future.

B.2.2 Approach

The proposed research is divided in four (partly overlapping) phases: a general optimization phase and three other phases all focusing on a different key objective. First, it is crucial to find the most optimal experimental set-ups to investigate our main and sub-questions. In **Work package 1**, one of the first and major problems is how to bypass the direct effect of the drought treatment on the microbial communities and soil properties. The goal of this research is to establish the scope with which plants can actively impact the microbial community composition under drought stress and the direct effects of a shortage of water is expected to cloud this response (Preece *et al.*, 2011; Sheik *et al.*, 2011). Several experimental set-ups will be tested, including split-root systems and artificial drought responses. A split-root system might give insight on the effect of drought on the microbial community. As the plant is not heavily subjected to drought due to the watered control pot in which half of the root system had developed, the plant induced changes in microbiome composition in the drought subjected pot should be neglectable. It is not possible to monitor the effect of drought on microbial communities in soil without plant roots, as the abundance of microbes decreases rapidly. Using an artificial method (e.g., mannitol or polyethylene glycol 6000 treatment; Hohl & Schopfer, 1991) to simulate drought stress in the plant (Earl, 2003; Macková *et al.*, 2013), the plant-induced response might be visible without the clouding of the direct drought effect. However, we expect it to be difficult to design an experimental set-up completely bypassing the direct effect of drought on microbial life, it is well possible that multiple different experimental set-ups have to be used to approach the intended results as close as possible, combining data from the different experiments and drawing conclusions from the joint results. Furthermore, work package 1 is meant to answer technical questions including the optimal duration and abundance of drought periods, growth period of *Arabidopsis* and ideal fitness measurements for *Arabidopsis* after drought stress. Initially, drought is meant to be subjected by withholding water completely for a certain amount of time, after which the watering regime will return to its normal amount. Soil water content is measured for monitoring of the severity of the drought period. If this method is somehow determined to be suboptimal, other methods will be tested.

WP.1: 'Drought stress and plant-microbe interactions'-protocol and experimental optimization

a. Determine and optimize the most suitable experimental set-up to investigate active promotion of beneficial microbes in the *Arabidopsis* rhizosphere and endosphere under drought stress

- i. Analyze the suitability of different experimental set-ups for rhizosphere and endosphere microbiome composition under drought stress (100 g soil per pot, 10 replicates per treatment):
 - a. 'Normal' experimental set-up consisting of a drought treatment and a control treatment with normal watering.
 - b. 'Split-root system' experiment consisting of a drought treatment and a control treatment per plant, with its root system divided over two pots.
 - c. 'Drought simulation' experiment, with an artificial drought response replacing the actual drought treatment.
- ii. Select the optimal growth period for *Arabidopsis*, based on the time from germination to full grown seed producing plant.

b. Analyze optimal duration and abundance of drought periods and growing time for *Arabidopsis*

- i. Determine what is the length of an optimal drought period for active promotion of beneficial microbes, based on the severity of the drought, the survival rate of the plants and the impact on the soil properties.
- ii. Select an optimal abundance of drought periods per growth period based on the optimal drought period duration.
- iii. Consider whether microbiome analysis is best to perform once at the end of the growth period, or after every period of drought (in the case of multiple drought periods).

c. Investigate most ideal fitness measurements for *Arabidopsis* after drought stress

- i. Analyze the suitability of different fitness measurements for *Arabidopsis* after being subjected to drought stress:
 - a. Plant growth (shoot fresh and dry weight, leaf area and root mass)
 - b. Photosynthetic performance (gas exchange, chlorophyll fluorescence)
 - c. Seed production (number and size)
 - d. Flowering time
 - e. Root architecture (imaged and measured at the end point)

Outcome: Experimental conditions for drought selected for WP.2-4.

Work packages 2 contains the first experiment monitoring the promotion of beneficial microbes by the plant to limit the damage of drought stress. *Arabidopsis thaliana* Col-0 is grown in pots with natural soil harboring natural communities of microbial life. The soil is obtained from a natural field site at Reijerscamp (the Netherlands) that supports an abundant endemic *Arabidopsis* population. The soil from this site was successfully used for earlier research by the group Plant-Microbe Interactions (Berendsen *et al.*, 2018). With an experimental set-up based on the results of Work package 1, rhizosphere and endosphere samples are collected, whereafter DNA is isolated (using DNA isolation kits specific for rhizosphere, bulk soil and endosphere samples) and analyzed on community composition using PhyloChip analysis (Second Genome Inc.; San Francisco, USA). Interesting individuals are isolated and identified, whereafter they are investigated on a possible link to drought tolerance and increased fitness in primary and secondary literature. Fitness experiments in sterilized soil with the selected isolates added are performed to monitor beneficial effects on drought tolerance,

plant growth and health, confirming the link between the selected microbes and drought tolerance, plant growth and health in Arabidopsis.

WP.2: Promotion of beneficial microbes in the rhizosphere and endosphere under drought stress

a. Analyze microbiome composition in rhizosphere and endosphere for promoted microbes

- i. Grow Arabidopsis Col-0 in natural soil (collected from Reijerscamp nature reserve, the Netherlands). Subject part of the plants to drought (as selected in WP.1).
- ii. Harvest the plants. Rinse the soil off the roots and collect for rhizosphere community analysis. Cut aboveground plant parts of and collect roots for endosphere community analysis. Take bulk soil samples as control. Surface-sterilize roots and grind with mortar and pestle (or macerate).
- iii. Isolate total genomic DNA from the rhizosphere samples, bulk soil samples and endosphere samples using DNA isolation kits for rhizosphere, bulk soil and endosphere samples and analyze bacterial and fungal community by amplicon sequencing.

b. Select and identify interesting isolates

- i. Plate suspensions of the rhizosphere, endosphere and bulk soil samples on a large number of different media, suitable for fungi and/or bacteria. Select fungi and bacteria with unique morphologies and store pure single cultures.
- ii. Amplify the 16S rRNA gene (bacteria) and 28S rRNA (fungi) with PCR using specific primers. Sequence PCR products with (External party).
- iii. Determine taxonomy of isolates through the Sequence Match function of the ribosomal database project. Perform BOX-PCR to determine if the isolated strains are identical on strain level.
- iv. Select interesting isolates based on PhyloChip analysis data and principal component analysis and perform whole-genome sequencing.

c. Test if selected isolates promote drought tolerance and increased fitness

- i. Search primary and secondary literature for links between promoted isolates and/or families, and drought tolerance in plants.
- ii. Perform fitness experiment in sterilized soil with selected isolates to monitor beneficial effects on drought tolerance.
- iii. Perform drought tolerance experiment in sterilized soil with selected isolates to monitor beneficial effects on plant growth and health.

Work package 3 focusses on finding the signal pathway and molecule(s) achieving the microbial community composition changes in the rhizosphere and endosphere (Gargallo-Garriga, 2018). Through metabolite fingerprinting of root exudates and root transcriptomics of Arabidopsis under drought stress, we hope to find the molecular signal responsible for the promotion of beneficial microbes (Strehmel *et al.*, 2014). Root transcriptomics are investigated for an effect on the endospheric communities, as both root exudates and transcriptomics might give insight regarding changes in the rhizosphere communities. Selection of interesting metabolites, other molecular signals and genes is made based on in- or decreases in abundance or expression between drought- and control-treated roots, analyzed by e.g., 1D-SOM clustering and prototype assignment (Stringlis *et al.*, 2018). To confirm the link between the plant-induced signal and the promoted microbes, we will perform experiments

monitoring the effect of these metabolites, other molecular signals or genes on bacterial and fungal isolates selected in WP.2.

WP.3: Changes in root metabolic activity and root exudates under drought stress

a. Metabolite fingerprinting of root exudates under drought stress

- i. Perform drought experiment in natural soil and analyze root exudates for changes between normal conditions and drought conditions (protocol based on Williams *et al.* (2019)).

b. Monitor root transcriptomics under drought stress

- i. Perform drought experiment in natural soil and analyze root transcriptomics for changes between normal conditions and drought conditions (protocol based on Fizames *et al.* (2004)).

c. Link signals to promotion of beneficial microbes

- i. Monitoring effects of selected metabolites, other molecular signals or genes on selected bacterial and fungal isolates.

Work package 4 contains a long-term soil-borne legacy experiment, monitoring the changes in community composition over several generations of Arabidopsis. This experiment is based on the protocol of Vismans *et al.*, (2021) and research carried out by Berendsen *et al.* (2018), where they performed a similar experiment to monitor disease-induced soil-borne legacy. To minimize the effect of drought on soil properties, microbial communities will be collected at the end of each generation and transplanted to newly sterilized soil with the initial properties in which the next generation will be grown. The experiment will contain a control treatment in which the next generation is grown in the same soil as the generation before. In this control we can determine the transferring effect drought has on the soil properties and consequently on the microbial communities.

WP.4: The Soil-Borne Legacy induced by drought stress

a. Multi-generation drought experiment monitoring changes in rhizosphere and endosphere microbial composition

- i. Perform a drought experiment in natural soil for six generations and analyze microbial compositions of rhizosphere and endosphere for changes between normal conditions and drought conditions, visualizing the Soil-Borne Legacy.

Table 1: Timetable of the project

WP	Objective	Specific activity	Year 1	Year 2	Year 3
WP.1 (a)	Experimental set-ups	Experimental set-ups (a-i)	■		
		Growth period (a-ii)		■	
WP.1 (b)	Drought periods (DP)	DP Duration (b-i)	■		
		DP Abundance (b-ii)		■	
WP.1 (c)	Fitness measurements		■		
WP.2 (a)	Microbiome analysis	Growth experiment (a-i)		■	
		DNA isolation (a-ii,-iii)		■	
WP.2 (b,c)	Isolate processing			■	
		Manuscript writing (WP.1-2)		■	
WP.3 (a)	Root exudates			■	
WP.3 (b)	Root transcriptomics			■	
WP.3 (c)	Microbe-signal linkage			■	
WP.4	Soil-Borne Legacy			■	
		Manuscript writing (WP.3-4)			■

B.2.3 Feasibility / Risk assessment

The proposed research is designed to test microbiome changes using newly described methods, investigated and optimized in work package 1. As these novel methods are based on existing experimental set-ups performed in previous research, we do not predict major difficulties in the feasibility and reliability of the new protocols. However, if problems occur concerning the experimental set-ups, we always have the possibility to perform the experiments of work packages 2-4 with techniques used in earlier research. The results might suffer in terms of validity, but we believe they would still contribute to the general pool of knowledge in this research field.

In work package 2, we assume to find a plant-induced microbiome composition change under drought conditions. If this active promotion of beneficials is not found, we can still change the course of the rest of the project. The experiments in search for the molecular signal from the plant (WP.3) and the monitoring of the long-term soil-borne legacy (WP.4) will be scratched and replaced by research concerning drought recovery and microbiome composition. As an alternative, we would like to investigate whether the direct effect of drought on the microbiome composition affect the recovery ability of the plant after a drought period, a hypothesis earlier presented by Fitzpatrick *et al*, (2018). In brief, we would examine the recovery of *Arabidopsis* after a period of drought if the rhizosphere and endosphere microbiome was also subjected to that drought, versus the microbiome composition at the start of the experiment. This could give us insight on the mutualistic properties of drought-tolerant microorganisms and survival of beneficial microbes differently than examined in our work package 1, possibly favored by the plant via other mechanisms. Moreover, more time and energy would be spent on optimizing experimental set-ups specialized for the research on plant-induced microbiome composition changes under drought stress.

As this research would be a combined effort of the research groups Plant-Microbe Interactions and Plant Ecophysiology, all the experience and equipment to perform both microbiome and drought experiments is present.

B.2.4 Scientific and societal impact

The direct effect of drought on soil properties and subsequently on the microbiome is one of the major difficulties when researching plant-induced microbiome changes under drought conditions. With work package 1, we investigate the best way to bypass these struggles and take a step towards a new protocol for this type of research on the *Arabidopsis* rhizosphere and endosphere. Future research can benefit from this new protocol, increasing the reliability of experiments and knowledge both long- and short-term. Furthermore, our proposed research will give more insight in the process of microbe promotion in general and microbe promotion under drought stress. As short-term effect, new questions will arise due to our research, enabling new projects on this subject, ultimately contributing to the step-by-step unravelling the different processes and pathways at play during microbe promotion. Long-term wise, this research might contribute to a change in agriculture, utilizing more and more of the beneficial effects that microorganisms have to offer. With a better knowledge of drought tolerance and microbe promotion, controlled agricultural systems might be optimized in terms of water use and drought stress, boosting yields. Further, linking microbe promotion to plant genes can enable plant breeding on these specific traits, improving our crops from inside out.

A more practical implication is the use of our results in the selection of desired genetic traits in crops, in which we expect plant breeders to be highly interested. The attraction and promotion of beneficial microbes in natural soils is a highly desired trait, letting the plant improve itself in terms of

growth and tolerance to both biotic and abiotic stresses. Furthermore, results from the root exudates and transcriptome experiments are of added value to molecular plant biology. Both the cooperating research groups Plant Ecophysiology and Plant-Microbe Interactions benefit from this proposed research.

B.2.5 Ethical considerations

As our proposed research consists solely of natural systems and organisms and does not include the use of genetic modified organisms, we foresee no ethical issues.

B.2.6 Literature/references

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