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Evaluation of stress protecting agents (SPAs) for crops

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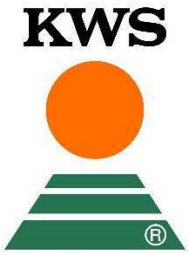

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Abstract

Agriculture is confronted with many problems in cultivation and harvesting due to climate changes. Crop yield is often reduced by extreme environmental conditions e.g. desiccation and heat. As it is known from previous studies, bacteria can promote plant growth and health and act as stress protecting agents in extreme conditions. Therefore, the goal of this study was to create bacterial consortia with stress protecting properties as biological seed treatment for maize, sorghum and oilseed rape with focus on sustainability and promoting diversity on host plants.

For consortia assembly preselected environmental strains were phenotypically characterized for beneficial abilities e.g. desiccation resistance, interaction with other bacteria, quorum sensing and antagonistic effects via volatile organic compound production. Their impact on germination, growth promotion and stress protection of the host plants in single and consortia application was observed in greenhouse trials. Seed and root colonization was tested using culture-dependent methods. Colonization of roots was also verified performing fluorescence *in situ* hybridization with family specific probes and confocal laser scanning microscopy for oilseed rape. Furthermore, to detect the influence of the particular stress protecting *Stenotrophomonas rhizophila* strain P69 on the microbial community, amplicon sequencing targeting bacteria and fungi of rhizosphere samples of treated and untreated maize plants grown on two different located fields was performed.

Bacterial characterization revealed many different characteristics, which might be useful in plant consortium interactions, like the ability to swim, swarm, inhibit other bacteria or produce acetylated homoserine lactones for quorum sensing. All strains showed high antagonistic potential in volatile organic compound assays against at least two out of four fungal phytopathogens tested. In greenhouse trials, germination and leaf formation was significantly improved for consortium treated oilseed rape seeds in germination filters. For consortium treated maize plants grown in germination pouches, fresh weight of green parts and roots was significantly increased. Significantly higher dry weight was observed for consortium treated sorghum plants grown in germination filters. Under extreme dehydration stress, consortium treated sorghum plants were found to have thicker stems and increased biomass production.

Bioinformatic analyses of bacterial and fungal amplicons of field grown maize rhizosphere samples showed strong differences between locations. Analysis showed, that *Stenotrophomonas rhizophila* P69 had no influence on the α diversity of microbial communities. This study showed positive effects of consortia on different plants and sets a headstone for further optimizations and future field trials.

Kurzfassung

Durch den Klimawandel ist die Landwirtschaft zunehmend mit Problemen wie Hitze und Trockenheit konfrontiert. Dies ist häufig mit Ertragsverlusten verbunden. In einigen Studien konnten Bakterien vermittelte, wachstumsfördernde und vor Stress schützende Effekte an Pflanzen gezeigt werden. Das Ziel dieser Studie war es daher, bakterielle Konsortien für eine Saatgut-Behandlung mit vor Stress schützender Aktivität für Mais, Sorghum und Raps zu entwickeln. Dabei sollte der Fokus auf Nachhaltigkeit und eine Förderung der Diversität an der Wirtspflanze gelegt werden.

Für die Erstellung eines Konsortiums wurden vorausgewählte Bakterien auf positive Eigenschaften wie Austrocknungsresistenz, Interaktion mit anderen Bakterien, Quorum sensing und antagonistische Effekte durch flüchtige organische Substanzen, getestet. Weiters wurde der Einfluss auf Keimung, Pflanzenwachstum und auf ihre Fähigkeit, Pflanzen vor Stress zu schützen, in Gewächshausversuchen überprüft. Die Kolonisierung der Samen und Wurzeln wurde mittels kultivierungsabhängigen Verfahren nachgewiesen. Für Raps wurde die Besiedelung der Wurzeln zudem unter der Verwendung spezifischer Sonden mittels Fluoreszenz *in situ* Hybridisierung und konfokaler Laser-Rastermikroskopie überprüft. Des Weiteren wurde der Einfluss des vor Stress schützenden Umweltstammes *Stenotrophomonas rhizophila* P69 auf die Zusammensetzung der Bakterien- und Pilzpopulation in Maisrhizosphären an zwei Standorten mittels Amplikonsequenzierung evaluiert.

Bei der Charakterisierung der Bakterienstämme wurden potentiell hilfreiche Eigenschaften, für eine nützliche Konsortium-Pflanzen-Interaktion detektiert. Darunter fanden sich beispielsweise die Fähigkeit zu schwimmen, schwärmen, andere Bakterien zu inhibieren oder Acyl-Homoserin-Lactone für Quorum sensing zu produzieren. Alle Stämme wiesen ein hohes antagonistisches Potential durch flüchtige organische Substanzen gegen mindestens zwei von vier pilzlichen Pflanzenpathogenen auf. Durch die Konsortium-Behandlung konnte die Biomasseproduktion und die Blattanzahl von Raps in Keimfiltern signifikant erhöht werden. Bei der Kultivierung von Mais in Keimtaschen zeigte sich ein signifikant gesteigertes Frischgewicht der Wurzeln und oberirdischen Pflanzenteile der mit Konsortium behandelten Pflanzen. Konsortium behandelter Sorghum wies ein signifikant höheres Trockengewicht nach Kultivierung in Keimfiltern auf. Des Weiteren wurden unter extremen Trockenstress eine erhöhte Biomasseproduktion und dickere Stämme bei Konsortium-behandeltem Sorghum nachgewiesen.

Bei den Feldversuchen wurden Unterschiede der Bakterien- und Pilzpopulationen zwischen den beiden Orten durch bioinformatische Analysen der Amplikons vorgefunden. Die Analyse zeigte zudem, dass *Stenotrophomonas rhizophila* P69 keinen Einfluss auf die α Diversität der mikrobiellen Gemeinschaften hatte. Diese Studie zeigt erste positive Effekte von Konsortien auf verschiedene Wirtspflanzen und legt somit einen Grundstein für weitere Optimierungen und Feldversuche.

1 Introduction

Due to climate changes, agriculture all over the world is increasingly confronted with problems like heatwaves, drought and desiccation (Newton *et al.*, 2011, Lesk *et al.*, 2016). Further, climatologists warn, that extreme weather tendencies will increase in the future (Meehl and Tebaldi, 2004, Schär *et al.*, 2004, Battisti and Naylor, 2009). Heavy changes or variability in climate and weather also causes yield loss. For example, Ray *et al.* (2015) showed, that climate variability caused 41-49% of yield variability for maize in the United States, France and Italy. Therefore, climate changes come hand in hand with the risk of food insecurity, especially in developing countries (Battisti and Naylor, 2009). Further, climate change might thereby not only have a huge impact on human health but according to Shaw (2009), will impair plant health and promote occurrence of plant pathogens.

To overcome the problem, plant-associated microorganisms, their important role for the environment and plants as well as possible biotechnological applications for environmental and plant protection take center stage in current research. Based on scientific progress in plant microbiome research, plants are increasingly recognized as metaorganisms, harboring specific microbes in various habitats like seeds, rhizospheres, phyllospheres or endospheres with functions improving plant health (Berg *et al.*, 2015). Researchers also showed that a plant specific core microbiome is transmitted to the next generation e.g. within the seeds for maize (Johnston-Monje and Raizada, 2011) or from the sporophytes to the gametophyte of *Sphagnum* moss (Bragina *et al.*, 2013). As secreted root exudates attract a highly specific diversity of microbiota, symbioses are promoted by the plants themselves (Badri and Vivanco, 2009). These microbiota can fulfill many beneficial functions like starting the germination process or protecting the host plant against pathogens (Berg *et al.*, 2015). Several studies have shown that those beneficial environmental microorganisms can be used as biological control agents (BCAs) for plant protection against biotic stresses like fungal phytopathogens (Krechel *et al.*, 2002, Haggag and Timmusk, 2008, Joo *et al.*, 2015). Therefore, they represent a sustainable solution to reduce the use of environment and health damaging pesticides like fungicides, which are under suspicion of causing cross-resistances in human associated fungal pathogens (Verweij *et al.*, 2009). As reviewed by Berg (2009), bacteria harbor several different mechanisms for plant protection and plant growth promotion (PGP). Some bacteria, so called stress protecting agents (SPAs) can help overcome abiotic stresses like salt stress due to salinated soil (Mayak *et al.*, 2004, Alavi *et al.*, 2013) or heat, drought and desiccation stress (Yang *et al.*, 2009, Naveed *et al.*, 2014, Rolli *et al.*, 2015). Furthermore, some bacteria harbor both, biological control and stress protecting effects and therefore can protect the plants from both, biotic and abiotic stresses (Egamberdieva *et al.*, 2011, Fürnkranz *et al.*, 2012). Also, plant defense responses, which are often induced by pathogens in form of so called systemic acquired resistances (SAR), can be induced by beneficial microbes via volatiles and thereby be useful to stimulate protective or resistance effects of the plants. This process is called induced systemic resistance (ISR) (Lugtenberg and Kamilova, 2009, Farag *et al.*, 2013).

Although that several studies present successful single strain treatments for plant stress protection, due to the progressive incrementalism of understanding and knowledge about plant-associated microbiomes

and microbe-plant interactions, the application of special assembled microbial consortia should be taken into consideration for sustainable agriculture (Berg *et al*., 2013). Using more than one bacterial strain, compatible consortia members can be chosen due to the required abilities for the respective situation and plant species, and thereby a broad range of protective or antagonistic tools can be included. Some studies already reported positive effects of microbial consortia and their protective abilities under greenhouse conditions e.g. regulation of stress response under drought stress in cucumber plants (Wang *et al*., 2012) or enhanced protection against pathogens in tomatoes or peas (Kannan and Sureendar, 2009, Jain *et al*., 2012). Also, plant growth promoting and antagonistic effects against *Rhizoctonia solani* of microbial consortium treated sugar beet cultivars was shown in field trials (Zachow personal communication).

The aim of this study was to characterize environmental strains, and develop and test consortia for biotic and abiotic stress protection of maize, sorghum and oilseed rape. A strategic overview is presented in Figure 1. Pre-selected environmental strains were tested for several abilities, which could be useful and beneficial in microbe-microbe or plant-microbe interactions. Screening and characterization included evaluation of quorum sensing, swimming and swarming abilities, as well as survival after dehydration for a long period of time. Also, interaction patterns between two bacterial strains were observed. Furthermore, strains were tested for their biocontrol ability, and therefore, for their ability to inhibit fungal pathogens via production of volatile organic compounds. In the next step, single strains were tested for their effect on host plants. Thus, abundance on seeds and roots, as well as influence on germination, leaf formation and biomass was examined. After consortium assembly with focus on maintaining and promoting diversity of the host plants, consortia were tested for their impact on plants and compared to the results of single strain testing. Additionally, colonization of the roots by the bacterial consortium was detected using fluorescent *in situ* hybridization and confocal laser scanning microscopy for oilseed rape. Further, consortia were tested for their ability of stress protection in *ad planta* dehydration experiments in the greenhouse. To evaluate the impact of a single SPA strain on the microbial rhizosphere community, *Stenotrophomonas rhizophila* P69 was applied to maize seeds used in two different located field trials in Austria. The culture-independent amplicon sequencing was used to identify shifts in taxonomic diversity of bacterial and fungal rhizosphere microbiomes of non-treated and treated seeds of the same sites.

The objectives of the study were: (i) characterization of environmental bacteria and development of beneficial consortia, (ii) assessment of advantages of consortium seed treatments compared to single strain treatments in cultivation of maize, sorghum and oilseed rape, (iii) stress protecting abilities of consortia during dehydration stress under greenhouse conditions and (iv) influence of the SPA *Stenotrophomonas rhizophila* P69 seed treatment on the microbiome communities of maize rhizospheres in field trials.

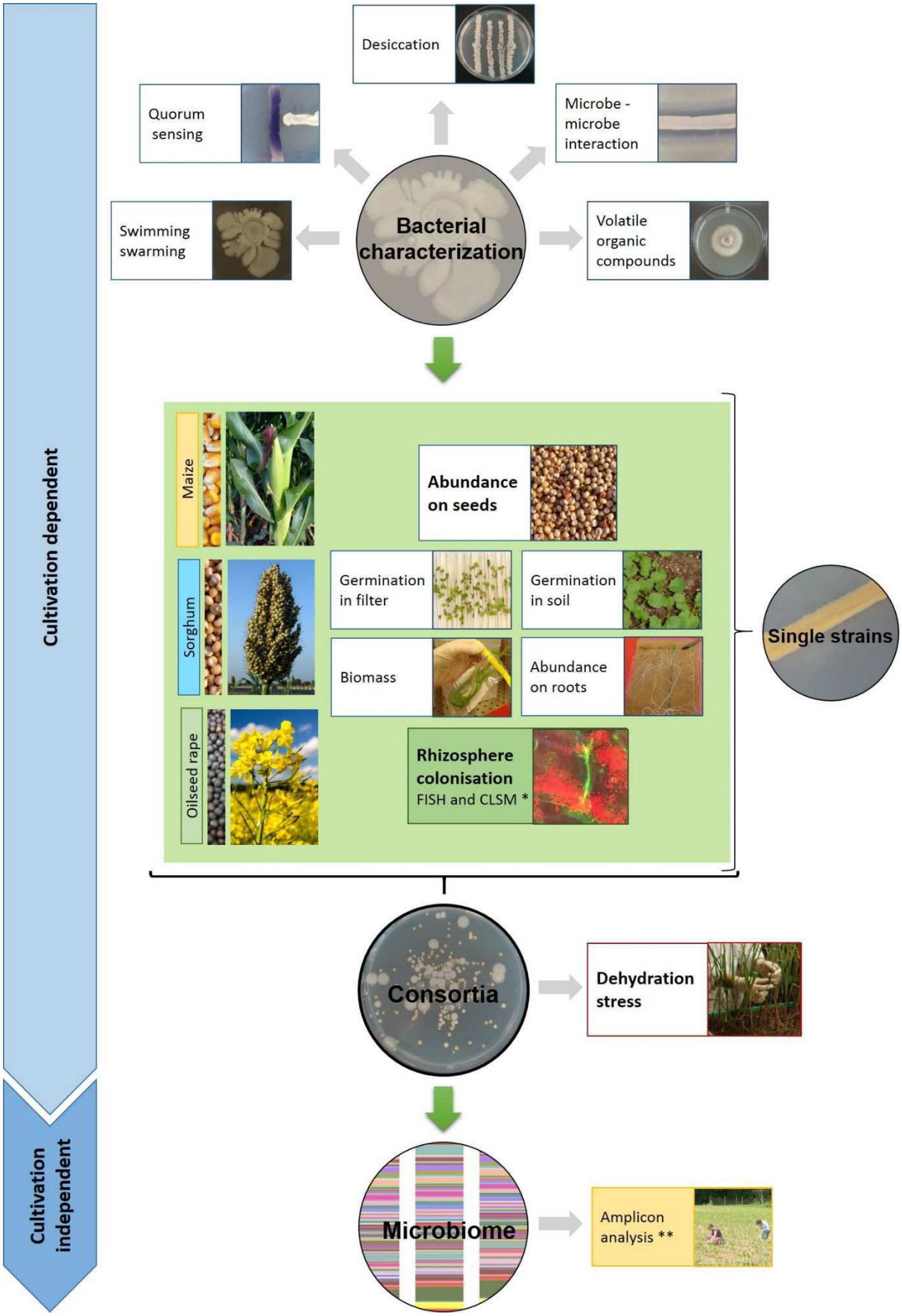

Figure 1: Strategic overview of experiments performed in this study.
* only for oilseed rape consortium
** maize rhizospheres treated with *S. rhizophila* P69, field trials

2 Material and methods

2.1 Bacterial strains

All strains considered for plant protecting consortia were isolated of natural habitats as shown in Table 1. As consortia for the *Poaceae* sorghum and maize three strains of the microbial strain collection of the Institute of Environmental Biotechnology (Graz University of Technology, Graz, Austria) were chosen. The consortium for sorghum only included the two *Stenotrophomonas rhizophila* strains e-p17 and P69. For the oilseed rape consortium assembly, eight strains from the strain collection of the study of Zachow *et al.* (2013) and the antagonistic endophyte *Pseudomonas poae* RE*1-1-14 were preselected due to their beneficial properties e.g. phosphate solubilization, nitrogen fixation, the production of siderophores, fast germination of oilseed rape and enzyme activity (Table 1).

Table 1: List of bacterial strains used for consortia assembly, containing strain abbreviations, species, origins and references. A – Austria, G - Germany

Abb.	Species	Origin	Reference
Strains considered for maize and sorghum consortia, * maize only			
B2g	*Bacillus subtilis**	Rhizosphere of oilseed rape, Biestow (G)	Marten *et al.*, 2000
e-p17	*Stenotrophomonas rhizophila*	Geocaulosphere of potato, Lüsewitz (G)	Minkwitz and Berg, 2001 Wolf *et al.*, 2002
P69 Syn.: e-p10, DSM14405^T	*Stenotrophomonas rhizophila*	Rhizosphere of oilseed rape, Rostock (G)	Minkwitz and Berg, 2001 Wolf *et al.*, 2002

Table 1: *Cont.*

Abb.	Species	Origin	Reference
Strains considered for oilseed rape consortium			
MF1-2-4	*Arthrobacter nitroguajacolicus*	Maize treated with lichen, Graz (A)	Zachow *et al*., 2013
RE*1-1-14	*Pseudomonas poae*	Endosphere of sugar beet, Hilprechtshausen (G)	Müller *et al*., 2013
RM1-1-4	*Pseudomonas corrugata*	Oilseed rape treated with moss, Graz (A)	
RM2-3-1	*Pseudomonas mediterranea*	Oilseed rape treated with moss, Graz (A)	
RP2-2-4	*Pseudomonas grimontii*	Oilseed rape treated with primrose, Graz (A)	
SF1-3-1	*Serratia* sp.	Sorghum treated with lichen, Graz (A)	Zachow *et al*., 2013
SF2-2-2	*Pseudomonas fluorescens*	Sorghum treated with lichen, Graz (A)	
ZM2-1-1	*Chryseobacterium wanjuense*	Sugar beet treated with moss, Graz (A)	
ZP2-1-3	*Bacillus* sp.	Sugar beet treated with primrose, Graz (A)	

2.2 Phenotype characterization of bacteria

Microbe-microbe interaction

For the microbe-microbe interaction tests bacterial strains were cultured overnight in 100 mL flasks containing 50 mL of nutrient broth II (SIFIN, Berlin, Germany) at 30 °C under agitation. Optical density 600 (OD_{600}) of the overnight cultures (ONC) was measured and 15 mL NBII media with OD_{600} 0.4 was prepared for each strain. Nutrient agar (NA, per liter: 15 g nutrient broth II and 15 g agar-agar) was prepared and cooled to 50 °C for 20 to 25 min. The bacterial suspension was added to 240 mL of agar. The agar was homogenized, poured into petri dishes and the plates were dried until solid under sterile conditions. Bacterial strains were streaked on each agar plate and incubated for two days at 30 °C and for another three days at room temperature. Each strain was tested both in and on the agar. The plates were monitored for interactions between bacteria each day.

Quorum-sensing

The ability of the consortium strains to communicate with each other using N-Acyl homoserine lactones (AHLs) was tested using the reference strain *Chromobacterium violaceum* CV026 (Ravn *et al.*, 2001). Tests were performed according to Pinto *et al.* (2007) with modifications. Four strains were streaked on NA in a 90° angle to the indicator strain (Figure 2). Plates were incubated at room temperature and 30 °C for one day. As a positive control *Serratia plymuthica* strain HRO-C48 (Müller *et al.*, 2009) was used. Violet coloring of *C. violaceum* next to the tested strain was interpreted as positive quorum-sensing reaction.

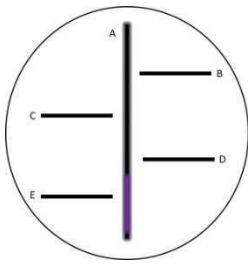

Figure 2: Test scheme for quorum-sensing assay. A: *C. violaceum*, **B-D:** test strains, **E:** *S. plymuthica* HRO-C48.

Motility test

The swimming and swarming ability of *Pseudomonas* and *Bacillus* strains (*P. fluorescens* SF2-2-2*, P. grimontii* RP2-2-4, *P. corrugata* RM1-1-4, *P. mediterranea* RM2-3-1, *P. poae* RE*1-1-14 and *B. subtilis* B2g) were monitored in the presence and absence of *Chryseobacterium wanjuense* ZM2-1-1 on three differently concentrated agar plates. *C. wanjuense* ZM2-1-1 was chosen due to its potential to induce swarming in other strains, as observed in microbe-microbe interaction tests. Therefore, all strains were cultured in 50 mL NBII over night at 30 °C under agitation. For the agar plates 800 mL NA with 1.5%, 0.6% and 0.2% of agar were autoclaved and cooled to 55 °C for at least one h. 50 mL of ZM2-1-1-NBII solution with an OD_{600} of 0.4 was prepared and added to each 800 mL agar-flask, homogenized using a magnetic stirrer and poured into petri dishes. Per test strain two 2 mL tubes were filled with ONC and centrifuged for 4 min at 8 °C and 4,000 × g. The supernatant was discarded. The cells of the two tubes were re-suspended in 500 µL of sterile sodium chloride (NaCl, 0.85%) each, mixed together and centrifuged at 10,000 × g for 1 min. Again the supernatant was discarded and the washing step was repeated two more times. Finally, the OD_{600} was measured, adjusted to 1.0 for each test strain with NaCl

and 5 µL or 10 µL of each suspension were dropped in the center of all three agar types containing ZM2-1-1 in three replicates. 3 plates without ZM2-1-1 each were used for comparison (1x 10 µL and 2x 5 µL). Plates were incubated at 23 °C for one to two days in the dark. Swimming (0.2% agar) and swarming ability (0.6% agar) was observed compared to normal agar plates (1.5% agar). Observation focus was also on increased or decreased swimming or swarming behavior in presence of ZM2-1-1.

Desiccation assay

To determine the survival capacity of the test strains, each strain was cultivated in 50 mL NBII at 30 °C under agitation overnight. OD_{600} was measured and half of a sterile 96 well plate was filled with 50 µL overnight culture per well per strain. The plates were dried in the clean bench for 24 h. Twelve different time points were chosen, from day 0, which was right after 24 h of desiccation to day 280. At each time point 20 µL of sterile NaCl (0.85%) were added to 4 wells per strain and incubated for 5 min. After incubation, the dried bacteria were re-suspended and dropped on NA plates. Plates were checked for bacterial growth after two days of incubation at room temperature. A bacterial strain was considered as having survived the desiccation at any time point, if at least three out of four replicates showed colonies on the NA.

Volatile organic compound assay

The volatile organic compound (VOCs) assay was prepared as described by Cernava *et al.* (2015) with modifications. 6 well plates (Greiner Bio-One, Frickenhausen, Germany) containing 5 mL Waksman agar (WA, per liter: 5 g tryptone (casein), 10 g glucose, 5 g NaCl, 3 g yeast extract and 20 g agar) per well were used. Each bacterial test strain was cultivated in a 100 mL flask containing 50 mL Waksman media overnight. The OD_{600} of the overnight cultures were measured and 100 µL of each culture were plated out in the wells and dried under the clean bench. The fungal phytopathogens were grown on WA and plugs with a diameter of 5 mm (*Botrytis cinerea* and *Alternaria alternata*) or 3 mm (*Sclerotium rolfsii* and *Rhizoctonia solani* AG4) were punched out using cork borers. The plugs were placed on 6-well plates (WA) in the center of each well. Using perforated silicon sheets between the bacterial and the fungal 6 well plate, the plates were clamped together using 4 clamps per sandwich, one on each side of the plates. Sterile WA without bacterial inoculants was used to observe fungal growth as a negative control. The assays were incubated at room temperature at normal light conditions until the control fungi fully covered the agar surface. The diameter of fungal growth was measured and the inhibition of the fungi (%) was calculated. VOCs assays were repeated two to three times (n=3 or 4) for statistical analysis.

2.3 Performance of greenhouse and field trials

For all greenhouse experiments seeds were provided by KWS SAAT SE (Einbeck, Germany). Varieties used were RONALDINIO for maize (*Zea mays* L.), ZERBERUS for sorghum (*Sorghum bicolor* L.) and TRAVIATA for oilseed rape (*Brassica napus* L.) experiments.

Bioassays

Seed preparation and bio-priming

For bioassays, seeds were surface sterilized using diluted sodium hypochlorite solutions (NaOCl, Carl Roth GmbH, Karlsruhe, Germany). Depending on the species, seeds were sterilized in a 4% or 2% NaOCl solution under agitation (4%: Maize, 10 min; Sorghum, 7 min; 2%: oilseed rape, 5 min). After the sterilization seeds were washed with sterile distilled water, dried and stored at room temperature in the dark until use (max. 3-4 days). Bacterial strains were plated on NA and grown at 30 °C for four days. Cells were harvested using sterilized slides and transferred into tubes containing 10 mL sterile 0.85% NaCl. After homogenization the OD_{600} was measured and bacterial suspensions were prepared as required. Seeds were primed in bacterial suspensions (OD_{600} = 1) for 4 hours at room temperature under agitation and dried in the clean bench before use. As a control sterilized or unsterilized seeds were primed in sterile NaCl. For colony forming unit (CFU) determination seeds were crushed in sterile Whirl-Paks (Nasco, Fort Atkinson, U.S.) using a hammer, sterile NaCl was added and the mass was homogenized (per bag: Maize: 6 seeds plus 6 mL NaCl; Sorghum and Oilseed rape: 10 seeds plus 2 mL NaCl). For maize the Whirl-Paks were additionally mixed in a homogenizer (BagMixer®, interscience, St. Nom, France) for 3 min each. Seed colonization was reviewed in 4 replicates per sample. Afterwards the solution was transferred to 2 mL tubes. Using 96 well plates, serial dilution was prepared for each replicate and 10 µL of 10^{-2} to 10^{-6} were dropped on NA in duplicates. Plates were incubated at room temperature for three to four days until CFU were countable. Average CFU per seed was calculated. Additionally, the CFU per mL OD_{600} = 1 was evaluated for each bacterial strain by dilution of the ONC and dropping on NA as described.

Germination approaches

To test seed germination capacity of treated and untreated seeds different environmental conditions were examined. On the one hand folded Rotilabo®-germ testing paper (Carl Roth GmbH, Karlsruhe, Germany) was used. Sterilized testing papers were added to ethanol cleaned plastic boxes (17 x 12.5 cm on top, 16 x 11.5 on bottom level) and watered with 45 mL of sterile distilled water. For testing maize seeds, the filters were cut into halves before sterilization and each half was placed in a separate box. Maize was watered with 30 mL per filter half. After allowing the water to distribute evenly for approximately 2 hours, two seeds were implanted per paper fold using a sterile tweezer. The boxes were incubated with the transparent lid closed. Lids were replaced by sterile plastic bags as plants grew

bigger. For oilseed rape the leaf rate was also documented. Due to the observation of some seeds, which germinated but then stagnated in further growth and therefor leaf building, counting of all grown leaves was considered a good parameter to rate plant health and growth in sterile environments. On the other hand, germination in soil was tested. Therefore, a 3:1:1 mixture of soil (Gramoflor Profi-Substrat, Kalsdorf, Austria) sand (Maxs Spielsand®, Scherf GmbH & Co KG, Hartberg, Austria) and vermiculite (3–6 mm, Ratioform, Vienna, Austria) was used. To each box (17.2 x 12 cm on top, 15.5 x 10 on bottom level) 250 to 300 g soil mixture, 125 to 150 mL sterile distilled water and the treated seeds (sorghum: 16-25, maize: 12-20, oilseed rape: 28-36) or control seeds (sterilized, Co st. or unsterilized, Co ust.) were added. Boxes, containing filters or soil, were incubated in the greenhouse at 23 °C at 16-8 h day-night rhythm and were treated equally. They were randomized at daily frequency and watered as required. Germination was monitored for two weeks and germination rates were calculated in % compared to the control seeds. Seeds were counted as germinated, if any part of the seedling was breaking through seed capsule. Further the biomass (fresh and dry weight, FW, DW) was evaluated for each soil and filter experiment. Green parts of the plants were collected into paper bags, weighted using an analytical balance and dried for a minimum of 3 days at 105 °C. When completely dried, the plants were acclimatized to room temperature for 2 h using desiccators and the DW was measured.

Root colonization

To detect colonization on plant roots, primed seeds were cultivated in germination pouches (Mega International, Minneapolis, MN) according to Zachow *et al.* (2010) with short modifications. Pouches were watered with 20 mL sterile deionized water. For maize pouches were folded according to company instruction for larger seeds (https://mega-international.com/tech-info/). For maize 4 seeds and for sorghum 8 seeds were set up per pouch. For oilseed rape seeds and pouches were treated as described in ‘Analysis with fluorescent *in situ* hybridization and confocal laser scanning microscopy’. After 2 weeks (maize and sorghum) or 22 days (oilseed rape) the roots of 2 pouches were harvested, weighted, mortared with 2 mL sterile NaCl and dropped out for CFU determination as described. Root colonization was observed in four replicates à two pouches.

Stress assay

For testing the effects of consortia on host plants in stress conditions dehydration tests were performed. Pre-tests have shown that the plant growth chamber APT.line® KBWF (Binder, Tuttingen, Germany) does not provide stable growth conditions. Therefore, stress assays were performed in the greenhouse under stable conditions at 23 °C with 16 h day, 8 h night rhythm. For desiccation tests, growing trays (L=55.5 cm, W=35 cm, D=6 cm) containing 5 kg of soil-mixture were used. For improved plant observation, trays were divided into 35 squares using cords. Trays were watered with 1400 mL deionized water and one (maize) or two seeds (sorghum and oilseed rape) were planted per square. CFU per seed was examined as described previously. As controls, sterilized and non-sterilized seeds were utilized in the experiment. Plants were grown without desiccation stress until they were tall enough. Before starting the stress test, the plants were adjusted to the same quantity and at a maximum of 1

plant per square. Water withdrawal started after 8 days of normal growth phase for maize, after 12 days for sorghum and after 14 days for oilseed rape plants. Trays were monitored and randomized in the greenhouse on a daily basis and watered at the last moment possible for the plants to recover, oriented on the poorest variety within one plant species. In watering, all plants from one species were treated equally. Plants were monitored in recovery and desiccation symptoms for a total amount of 45 days from planting to harvest. On day 45, green parts of the plants were harvested and their vitality was rated. Further parameters like plant length, thickness of the stem and amount and color of the leaves were documented. Fresh and dry weight was measured as described in 'Germination approaches'.

Field trials and sampling for amplicon analysis

Field trials were performed with the same set-up in two different Austrian locations: Mitterdorf an der Raab (N 47.178464, E 15.612477) and Melk (N 48.155633, E 15.512528). On each field four different treatments were applicated to the maize cultivar LG3258. Treatments were planted in 3 replicates, resulting in 12 randomized plots per field (Figure 3). The treatments included stripper coated seeds (Maxim XL+Captan/Korit: 1 L plus 0.3 L in Sacrust as carrier substance) and uncoated seeds, as well as *S. rhizophila* P69 primed stripped and non-stripped seeds. Rhizosphere sampling 43 (Mitterdorf) to 44 days (Melk) old plants was performed after Kröber *et al.* (2014) with modifications. Four plants were excavated and pooled as one sample per treatment and field. Each treatment was sampled in 4 replicates, resulting in 16 rhizosphere samples per location. Soil samples from the respective field served as controls. Whole plants were transported at 4 °C to minimize desiccation of roots and were processed in the lab immediately.

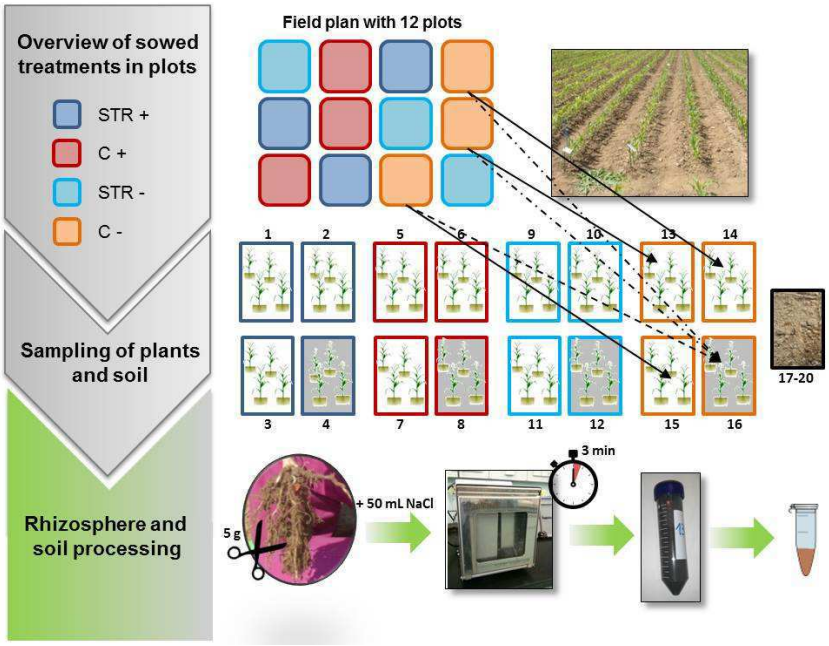

Figure 3: Overview of field setup (example given: Mitterdorf) and sampling procedure of maize rhizospheres.

For each replicate, 4.8 g (±1.7 g) rhizosphere or soil were mixed for 3 min with 50 mL sterile NaCl in sterile plastic bags using a BagMixer®. Homogenized fluids were transferred to 50 mL tubes, mixed

again and 2 mL of the solution were centrifuged in two separate Eppendorf tubes (2 mL) per sample at 4 °C and 16,000 × g for 20 min. Pellets were stored at -70 °C until use. Field trials were sown and maintained by Saatzucht Gleisdorf GmbH and evaluated by Henry Müller and Eveline Adam.

2.4 Analysis with fluorescence *in situ* hybridization and confocal laser scanning microscopy

For fluorescence *in situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM) of roots, oilseed rape seeds were approached as described in 'Seed preparation and bio-priming' with 24 h of incubation time for seed priming. 8 seeds were transferred to 12 pre-watered germination pouches as described in 'Root colonization' without drying. For minimizing appearance of autofluorescence, open sides of the pouches were covered with aluminum foil to darken the root growth area. Roots were harvested after 12 days, fixed according to Cardinale *et al*. (2008) via paraformaldehyde (PFA) fixation and stored at -20 °C until use. Probes for FISH, fluorochromes (FC) and formamide concentrations (FA) at 41 °C are listed in Table 2. CFU of seeds and roots were examined. For FISH, the first 1.5 cm of the roots after the stem were used. FISH procedure was performed in three different probe combinations, as described by Erlacher *et al.* (2015): **1)** Actinobacteria and Firmicutes, **2)** Proteobacteria and Enterobacteria and 3) Bacteroidetes, all combined with labeling of all bacteria present (EUB probes). For staining of the roots, 350 µL of 0.15% calcofluor white staining (Sigma-Aldrich) was applied to the samples, incubated at room temperature in the dark for 10 min and rinsed with ice cold double distilled water (ddH_2O). Samples then were further washed with 1 mL ice cold ddH_2O for 5 min in the dark. For fixation on slides, roots were dried with compressed air and mounted with ProLong Diamond Antifade Mountant (molecular probes, Life Technologies) immediately. Coverslips were sealed with nail polish. NONEUB probes with the corresponding FC were used as negative control on primed roots.

Table 2: FISH probes used in this study.

Name	Sequence (5'-3')	FC	Target	Int. target	FA (%)	Reference
EUB338*	GCTGCCTCCCGTAGGAGT	Cy3	Most bacteria	All	10-20	Amann *et al.* (1990)
EUB338II*	GCAGCCACCCGTAGGTGT	Cy3	Planctomycetales	/	10-20	Daims *et al.* (1999)
EUB338III*	GCTGCCACCCGTAGGTGT	Cy3	Verrucomicrobiales	/	10-20	Daims *et al.* (1999)
NONEUB**	ACTCCTACGGGAGGCAGC	Cy3, Cy5, ATTO488, FITC	/	/	**	Wallner *et al.* (1993)
HGC236	AACAAGCTGATAGGCCGC	Cy5	Actinobacteria	MF1-2-4	10-20	Erhart *et al.* (1997)
GAM42a	GCCTTCCCACATCGTTT	Cy5	Gammaproteobacteria	RE*1-1-14	35	Manz *et al.* (1992)
Enterobac_D	TGCTCTCGCGAGGTCGCT TCTCTT	ATTO488	Enterobacteriaceae	SF1-3-1	25-30	Ootsubo *et al.* (2002)
BAC303	CCAATGTGG GGGACCTT	Cy5	Bacteroidetes	ZM2-1-1	10	Manz *et al.* (1996)
LGC354A***	TGGAAGATTCCCTACTGC	FITC	Part of Firmicutes	ZP2-1-3	35	Meier *et al.* (1999)
LGC354B***	CGGAAGATTCCCTACTGC					
LGC354C***	CCGAAGATTCCCTACTGC					

*/*** Equimolar concentration of probes was used.
** NONEUB probes were used as negative control with FA concentrations adapted to positive probes.

Leica TCS SPE confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany) was used for visualizing root colonization. Fluorochromes were excited with 532 nm (Cy3), 635 nm (Cy5) and 488 nm (ATTO488 and FITC) laser beams. Calcofluor white was excited with a laser beam of 405 nm. For imaging, software Imaris 7.3 (Bitplane, Zurich, Switzerland) was used.

2.5 Molecular analysis

DNA isolation, 16S rRNA gene amplification and sequencing for strain identification

For DNA isolation, 500 µL lysis buffer (1.4% CTAB, 1 M NaCl, 7 mM Tris, 30 mM EDTA, pH 5.5) was added to several colonies in 2 mL micro tubes with screw caps (Sarstedt, Nümbrecht, Germany) containing approximately 200 µL of 0.25-0.3 mm sized glass beads (Sigma) and 6 pieces of 1.5 mm glass beads and incubated at 65 °C for 1 h. After incubation cells were disrupted using FastPrep®-24 (MP Biomedicals, Eschwege, Germany, 20 s, 4m/s) and 500 µL chloroform/isoamyl alcohol (CI, 24:1) was added. Tubes were vortexed and centrifuged for 5 min at 13,500 rpm. The upper phase was transferred to a new tube and CI extraction was repeated. 1 mL of precipitation buffer (0.5% CTAB, 40 mM NaCl) was added and tubes were incubated at room temperature for 1 h. Samples were centrifuged for 15 min at 13,500 rpm and the supernatant was discarded. Pellets were resuspended in 1.2 M NaCl and one CI extraction step was repeated. The upper phase was transferred into a new tube, mixed with 210 µL ice cold isopropanol and stored at -20 °C overnight. The following day tubes were incubated for 5 min at room temperature and centrifuged for 20 min at 4 °C. After discarding the supernatant, 200 µL

80% ethanol was added to the pellets on ice. Tubes were centrifuged again for 5 min at 4 °C. Pellets were dried under the clean bench and resuspended in 25 µL PCR water.

For amplification of the 16s rRNA gene, 0.3 µL 27F and 1492r primer (10 µM) each, 6 µL Taq-&Go Mastermix (MP Biomedicals, Eschwege, Germany) plus 1 µL DNA template were used in a total reaction volume of 30 µL. For SF1-3-1 and SF2-2-2 338bF was used as forward primer. Initialization step of the PCR reaction was performed at 95 °C for 5 min, followed by 30 cycles of denaturing (95 °C, 30 s), annealing (57 °C, 30 s) and extending (72 °C, 90 s). Final elongation took place for 5 min at 72 °C. For control of PCR fragments, 3 µL product mixed with 1 µL loading dye (6x) were run on a 0.8% agarose gel in 1x TAE buffer at 100-110 V for 40 min. As a standard GeneRuler™ 1 kb DNA Ladder (Thermo Scientific, Wien, Austria) was used. For imaging gels were stained in 0.0001% ethidium bromide (EtBr) solution for 20 to 25 min. Results were evaluated by comparing fingerprints with re-isolated and originally applied strains.

PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Mannheim, Germany). Double amount of Membrane Binding Solution was used in the clean-up process. Sanger sequencing of 16s rRNA genes was performed by LGC Genomics using the forward primer (27F or 338bF). Identification of strains was observed via BLAST analysis.

Quick DNA isolation and BOX-PCR

Amplification of the BOX-A fragment was used as a method of comparing and verifying re-isolated strains e.g. from roots or seeds to confirm identity with the biocontrol strains originally used. For bacterial DNA isolation several colonies of a strain were re-suspended in 300 µL PCR-water (Carl Roth GmbH, Karlsruhe, Germany) in 2 mL micro tubes with screw caps containing glass beads (as described in DNA isolation, 16S rRNA gene amplification and sequencing for strain identification). Cells were mechanically disrupted using FastPrep®-24 (30 s, 6m s^{-1}). Afterwards, the tubes were frozen for a minimum of 30 min at -70 °C until frozen solid and heated at 95 °C for 10 min. This step was performed twice. Supernatant containing DNA was collected after 5 min centrifugation at 4 °C (3,000 rpm).

BOX-PCR was performed using 1.5 µL 10 µM BOXA1R primer (5′-CTA CGG CAA GGC GAC GCT GAC G-3′), 3 µL DNA template and 3 µL Taq-&Go Mastermix in a total reaction volume of 15 µL. PCR program was performed according to Berg *et al*. (2005) with initialization step for 6 min at 95 °C, followed by 35 cycles of 1 min denaturing at 94 °C, 1 min annealing at 53 °C and 8 min elongation at 65 °C. Final elongation step was performed at 65 °C for 16 min. Products were stored for 4 °C until use. 15 µL of product was mixed with 3 µL loading dye (6x) and applied to a 1.5% agarose gel in 0.5 Tris/Borate/EDTA (TBE) buffer. Gel electrophoresis was performed for 4 h with 90 V. 1 kb DNA Ladder GeneRuler™ was used as standard. For imaging, gels were processed as described.

Amplicon sequencing of maize rhizosphere microbiome

The rhizosphere microbiome of four differently treated maize plant varieties (seeds: P69 primed with and without stripper and non-primed controls with and without stripper) from two sites in Austria were analyzed. Detailed description of the varieties and the locations is shown in the section 'Design of greenhouse and field trials – Field trials'. As a control for the influence of the local site itself, soil samples were also analyzed for each site.

DNA extraction of soil or rhizosphere samples was performed using FastDNA™ SPIN Kit for soil and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA). Per variety, four DNA extractions were performed as individual replicates. For soil analysis, DNA of two tubes each was extracted and split into four replicates per location. For amplification of 16S rRNA genes barcoded 515f and 806r primers (Caporaso *et al.*, 2011) were used. Amplification was performed according to Lundberg *et al.* (2013) with modifications. PCR reaction mixture contained 6 µL Taq-&Go Mastermix, 1.2 µL primer (5 µM) each, 0.45 µL mixed peptide nucleic acids (PNA, 1:2 mix of 100 µM anti-mitochondrial and anti-plastid PNA) and 1 µL template in a total reaction volume of 30 µL. PCR was performed with initialization step at 96 °C for 5 min followed by 30 cycles of denaturation at 96 °C for 1 min, PNA annealing at 78 °C for 5 s, primer annealing at 54 °C for 1 min and extending at 74 °C for 1 min. Final elongation was performed for 10 min at 74 °C. For fungi barcoded ITS1f and ITS2rP primers were used according to Mahnert *et al.* (2015) with modifications. PCR reaction contained 0.9 µL magnesium chloride (20 mM), 6 µL Taq-&Go Mastermix, each 1.5 µL primer (5 µM) and 1 µL community DNA template in a total reaction volume of 30 µL. PCR reaction was modified as follows: initial denaturation 5 min at 95 °C, followed by 30 cycles of denaturation at 95 °C (30 s), annealing at 58 °C (35 s), extending at 72 °C (40 s) and final elongation for 10 min at 72 °C. After each PCR, a 1% agarose control gel in 0.5% TBE buffer was run for 75 min using 110-150 V. As a standard 100 bp DNA Ladder (Thermo Scientific, Wien, Austria) was used. Gels were stained in EtBr as described. Products were amplified in 3 replicates per sample, pooled and cleaned up using Wizard® SV Gel and PCR Clean-Up System. Further, all samples were pooled equimolar with consideration of required parameters in separate pools for fungi and bacteria and sent to Eurofins Genomics GmbH (Ebersberg, Germany) for further processing and Illumina MiSeq sequencing. Data analysis and filtering was performed according to Mahnert *et al.* (2015) by using QIIME (Caporaso *et al.*, 2010).

2.6 Statistics and data analysis

For statistical data analysis, t-test (http://graphpad.com/quickcalcs/ttest1/) was used for pairwise comparison of Co st. or Co ust. samples and treatments in greenhouse trials, as well as in VOCs assays. Each experiment was performed in a total of at least 4 replicates unless otherwise stated. Data was illustrated as arithmetic mean ± standard deviation. For all analyses a P value <0.05 was considered as statistically significant. For creation of graphs, Microsoft Excel and Mircrosoft Powerpoint were chosen as working tools.

3 Results

3.1 Bacterial strain characterization and designing consortia

Sequencing of 16S rRNA genes and BLAST alignment confirmed the species identity of bacterial strains isolated by Zachow *et al.* (2013), as previously shown in Table 1. Only strains of risk group 1 were considered as possible consortium strains.

For characterization of bacterial strains, interaction was examined between two partners in all possible combinations. Microbe-microbe interaction tests showed a number of different reaction patterns. It was noticeable, that most reactions differed, depending on the location of the same interacting partners – in or on the agar plates. An overview of all observed reactions can be found in Figure 4. Example pictures of the interactions are shown in Figure 5. Very strong swarming of the bacteria on the agar was observed for *Bacillus subtilis* B2g and *Pseudomonas poae* RE*1-1-14 in presence of *Chryseobacterium wanjuense* ZM2-1-1 or *Stenotrophomonas rhizophila* e-p17 in the agar (Figure 5B).

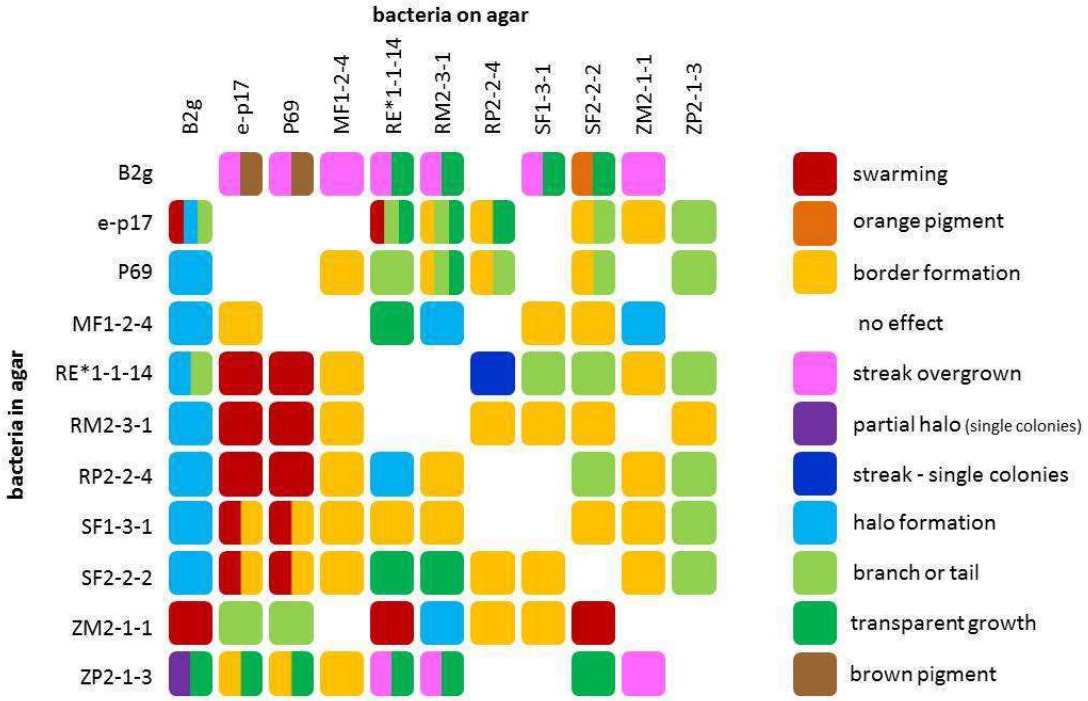

Figure 4: Reactions observed in microbe-microbe interaction test.

Strong pigment formation (Figure 5J) was only observed for *S. rhizophila* strains e-p17 and P69 in presence of *B. subtilis* B2g in the agar. In general, B2g showed a strong tendency to overgrow other strains if grown in the agar (Figure 5D) or to inhibit growth of other bacteria if streaked on the agar (Figure 5G). All reaction patterns are considered as potential positive interactions in consortia communities.

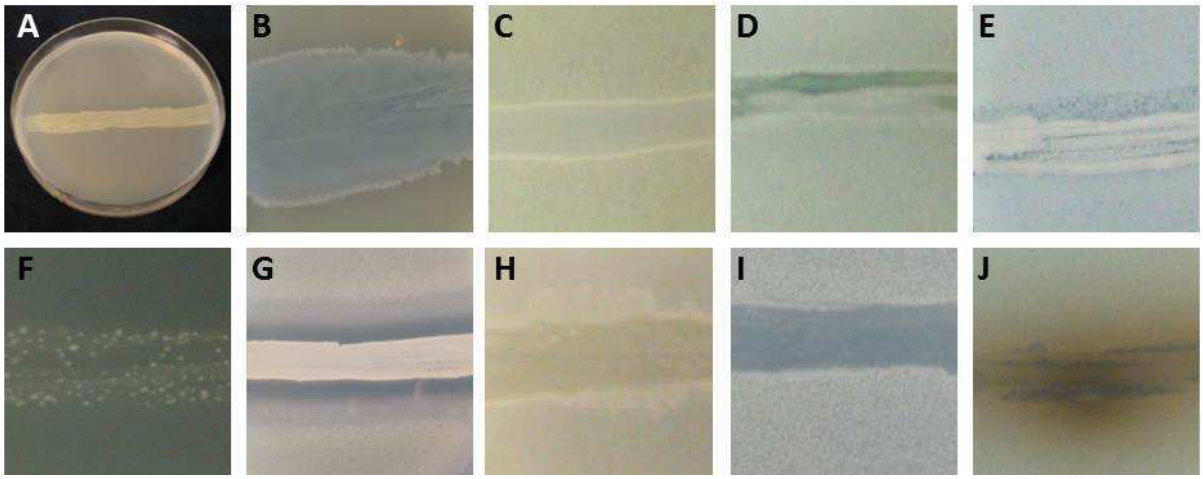

Figure 5: Examples of observed interaction types. A: no effect, e-p17^{i} and MF1-2-4^{o}; **B:** swarming, ZM2-1-1^{i} and RE*1-1-14^{o}; **C:** border formation, MF1-2-4^{i} and SF2-2-2^{o}; **D:** streak overgrown, B2g^{i} and ZM2-1-1^{o}; **E:** partial halo, ZP2-1-3^{i} and B2g^{o}; **F:** streak - single colonies, RE*1-1-14^{i} and RP2-2-4^{o}; **G:** halo formation, MF1-2-4^{i} and B2g^{o}; **H:** branch or tail formation, e-p17^{i} and RM2-3-1^{o}; **I:** transparent growth, B2g^{i} and SF2-2-2^{o}; **J:** brown pigment, B2g^{i} and P69^{o}; ^{i} bacteria in agar, ^{o} bacteria streaked on agar.

For better understanding of the strains properties and possible beneficial features in the consortium, the ability for quorum sensing (production of N-Acyl homoserine lactones, AHLs), the motility in swarming (0.6%) and swimming agar (0.2%) and the survival of desiccation in microwell plates up to 280 days were investigated (Table 3). The two strains *Pseudomonas mediterranea* RM2-3-1 and *Serratia* sp. SF1-3-1 showed the ability to communicate via AHL production at room temperature and at 30 °C. All strains tested were able to swim or swarm, except for *P. grimontii* RP2-2-4, which only showed swimming but no swarming ability. Examples are given in Figure 6.

Table 3: Overview of ability test results for consortium strains.

Species	Abb.	Quorum sensing	Swarming	Swimming	Desiccation (last day survived)
Bacillus subtilis	B2g	-	+	+	70
Stenotrophomonas rhizophila	e-p17	-	nt	nt	21
Stenotrophomonas rhizophila	P69	-	nt	nt	21
Arthrobacter nitroguajacolicus	MF1-2-4	-	nt	nt	70
Pseudomonas poae	RE*1-1-14	-	+	+	70
Pseudomonas corrugata	RM1-1-4	nt	+	+	15
Pseudomonas mediterranea	RM2-3-1	+	+	+	49
Pseudomonas grimontii	RP2-2-4	-	-	+	70
Serratia sp.	SF1-3-1	+	nt	nt	28
Pseudomonas fluorescens	SF2-2-2	-	+	+	70
Chryseobacterium wanjuense	ZM2-1-1	-	nt	nt	3
Bacillus sp.	ZP2-1-3	-	nt	nt	280

nt: not tested.

Further, no increased or decreased motility was observed in presence of *C. wanjuense* ZM2-1-1. Moreover, *P. mediterranea* RM2-3-1 and *P. corrugata* RM1-1-4 were observed to inhibit *C. wanjuense* ZM2-1-1 if present in the agar (Figure 6D).

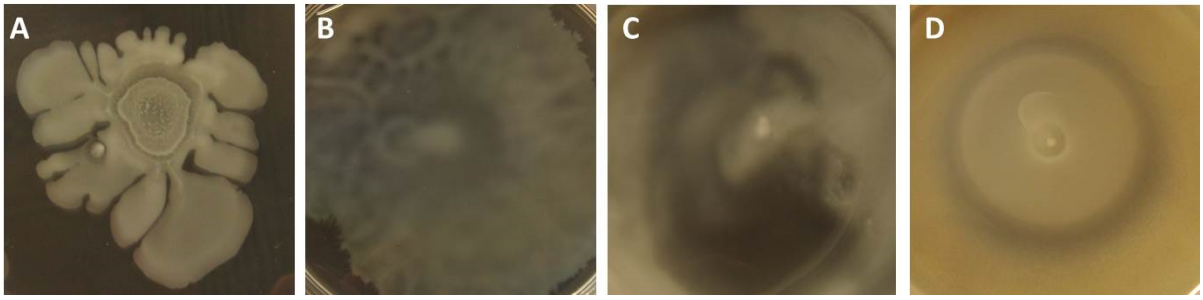

Figure 6: Swarming and swimming of tested bacteria. **A:** swarming B2g, **B:** swarming SF2-2-2, **C:** swimming RE*1-1-14, **D:** swimming RM1-1-4 with inhibition of ZM2-1-1 in the agar.

Concerning desiccation in microwell plates and revitalization on NA, *C. wanjuense* ZM2-1-1 survived the shortest with last growth after 3 days of desiccation, followed by *P. corrugata* RM1-1-4 with 15 days and *S. rhizophila* e-p17 and P69 with 21 days. *Serratia* sp. SF1-3-1 was able to survive until day 28 followed by *P. mediterranea* RM2-3-1 with survival until day 49. Four strains, *B. subtilis* B2g, *A. nitroguajacolicus* MF1-2-4, *P. poae* RE*1-1-14 and *P. fluorescens* SF2-2-2, survived at least until day 70. *Bacillus* sp. ZP2-1-3 survived until day 280 which was the last date tested. As this strain belongs to the family *Bacillus*, this might be due to the ability of sporulation, although *B. subtilis* B2g did not survive until day 280. Nevertheless, regarding these results, one should be kept in mind that there are no data measurements between day 70 and day 280.

Effect of bacteria on biotic stress

The effect of bacteria on biotic stress factors such as phytopathogens was evaluated using VOCs assay. All three bacterial strains for maize or sorghum consortia had statistically high antagonistic effects ($P<0.006$) on the mycelial growth via production of volatile organic compounds compared to the control (Figure 7). Growth of *Botrytis cinerea* and *Sclerotium rolfsii* was strongly inhibited by all strains, whereas *Alternaria alternata* was reduced in mycelial growth only by *B. subtilis* B2g and *S. rhizophila* P69 volatiles, but stimulated by *S. rhizophila* e-p17 volatiles. *Rhizoctonia solani* was reduced 28.9 to 41.4% in its growth by all three strains.

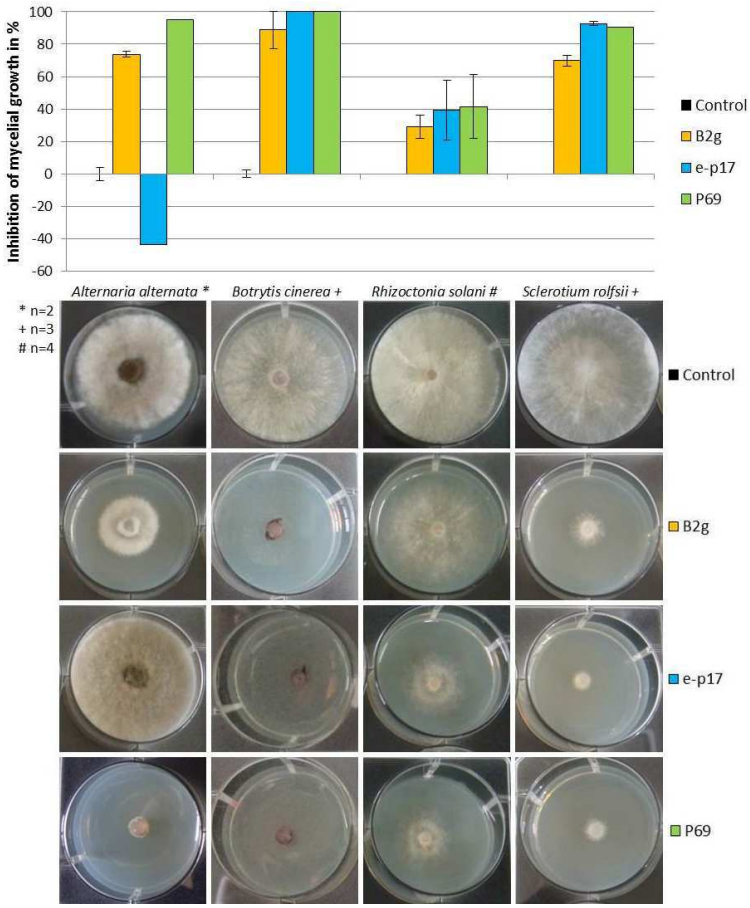

Figure 7: VOCs assay of sorghum and maize consortium strains against phytopathogens.

All considered consortium strains for oilseed rape showed a high inhibition of mycelial growth in tests with *B. cinerea* (Figure 8). Regarding *A. alternata*, four strains, namely *P. poae* RE*1-1-14, *P. corrugata* RM1-1-4, *Serratia* sp SF1-3-1 and *P. fluorescens* SF2-2-2, showed a significant growth inhibiting influence (P≤0.02), whereas other two strains, namely *P. mediterranea* RM2-3-1 and *Bacillus* sp. ZP2-1-3, significantly promoted growth of the fungi (P≤0.02). All strains were observed to significantly inhibit growth of *B. cinerea* (P<0.0001). All strains except *P. mediterranea* RM2-3-1, showed intermediate to slight mycelial growth inhibition of *R. solani*. All results, with exception of *P. poae* RE*1-1-14 and *P. grimontii* RP2-2-4, showed to be highly significant (P≤0.013). Growth of *R. solani* mycelia was not influenced by RM2-3-1. All strains showed high (MF1-2-4, RE*1-1-14, RM1-1-4, RM2-3-1, SF1-3-1 and ZM2-1-1, P≤0.0002) to intermediate (RP2-2-4 and ZP2-1-3, P≤0.0091) antagonistic effects via volatiles in testing against *S. rolfsii*. Inhibition caused by SF2-2-2 was not significant (P=0.0732). Due to these results, all strains were considered as consortium partners and were further tested for their influence on plant germination.

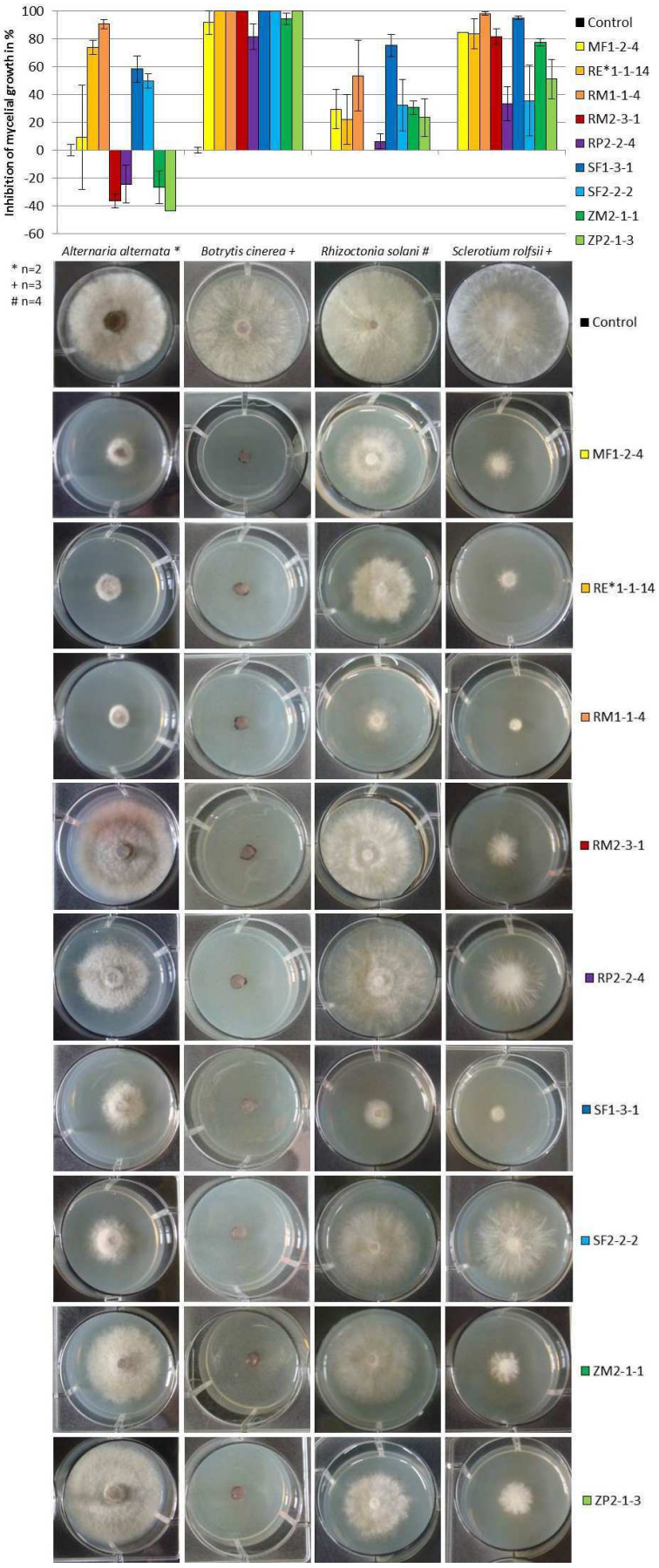

Figure 8: VOCs assay of strains considered for the oilseed rape consortium.

3.2 Influence of bacteria on oilseed rape plants

For the assembly of a consortium for oilseed rape plants, several strains, as shown in Table 1, were considered. Therefore, 5 different Mix compositions were tested for their effect on germination in germination filters, to get an idea of beneficial tendencies of the strains for this particular plant species. The Mixes were compiled due to previously considered criteria and coherences, as can be seen in Table 4. Germination was observed for 2 weeks and is shown in Figure 9. Further, seedlings were observed for formation of leaves (Figure 10).

Table 4: Mix compositions for pretrial with oilseed rape seeds.

Mix number	No. of strains	Selection criterion	Strains
Co st.	0	-	-
Mix 2	5	all *Pseudomonas* spp.	RE*1-1-14, RM1-1-4, RM2-3-1, RP2-2-4, SF2-2-2
Mix 3	9	all preselected strains	MF1-2-4, RE*1-1-14, RM1-1-4, RM2-3-1, RP2-2-4, SF1-3-1, SF2-2-2, ZM2-1-1, ZP2-1-3
Mix 4	3	only oilseed rape re-isolated strains (Zachow *et al.*, 2013)	RM1-1-4, RM2-3-1, RP2-2-2
Mix 5	5	one strain from each bait plant (Zachow *et al.*, 2013), plus the strain from the favorite collection	MF1-2-4, RE*1-1-14, RP2-2-4, SF1-3-1, ZM2-1-1
Mix 6	5	one strain from each family	MF1-2-4, RE*1-1-14, SF1-3-1, ZM2-1-1, ZP2-1-3

After 7 days (Figure 9A), Mix 4 and Mix 6 showed to have increased germination rate compared to sterilized control seeds (Co st.) (SD ± 2.6) with 106% (SD ± 4.3. P=) and 103% (SD ± 11.5). Mix 2, 3 and 5 showed a slightly decreased germination rate compared to the control, with a rate of 90% (SD ± 4.4) for Mix 2, 99% (SD ± 12.2) for Mix 3 and 97% (SD ± 8.1) for Mix 5. Germination rate of Mix 2 (P=0.0069) and Mix 4 (P=0.0436) were significantly different than germination rate of Co st. seeds.

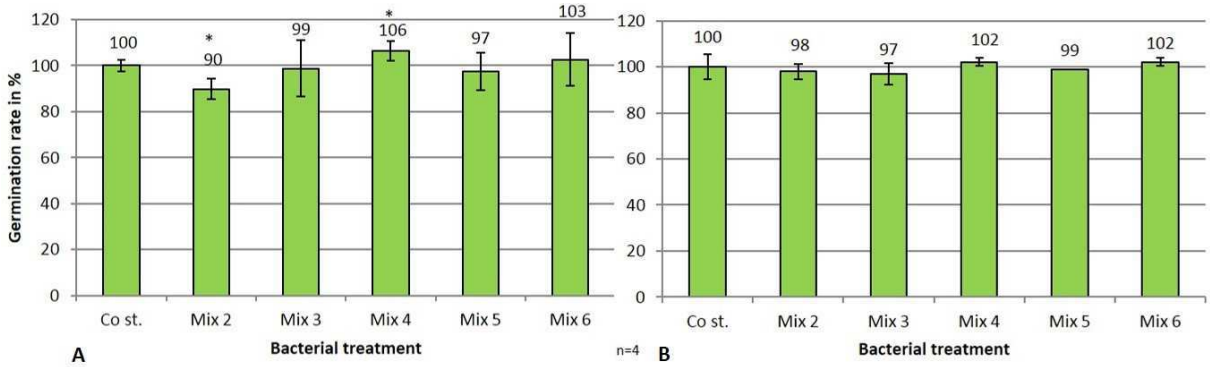

Figure 9: Germination rate of Mix treated oilseed rape seeds after 7 (A) and 14 days (B) in germination filters. * statistically significant difference, P≤0.0436.

After 14 days (Figure 9B), no statistically significant difference in germination rate was found, with Mix 4 and 6 showing a slightly increased, and Mix 2, 3 and 5 a slightly decreased germination rate compared to Co st. seeds. Nevertheless, extreme differences both in calculation and in optic appearance were observed in leaf formation rate of different treated oilseed rape seedlings (Figure 10). Differences between treatments and Co st. were enormous (values normalized to Co st. equals 100%, SD ± 63.6). Mix 6 and Mix 5 had the highest leaf rate with 473% (SD ± 83) for Mix 6 and 407% (SD ± 22.1) for Mix 5 compared to Co st. (P≤0.0004). Also the optical appraisal resulted in Mix 6 and 5 plants looking healthiest compared to other treatment or control plants (Figure 10, right). Mix 3 with a leaf rate of 220% (SD ± 34.6) and Mix 4 with 207% (SD ± 43.7) showed to have a significantly higher leaf rate than Co st. (P≤0.03). No significant differences between Mix 2 (187%, SD ± 53.3) and Co st. were found.

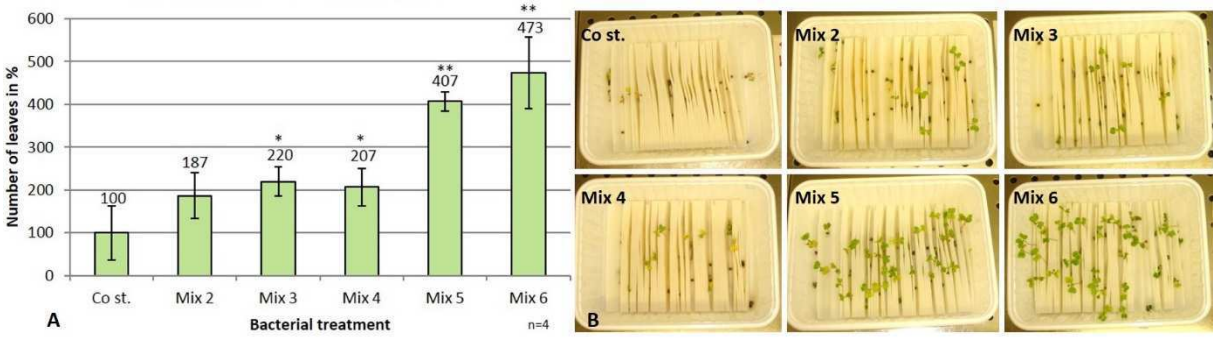

Figure 10: Leave formation of 14 days old, Mix treated oilseed rape seedlings in germination filters. Calculated leaf rate in comparison to the control (A) and optic appearance (B). Statistically significant difference: * P≤0.03, ** P≤0.0004.

Due to the results of the pretrial, the best Mix, Mix 6, was chosen as consortium treatment and therefore used in all further oilseed rape experiments. In the following, Mix 6 will only be referred to as Mix or consortium.

Alteration of bacterial abundance on bio-primed seeds and roots of oilseed rape

CFU of OD_{600}=1 of all five consortium strains were determined and varied only slightly between strain families (Figure 11).

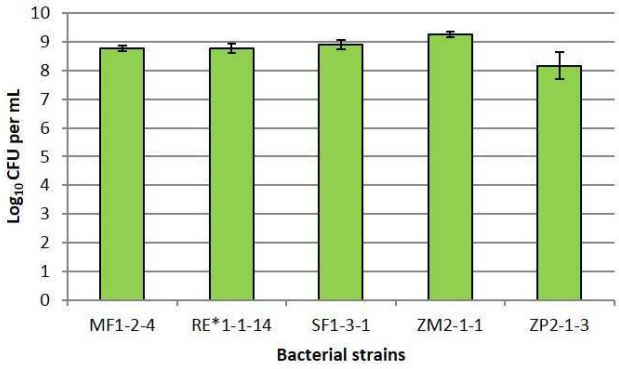

Figure 11: Colony forming units per mL of five consortium strains for oilseed rape at OD_{600}=1. n=4.

MF1-2-4 and RE*1-1-4 showed to grow on average Log_{10} 8.8 (± 0.1 or ± 0.16) CFU per mL bacteria suspension. For SF1-3-1 Log_{10} 8.9 (± 0.17) and ZP2-1-3 Log_{10} 8.2 (± 0.48) CFU per mL were counted. Highest amount of CFU per mL was found for ZM2-1-1 with Log_{10} 9.2 (± 0.1).

Concerning bacterial abundances on consortium treated seeds and roots, strains could be distinguished phenotypically very easily and were therefore counted separately. Bacterial composition on normal primed seeds (4 h) and for FISH primed seeds (24 h) as well as for FISH used roots is shown in Figure 12. Total number of CFU per 4 h primed seed (equals Mix column) was counted to be Log_{10} 5.6 (± 0.24) per oilseed rape seed. This number consists of the sum of the CFU of all five Mix strains. On average, RE*1-1-14 and ZM2-1-1 showed the highest abundance with Log_{10} 5.0 (± 0.53 or ± 0.09) CFU per seed, followed by MF1-2-4 with Log_{10} 4.9 (± 0.32) and SF1-3-1 with Log_{10} 4.7 (± 0.46) CFU. The lowest abundance of CFU per seed was found for ZP2-1-3 with Log_{10} 3.6 (± 0.5). Abundances on seeds that were primed for 24 h differed slightly from normal primed seeds (Figure 12B). Total number of CFU was Log_{10} 5.7 per seed, consisting of Log_{10} 4.8 MF1-2-4, each Log_{10} 5.2 RE*1-1-14 and SF1-3-1, Log_{10} 5.1 ZM2-1-1 and Log_{10} 3.3 CFU ZP2-1-3. For a statistical interpretation 24h seed priming needs to be repeated. Abundances on roots from 24 h primed seeds were detected after 22 days of growth in pouches and calculated per g root. The distribution of strains per g root differed from the distribution patterns found on seeds. RE*1-1-14 was found to be most abundant with Log_{10} 8.7 (± 0.4) CFU per g root followed by SF1-3-1 with Log_{10} 7.8 (± 0.2) and ZM2-1-1 with Log_{10} 6.6 (± 0.4). Oilseed rape roots showed to have Log_{10} 6.5 (± 0.4) CFU ZP2-1-3 per g. The least abundant strain in this composition was MF1-2-4 with Log_{10} 6.2 (± 0.9) per g root. All strains could be re-isolated from oilseed rape roots and were therefore considered as rhizosphere competent.

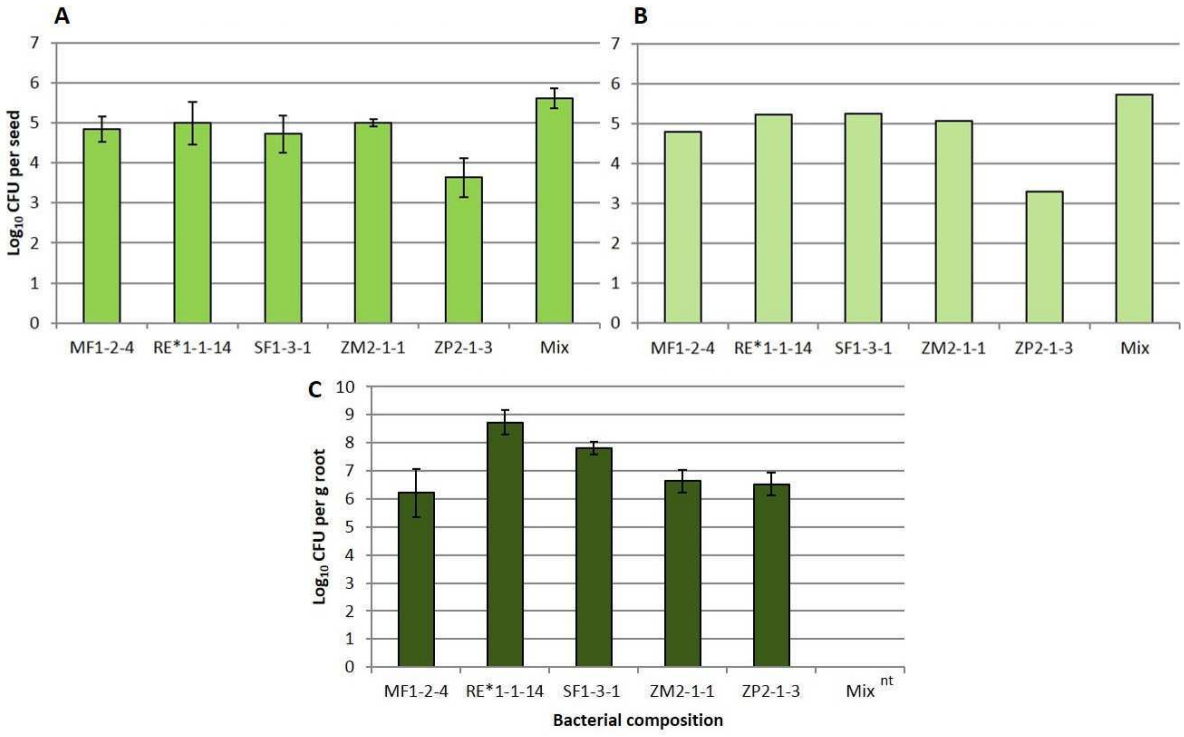

Figure 12: Bacterial abundance on oilseed rape seeds (A: primed for 4 h, B: primed for 24 h) and roots (C, 22 days) after consortium bio-priming. nt: not tested.

Root colonization and confocal laser scanning microscopy

Twelve-days old oilseed rape seedlings were investigated for colonization patterns of consortium strains after bio-priming. Therefore, FISH was performed with the oldest part of the roots (about 1.5 cm). Different colonization pattern on the oilseed rape roots were observed via CLSM. Figure 13 shows microcolony formation on root epithelia cells (A) and biofilm formation around root hairs (B) under use of FISH combination 1 (HGC236, LGC354, EUB338). EUB probes labeled cells non-specifically and therefore labeled all bacteria (A). *Bacillus* sp. ZP2-1-3 is labeled with EUB and LGC354 mix probes and therefore appears pink (Figure 13B). In the negative control under same FISH and CLSM specification NONEUB probes with same fluorophores as in the respective combination did not result in interfering signals, as is shown exemplary in Figure 14.

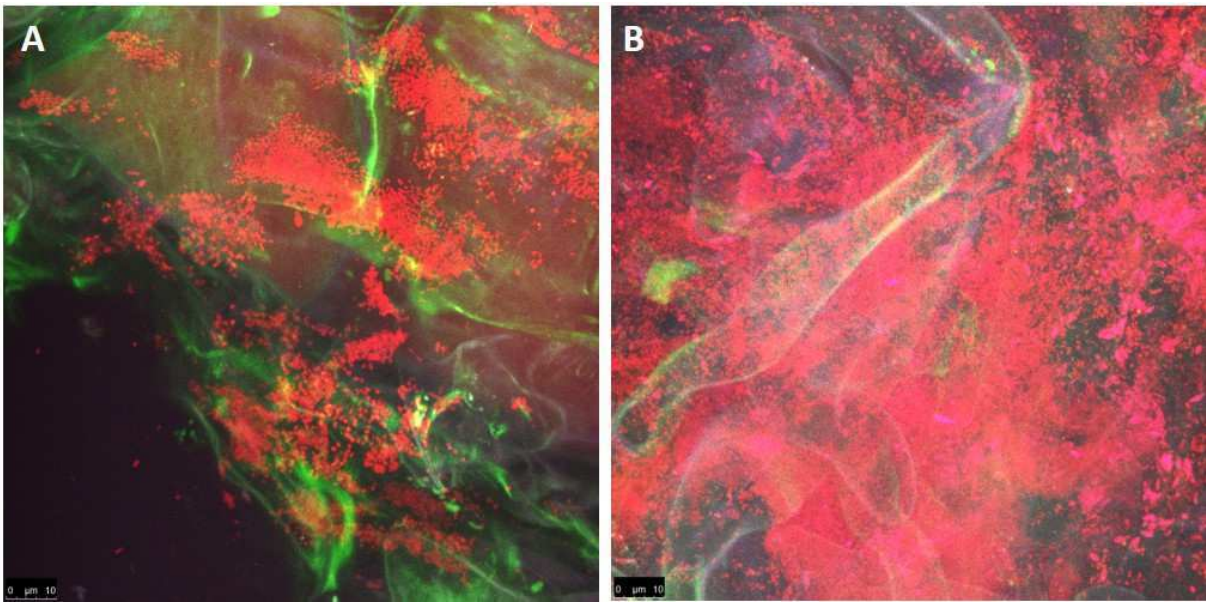

Figure 13: Microcolony (A) and biofilm formation (B) of consortium bacteria on 12 days old oilseed rape roots. A: bacteria labeled with EUB probes (red) on root epithelial cells, **B:** biofilm formation of EUB labeled bacteria (red) and *Bacillus* sp. ZP2-1-3 (pink) on root hairs. Scale bars: 0-10 µm.

Further, via observation of combination 1, bacterial cluster formation of *Bacillus* sp ZP2-1-3 or other EUB labeled bacteria was observed (Figure 15). Clusters of ZP2-1-3 were often found to be surrounded by other bacteria (Figure 15B and C). Also single cells of ZP2-1-3 or HGC236 labeled *A. nitroguajacolicus* MF1-2-4 were found (Figure 15A). FISH combination 2 revealed microaggregate formations of GAM42a labeled *P. poae* RE*1-1-14 with lose and low accumulation of GAM42a and Enterobac_D labeled *Serratia* sp. SF1-3-1 (Figure 16). A picture of the negative control, labeled with NON-EUB probes and fluorophores of combination 2 is given in Figure 14. Four out of five strains in the mixture could be observed using CLSM. Combination 3 (BAC303, EUB338) still needs to be performed, to reveal colonization patterns for Bacteroidetes, particularly for the visualization of *C. wanjuense* ZM2-1-1.

Figure 14: NON-EUB labeled negative control.

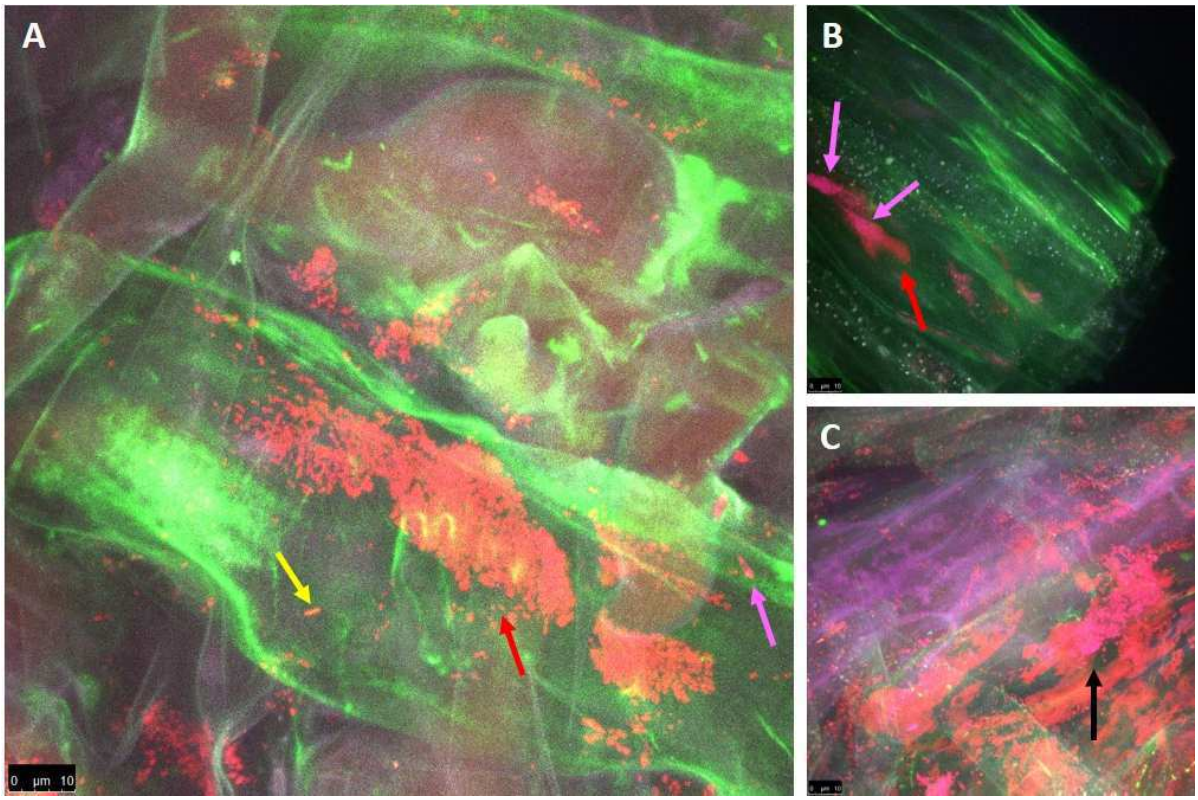

Figure 15: Cluster formation of bacteria on oilseed rape roots. A: Cluster formation of bacteria (red), and single cells of *Arthrobacter nitroguajacolicus* MF1-2-4 (yellow) and *Bacillus* sp. ZP2-1-3 (pink arrow). **B** and **C:** ZP2-1-3 cluster surrounded by other bacteria. Scale bars: 0-10 µm.

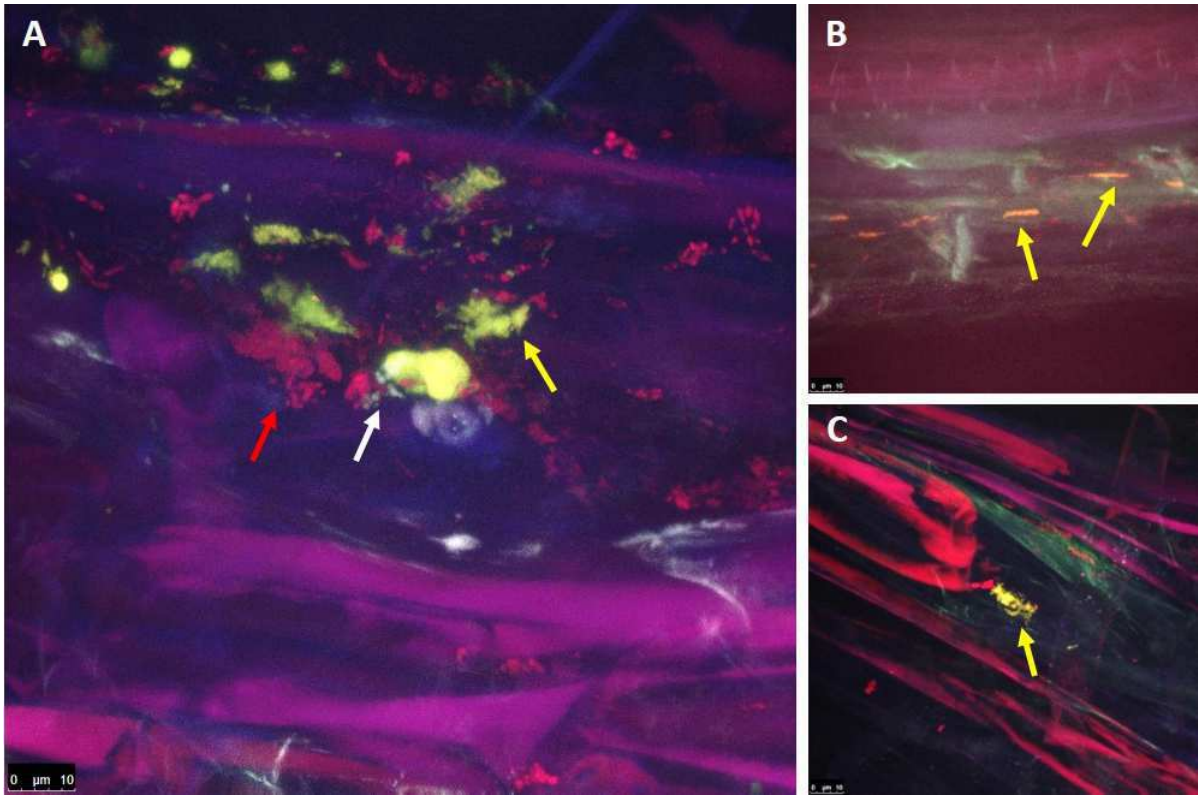

Figure 16: Microaggregate formation on oilseed rape roots. A: lose accumulation of low abundant *Serratia* sp. SF1-3-1 cells (white), microaggregate formation of *Pseudomonas poae* RE*1-1-14 (yellow) and other bacteria (red). **B** and **C:** microaggregates of RE*1-1-14. Scale bars: 0-10 µm.

Effect of consortium strains on germination and biomass production in different habitats

Germination of oilseed rape seeds treated with single strains or consortium (Mix) was tested in germination filters and soil mixture. Results were normalized to sterilized control seeds, Co st. equals 100%. Further, as shown in pre-trials, formation of leaves was observed in germination filters. All single strain or consortium treated seeds showed on average an increased germination compared to Co st. (SD ± 4.8%) and non-sterilized, NaCl primed control seeds (Co ust., 101% ±6.2) Figure 17A. Only treatment with *Serratia* sp SF1-3-1 (112% ± 2.4) and Mix (115% ± 2.1) showed significantly higher germination rate than the sterile control (P≤0.004). Although all treated seeds showed better germination in sterile filters, drastic differences were observed in plant health and leaf formation (Figure 18).

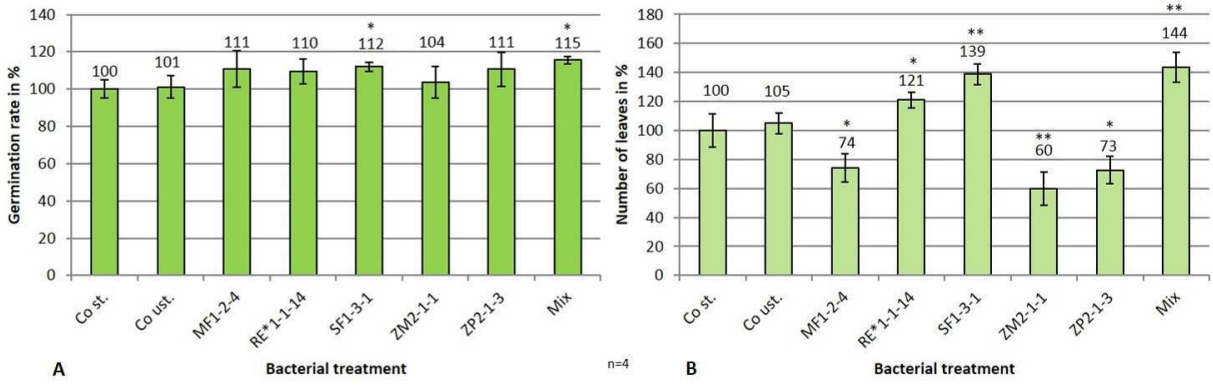

Figure 17: Germination rate (A) and leaf rate (B) of oilseed rape after 9 days in sterile germination filters.
*** P<0.02, ** P<0.003.** Co st = sterilized control seeds, Co ust = not sterilized, NaCl-primed control seeds.

As shown in Figure 17B, leaf formation in *A. nitroguajacolicus* MF1-24 (74% ± 9.7) and *Bacillus* sp ZP2-1-3 (73% ± 9.5) treatments was significantly (P<0.02) and of *C. wanjuense* ZM2-1-1 (60% ± 11.5) highly significantly (P=0.003) decreased in number compared to Co st.. Although, taking a closer look at the appearance of the plants (Figure 18), also plants of both controls, as well as some plants of MF1-2-4 and ZP2-1-3 treatments looked ill and leaves showed yellow coloring. Leaf rate of *P. poae* RE*1-1-14 treated plants was significantly higher (P<0.002) with 121% (± 5.3), which also resulted in healthier looking plants (Figure 18). Treatments with best leaf rate were found to be the treatments that had also the best germination rate: *Serratia* sp SF1-3-1 treatment with a leaf rate of 138.7% (± 7.2) and consortium treatment with a rate of 143.5% (± 10.6). Further, these positive effects showed to be highly significant compared to Co st. (P<0.002). The positive effect of the applied strains on germination and leaf formation was also reflected in optical appearance and health of the plants.

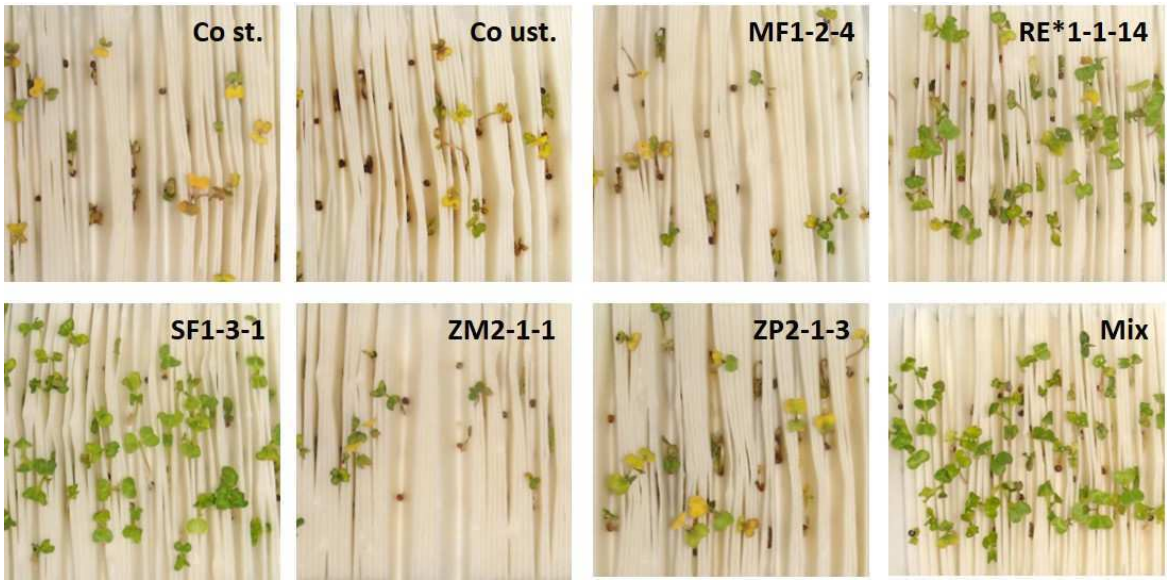

Figure 18: Plant health of different oilseed rape treatments after 14 days in sterile germination filters.

Results for germination in soil varied strongly. As shown in Figure 19, most single strain treatments, except for treatment with RE*1-1-14, resulted in decreased germination rate compared to Co st..

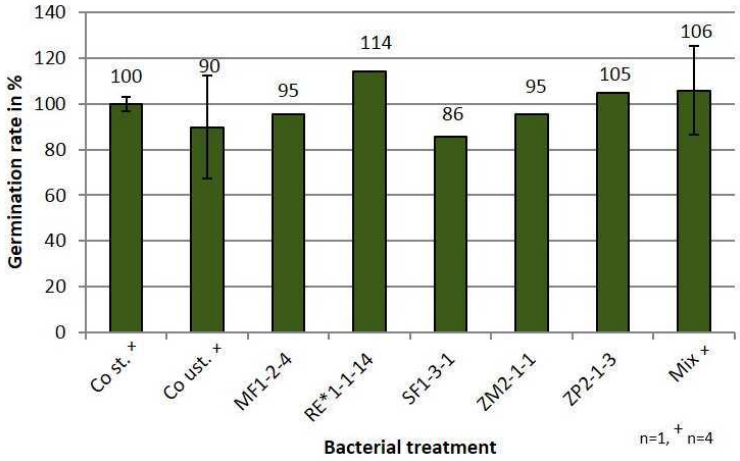

Figure 19: Germination of single strain and Mix treated oilseed rape seeds in soil mixture after 9 days. Co st = sterilized control seeds, Co ust = not sterilized, NaCl-primed control seeds.

As results need to be interpreted carefully all experiments need to be repeated to receive meaningful data. Concerning controls and Mix treatment, experiments were performed 4 times with results varying strongly. Interpreting data, on average Mix showed best germination behavior with 106% although results varied strongly which can be observed in a high standard deviation (± 19.3%). Also, fluctuations in germination rate of Co ust. with an average of 90% compared to Co st. (100% ± 3.2) was very high with ± 22.6%. Differences were not significant. Fluctuations in these results might be due to unknown influence factors present in the soil.

Optic evaluation of plant health and growth showed to be difficult, as differences between treatments were, except for number of plants, not obvious (Figure 20). Co st. and consortium plants were rated to look the healthiest.

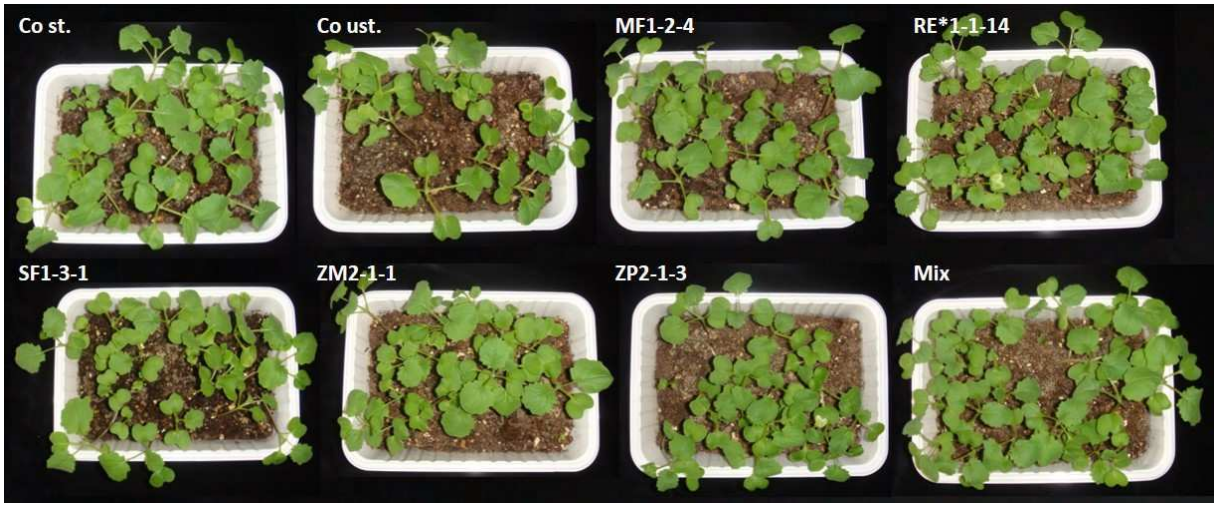

Figure 20: Different treated oilseed rape plants grown for 14 days in soil mixture. Co st = sterilized control seeds, Co ust = not sterilized, NaCl-primed control seeds.

This tendency was also observed in a first biomass evaluation (Figure 21). It was shown that, Co st. and Mix plants had grown more biomass compared to other treated plants. This results were observed for fresh weight (FW, A) as well as for dry weight (DW, B) with 3.0 g FW and 0.2 g DW for Co st. and 2.6 g FW and 0.2 g DW for Mix. Differences between consortium plants and other treatments emerged stronger in comparison of DW. Another interesting observation was the improved biomass production of sterilized control plants compared to non-treated seeds. Clearly, for a powerful statement and statistic data interpretation experiments need to be repeated.

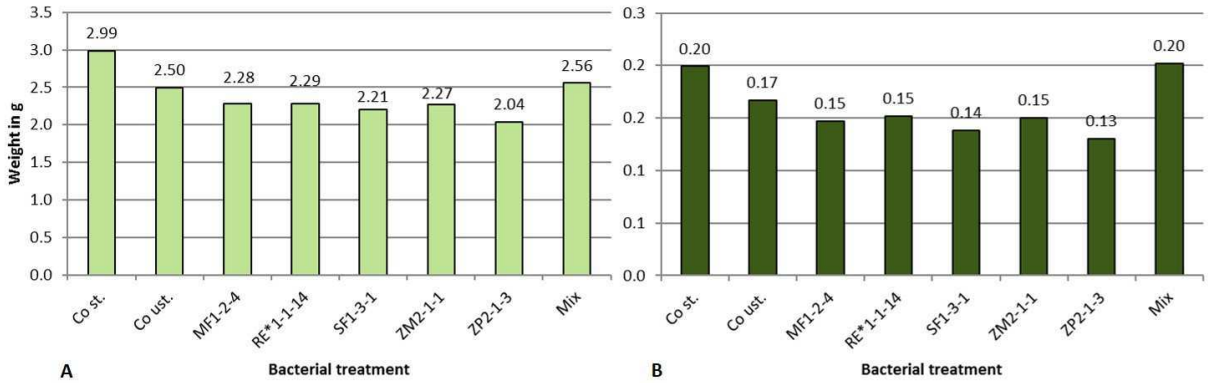

Figure 21: Biomass measurement of 14-day old oilseed rape plants. Fresh (A) and dry weight (B) was evaluated. Co st = sterilized control seeds, Co ust = not sterilized, NaCl-primed control seeds.

Effect of consortium strains on oilseed rape plants with dehydration stress

Different treated oilseed rape seedlings were tested for plant performance under dehydration stress. At the start of the experiment, it was observed, that germination rate in this particular experiment was lower than observed in other experiments before with 81.3% germination of Mix seeds and 89.1% for Co ust. seeds normalized to Co st. equals 100%. Figure 23 shows a timeline of oilseed rape plants during the experiment, at different days after sowing, from early stage (I) to late stage (VI). Plants of Co ust. turned out to be growing faster and larger than Co st. or Mix plants even within one week of water withdrawal (row II). It was also observed, that in this early stage after 8 days of starting the first dehydration stress, cotyledons of Mix and Co st. plants started to loose color. Cotyledons of Mix plants started to pale out, whereas Co st. cotyledons stained yellowish. After four more days of water withdrawal germ layers of Co st. and Mix had fallen off (row III, A and C). Also, Co ust. plants showed first symptoms of severe dehydration and wilt (B). Leaves started to enroll, hang and develop a silvery color. These symptoms were not noticed for other treatments. After watering, Co ust. plants were able to regenerate fully (Figure 23, IV, B). Exposed to continuing stress after short regeneration periods, same symptoms or behaviors as in early stages were observed for the treatments, although symptoms worsened. Co ust. plants wilted after the third stress period (row V, B), whereas leaves of Co st. and Mix plants kept losing the healthy dark green color and stained in light yellow and purple shades (row V and VI, A and C). Also, after continuing stress, leaves of Co ust. plants started to lose color (row VI, B). In general, Co st. and Mix plants stayed much smaller than plants of Co ust., which must be considered in the interpretation of occurrence of stress symptoms. Due to the difference of leave surfaces between treatments, transpiration was much higher for Co ust. plants and therefore is highly likely to have caused the appearance of wilt symptoms at time points, where Co st. and Mix plants did not show wilt symptoms at all. This might be supported by the results of biomass evaluation at the end of the experiment, after four stress phases (Figure 22). Co ust. was observed to have more FW (A) or DW (B) than other treatments, although only differences between Co ust. and Mix in both measurements were significant ($P<0.03$).

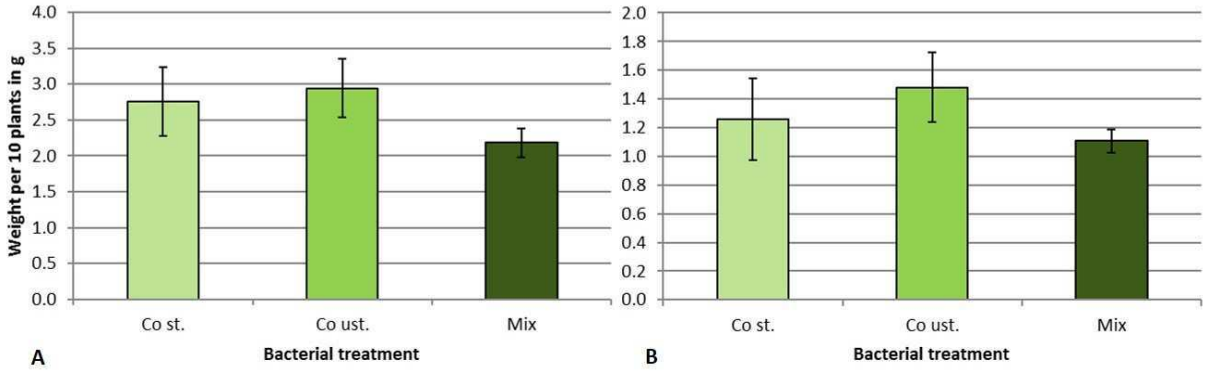

Figure 22: Biomass evaluation of oilseed rape plants after dehydration stress assay. A: fresh weight**, B:** dry weight. Co st = sterilized control seeds, Co ust = not sterilized, NaCl-primed control seeds.

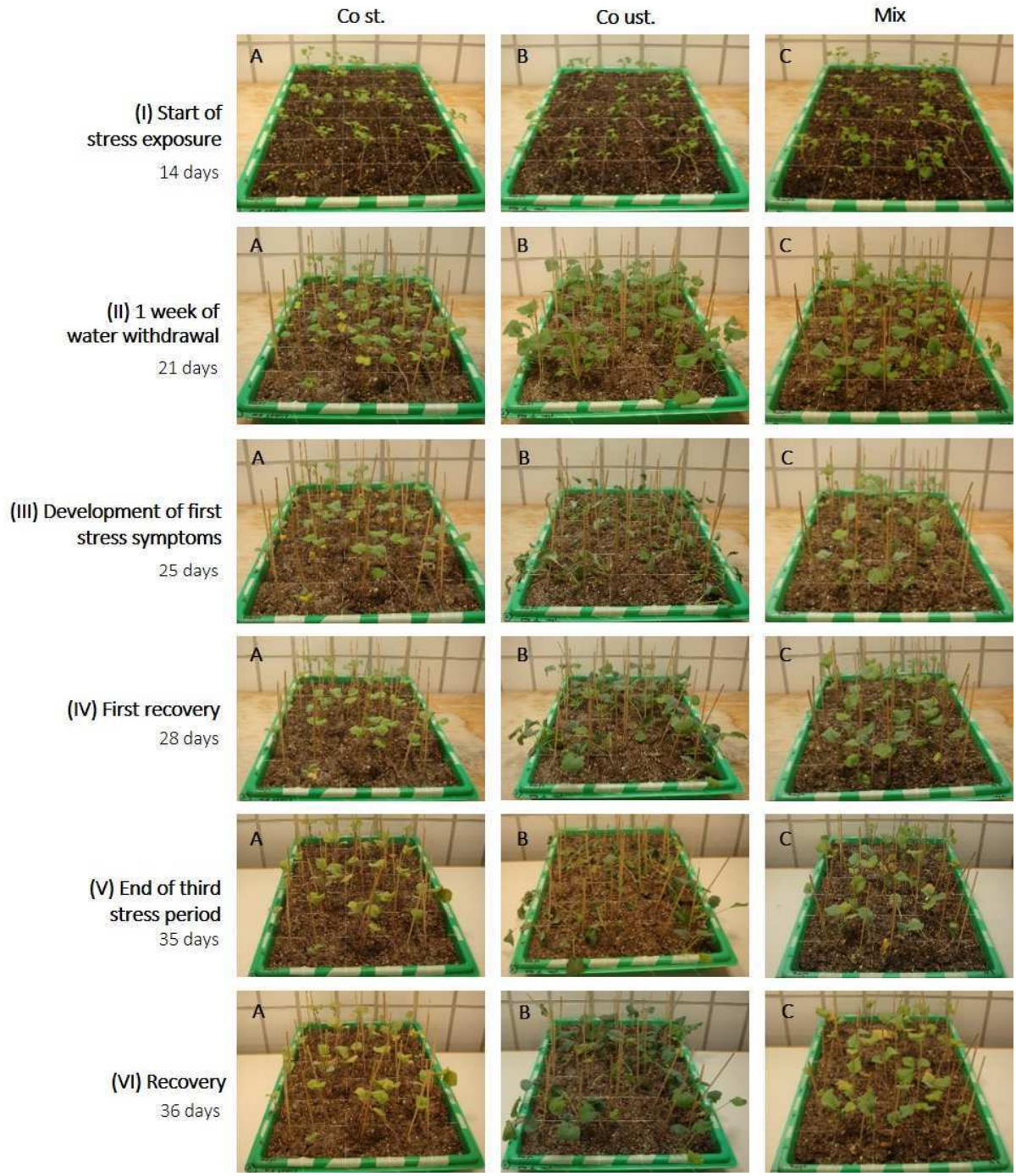

Figure 23: Observation of oilseed rape plants exposed to dehydration stress during different stages. Column **A**: sterile control, column **B**: non-sterile control, column **C**: Mix treatment. Rows **I** to **VI** show different time points after sowing during the experiment.

By comparison of other parameters, like number of leaves counted, Co ust. plants were found to have significantly more leaves with 5 on average (SD ± 0.19), compared to Co st. with 4 (SD ± 0.05) or consortium plants with 4 leaves (SD ± 0.24) per plant ($P \leq 0.001$). Further, evaluation of plant length (Figure 24A) also resulted in significant differences between Co ust. and both other treatments. Co ust.

plants were longest with 12.5 cm (SD ± 0.8) on average, followed by Mix plants with 11.14 cm (SD ± 0.74, P<0.05) and Co st. plants with 10.2 cm (SD ± 0.22, P<0.002) per plant. Measurement of stem thickness (Figure 24B) showed, that stems of Co ust. plants were thickest with a diameter of 1.15 mm (SD ± 0.14), followed by Co st. plants with 1.06 mm (SD ± 0.04) and Mix plants with the smallest diameter of 0.97 mm (SD ± 0.03). Differences between both controls and Mix plants were statistically significant (P<0.05).

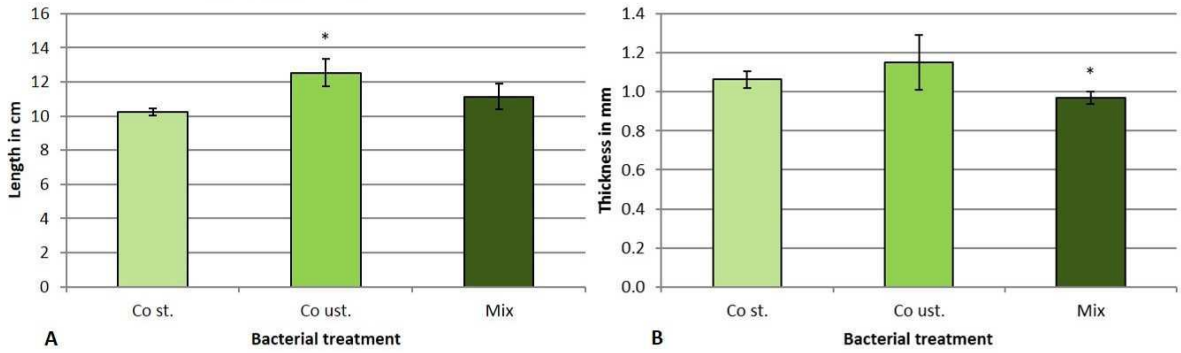

Figure 24: Evaluation of growth and vitality parameters of stressed oilseed rape plants. Average plant length (**A**) and stem thickness (**B**) at the end of dehydration stress experiment. * statistically significant, P≤0.0456.

3.3 Influence of bacteria on maize and sorghum plants

In pre-trials it was observed, that sorghum seeds are more sensitive to dehydration in the first days of germination than maize or oilseed rape and therefore sufficient amount of water is needed to enable evenly germination in germination filters. Germination tests in bell jars showed the best germination rate for seeds primed with OD_{600}=1. Therefore, OD_{600} of 1 was considered as standard priming density in all further plant experiments. In all following experiments, *S. rhizophila* e-p17 and P69 were used for sorghum seed treatment and for maize e-p17, P69 and *B. subtilis* B2g.

Alteration of bacterial abundance on bio-primed seeds and roots of maize and sorghum

CFU of OD_{600}=1 of all three strains were determined and varied between strain families. Both *Stenotrophomonas* strains were calculated to have log_{10} 8.9 CFU per mL at an OD_{600} of 1, with a standard deviation of ± 0.37 for P69 (n=9) and ± 0.5 for e-p17 (n=12). For *B. subtilis* B2g, on average log_{10} 8.2 CFU per mL OD_{600}=1 were observed (SD ± 0.2, n=13).

Bacterial abundance on seeds and roots were detected for single strain application and consortium application, further called Mix. Abundances on roots were detected after 2 weeks of growth in pouches. For maize, on average one seed was shown to host between log_{10} 6.5 to 6.8 bacteria (Figure 25A). Optically, the consortium strains could only be differentiated into *Stenotrophomonas* spp. and *Bacillus subtilis* B2g. Counting of the Mix resulted in an average abundance of log_{10} 6.6 CFU (SD ± 0.40) of *Stenotrophomonas* spp. and 6.1 CFU (SD ± 0.25) of B2g per maize seed. By observing maize roots, single strain priming with B2g resulted in the lowest abundance with log_{10} 4.5 (SD ± 0.48) CFU per g root. *Stenotrophomonas* strains had the highest abundance if applied as single strains with log_{10} 6.8 CFU per g root for e-p17 (SD ± 0.21) and log_{10} 7.0 (SD ± 0.24) for P69 (Figure 25B). Strains applied in consortium showed also a high total abundance of log_{10} 6.2 (SD ± 0.3) consisting of log_{10} 6.2 CFU of *Stenotrophomonas* spp. (SD ± 0.32) and log_{10} 5.2 CFU of B2g (SD ± 0.43) per g root.

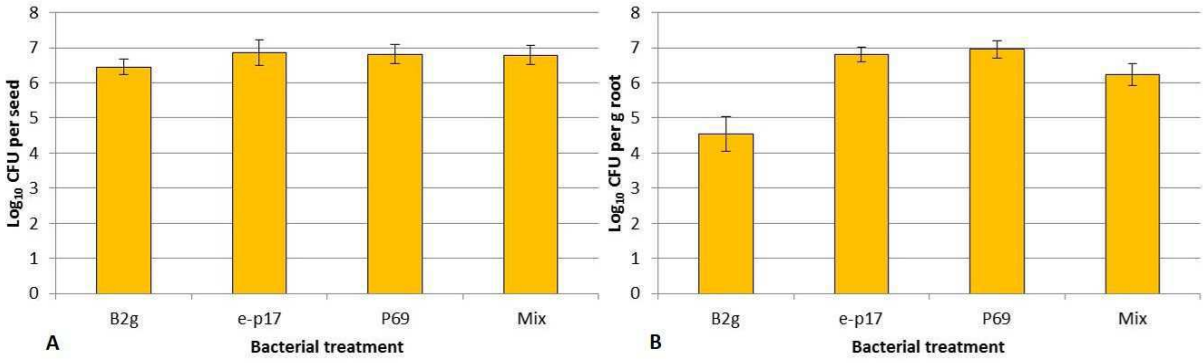

Figure 25: Bacterial abundance on maize seeds (A) and roots (B) after single strain or consortium treatment.

To verify identity, strains of maize seeds and roots were re-isolated, BOX-PCR was performed and the patterns were compared to those of the original consortium strains. Therefore, presence of e-p17 and B2g on roots and seeds could be confirmed (data not shown).

For sorghum, log_{10} 6.0 to 6.2 CFU could be re-isolated per seed after priming (Figure 26A). A phenotypic differentiation between e-p17 and P69 for consortium application was not possible. Counting of re-isolated CFU of two-weeks old sorghum roots primed with *Stenotrophomonas* spp. resulted in very similar numbers per g root, with log_{10} 7.0 (SD ± 0.37) CFU for e-p17 and log_{10} 7.2 (SD ± 0.95) CFU for P69 (Figure 26B).

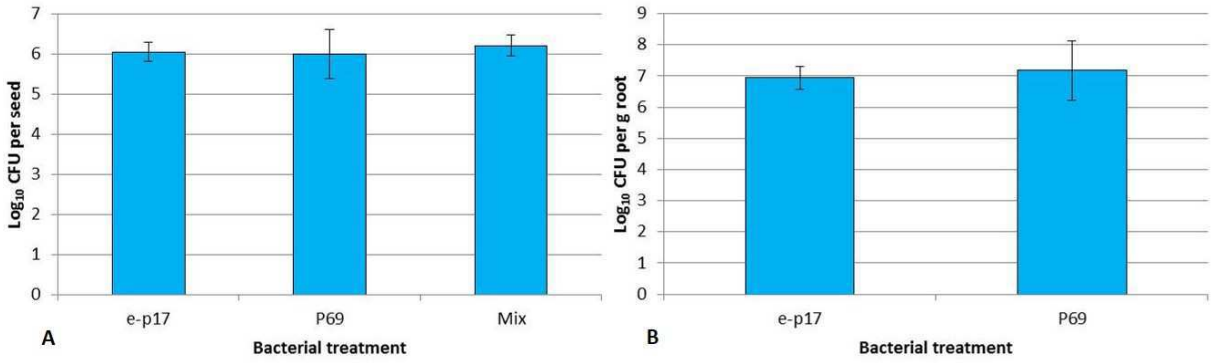

Figure 26: Bacterial abundance on sorghum seeds (A) and roots (B) after single strain or consortium bio-priming.

Effect of consortium strains on germination and biomass production in different habitats

Germination of treated and untreated maize and sorghum seeds was observed in germination filters and soil. All results were normalized to sterile and NaCl-primed control (Co st.) seeds with 100%, which were sterilized and treated the same way bacteria treated seeds were. Single strain treated maize seeds showed no significant in- or decrease in germination rate compared to control seeds if incubated in germination filters. For Mix treated seeds germination rate decreased significantly (P=0.04) in sterile environment (Figure 27A).

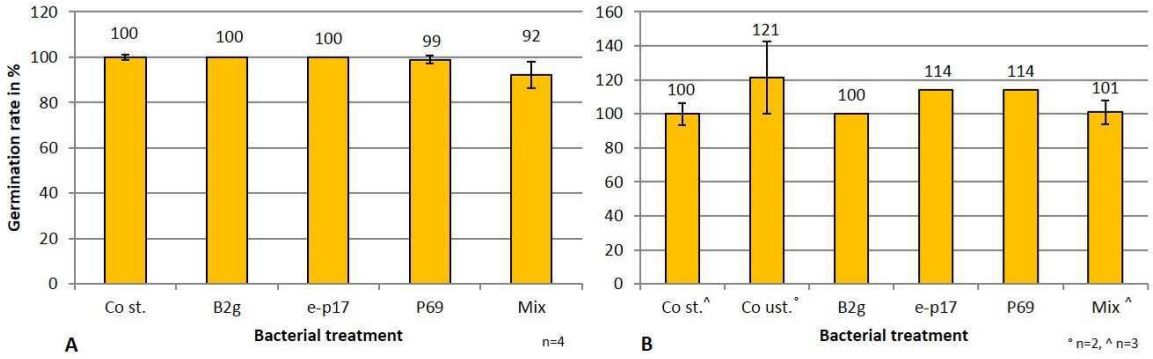

Figure 27: Germination of different treated maize seeds in germination filters after 3 days (A) and in soil after 1 week (B). Co st = sterilized control seeds, Co ust = not sterilized, NaCl-primed control seeds. * P<0.04 Note: scales are different for germination pouch and soil experiments

Whereas, germination experiments in soil mixture showed same (B2g) or increased germination for treated seeds compared to sterile control seeds (100%, SD ± 6.4) (Figure 27B). Best germination rate was observed by untreated control seeds with 121% (SD ± 21.4) followed by e-p17 and P69 treated seeds. Mix treatment resulted in an average germination rate of 101% (SD ± 6.9). Comparison of sterile and unsterile control seeds with Mix showed no significant differences in germination rates (P>0.05). For single strain treatment, no statistical statements can be made.

Germination of treated sorghum seeds in comparison to sterile and NaCl-primed Co st. seeds (100%, SD ± 1.9) in germination filters, resulted in best germination rate with 101% (SD ± 5.0) for Mix primed seeds followed by e-p17 primed seeds with 99% (SD ± 7.1) and unsterile control and P69 primed seeds with each 96.6% (Co ust: SD ± 2.2, P69: SD ± 12.1) germination rate (Figure 28A). Differences in germination rate in filters are not significant for all applications tested. Germination rate of sorghum seeds differed if seeds were planted in soil. Germination was evaluated after one week in soil-sand-vermiculite mixture (Figure 28B). Best results were obtained for Co ust. with 106% (SD ± 10.5) followed by P69 treated seeds with 105% and Mix seeds with 102% (SD ± 12.3). Germination rate of e-p17 primed seeds was equal to Co st. (100%, Co st: SD ± 2.8). For single strain treatment no statistics could be performed. Comparison of both controls and Mix showed no statistically significant difference for sorghum germination in soil.

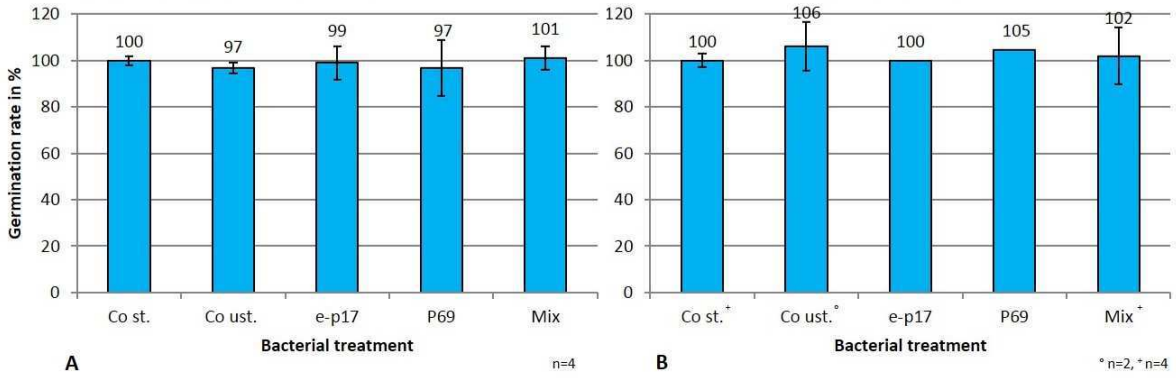

Figure 28: Germination of different treated sorghum seeds in germination filters after 3 days (A) and soil after one week (B) cultivation. Co st = sterilized control seeds, Co ust = not sterilized or primed control seeds.

Further, the biomass production of different treated sorghum or maize plants was investigated in different growth habitats. After 14 days, biomass of green parts and roots in pouches was investigated. Therefore, the fresh weight (FW) of green parts and roots was measured (Figure 29). Taking a closer look on the maize plants, consortium plants showed the highest weight concerning green part biomass production with 6.5 g per 10 plants. Also, this weight differed significantly from the control plants (P=0.02), which were observed to have the second highest biomass for green parts with 5.4 g per 10 plants. Green parts of P69 primed plants weighted 5.2 g and B2g primed plants 5.1 g per 10 plants. Green part and root growth of e-p17 primed plants was decreased, but only lower root biomass was calculated as statistically significant (P<0.04) compared to Co st. plants. Roots of P69 primed plants showed a high significant increase in root biomass with 14.7 g per 10 plants compared to the control

with 7.9 g per 10 plants (P<0.0009, Figure 29A). B2g root biomass did not significantly differ from control plants. For sorghum (Figure 29B) no significant difference between control plants in either green part biomass production or root biomass production was found, with both single strain treatments showing higher biomass production than the control.

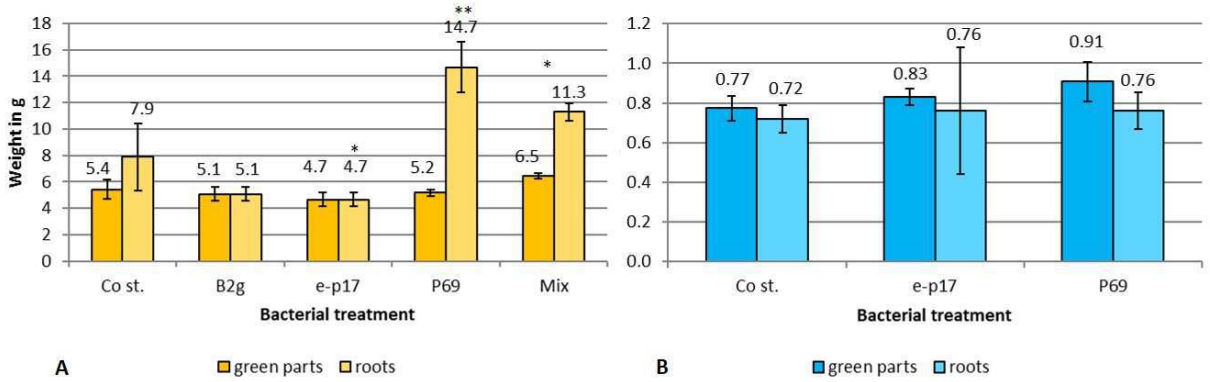

Figure 29: Biomass evaluation of green parts and roots per 10 plants for maize (A) and sorghum treatments (B) after 14 days of growth in pouches. Co st = sterilized control seeds, Co ust = not sterilized or primed control seeds. * P<0.04, ** P=0.0009. Note: scales are different for maize and sorghum experiments.

In addition, differences in biomass production of green parts in germination filters and soil was tested for several maize and sorghum treatments. Figure 30 gives an overview of biomass production for maize plants in germination filters (A+B) and soil (C+D).

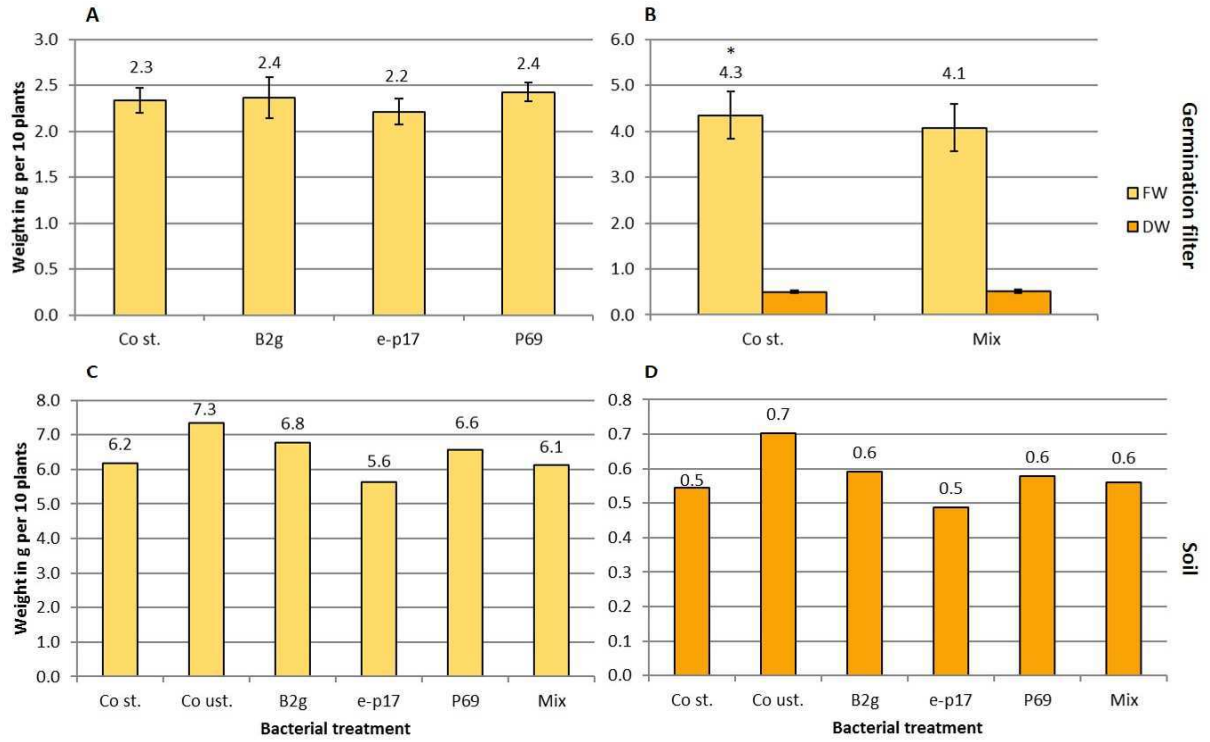

Figure 30: Biomass evaluation of green parts of different treated maize plants in germination filters (A+B) and soil (C+D). **A:** fresh weight of 9 day old seedlings grown in germination filter. **B:** fresh and dry weight of 14 day old seedlings grown in germination filter (P<0.04). Fresh weight (**C**) and dry weight (**D**) of 14 day old seedlings grown in soil mixture. Note: scales are different for experiments.

As weight evaluation after 9 days in germination filters (Figure 30A) has shown, P69 and B2g priming result in a slight, but not significant increase in fresh weight, whereas e-p17 priming showed a slightly decreased biomass production compared to the control. Comparison of FW for Mix and control after 14 days (Figure 30B) showed significantly higher biomass for control plants (P<0.04), whereas dry weight (DW) evaluation resulted in higher weight for Mix plants. Although, differences in DW measurements were not significant. For biomass production in soil no statistical analysis could be performed. First results showed, that concerning FW, non-sterilized plants had highest biomass values, followed by B2g and P69 primed plants and sterile control plants (Figure 30C). Mix and e-p17 primed plants had the lowest biomass. Whereas, in terms of DW, all treatments, except for e-p17 plants, resulted higher biomass weights than Co st. plants (Figure 30, D).

Testing different treatments for sorghum, results of biomass measurements showed that fresh weight of e-p17 and P69 primed plants, as well as unsterile control plants was significantly higher (P<0.02) if grown for 14 days in germination filters compared to the weight of sterile control plants (Figure 31A). Fresh weight of Mix treated plants was found to be higher than Co st. plants but the difference was not significant. Regarding DW values of the same plants, although all treatments showed higher biomass production than the sterile control, only the increase of dry weight for Mix plants was statistically significant (P<0.02, Figure 31B). For biomass production of plants, that were grown for 21 days in soil, no statistics could be calculated. However, first results show an increased biomass production for Mix treatment compared to both controls in FW (Figure 31C) and DW (D). Single strain treatments resulted in slight reduced biomass production compared to both controls.

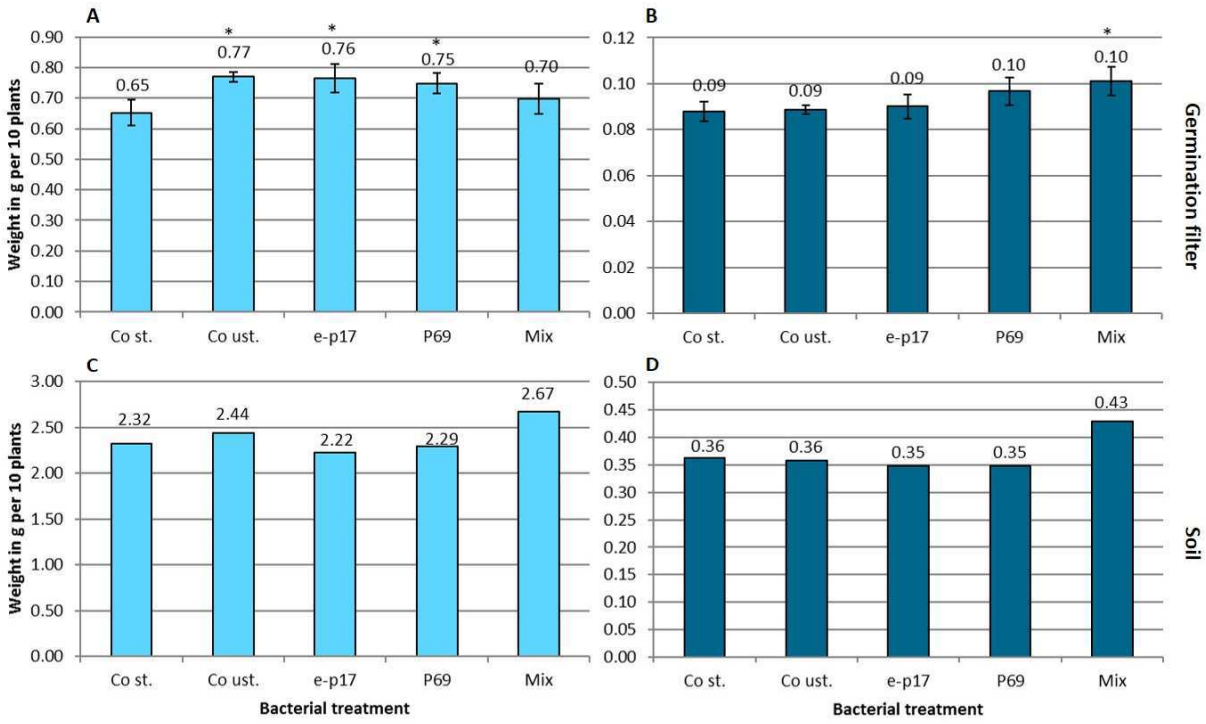

Figure 31: Biomass evaluation of green parts of different treated maize plants in germination filters (A+B) and soil (C+D). Fresh weight (**A**, P<0.02) and dry weight (**B**, P<0.02) of 14 day old seedlings grown in germination filter. Fresh weight (**C**) and dry weight (**D**) of 3 weeks old seedlings grown in soil mixture. Note: scales are different for experiments.

Effect of consortium strains on maize and sorghum plants with dehydration stress

As pre-trials had shown, desiccation stress assay could not be performed in a plant growth chamber due to the unstable conditions for dehydration. Therefore, stress assays were performed in the green house with daily randomization of plants to enable equal conditions for all treatments. Plants were observed for 45 days under stress conditions. Focus was on emergence of stress symptoms like rolled up or kinked leaves, the possibility of regeneration after stress via watering and mainly differences in the categories mentioned between treatments.

Figure 33 gives an optic overview of the changes in shape of maize plants during the experiment, counting the days from sowing at an early time point (I start of stress exposure) to a late time point (VI recovery after six stress periods). From the start, it was observed, that Mix treated plants were smaller and had a paler green color than control plants which could best be seen in I, showing plants 1 week and II, showing plants 12 days after of sowing (Figure 33). For the first stress period, trays were watered last at time point I and did not start to show stress symptoms until one week passed by. Plants showed heavy dehydration symptoms after 10 days of dehydration (Figure 17, III A-C). It could be seen that both controls show clear symptoms of stress: plants looked wilted, had enrolled, silvery or colorless looking leaves, whereas Mix plants didn't show any stress symptoms. Although, it was noticed, that soared up from the stem (Figure 32, A and C) to the nervures (A and B) consortium plants were starting to stain purple. Plants were watered equally and control plants were able to fully regenerate the day after watering (IV). 32 days after sowing and repeated dehydration stress (six times) showed severe stress symptoms (V). Control plants as well as Mix plants had enrolled leaves. Conspicuous was, that leaves of both controls kinked, whereas leaves of consortium plants stood upright even under strong dehydration stress. Also red or purple coloring of the Mix plants had spread heavily into the whole plants. It was observed, that with continuous stress phases, plants were permanently damaged, although they were able to recover to a certain degree after watering (VI). Kinked leaves of both control treatments stayed kinked after watering, peaks of leaves started to turn brown and green color of leaves started to fade out, especially for sterile control and Mix plants.

Figure 32: Purple coloration of Mix treated plants. A: whole plants in a row, **B:** tip of a leaf, **C:** coloration of stem.

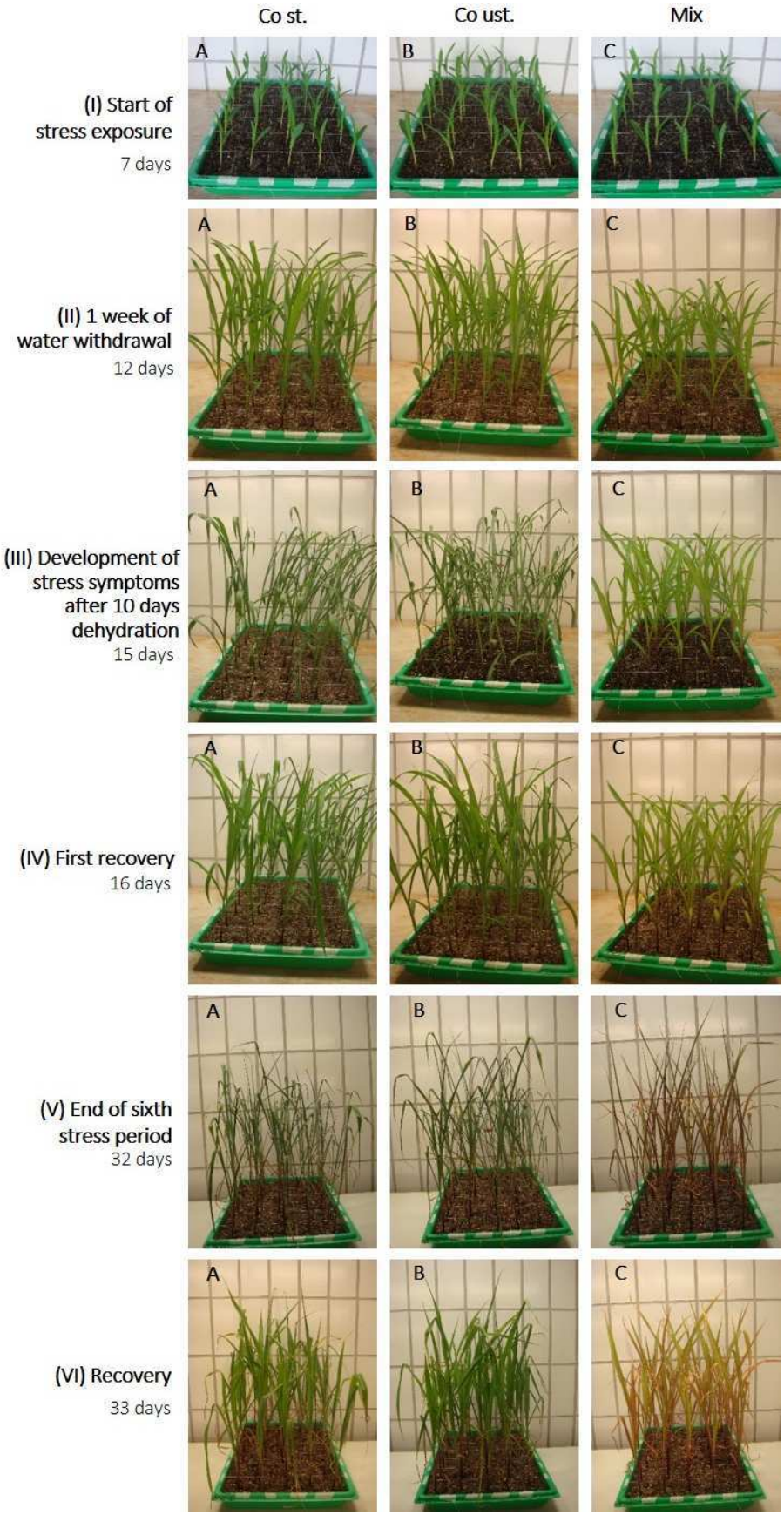

Figure 33: Time series of maize plants exposed to dehydration stress. Column **A**: sterile control, column **B**: non-sterile control, column **C**: Mix treatment. Rows **I** to **VI** show different phases during the experiment. Days show time point after sowing.

On day 45 after six times stress induction plants were rated for vitality parameters and harvested to determine biomass differences. The number of leaves per plant was counted. Mix treated plants had significantly more leaves per plant (P<0.003), with 6 leaves (SD ± 0.06) in average than sterile control plants with 5 leaves (SD ± 0.32) or non-sterile control plants with 5 leaves (SD ± 0.19). Figure 34 shows the average plant length (A) and stem thickness (B) of the tested maize plants. Plants of both controls were measured to be higher (Co st.: 51.91 ± 2.07 cm, Co ust.: 61.03 ± 4.14 cm) and have thicker stems (Co st.: 4.16 ± 0.18 mm, Co ust.: 4.31 ± 0.06 mm) than Mix plants (length: 55.7 ± 0.56 cm, thickness: 3.69 ± 0.14 mm). Differences between both controls and Mix plants were statistically significant (P<0.05).

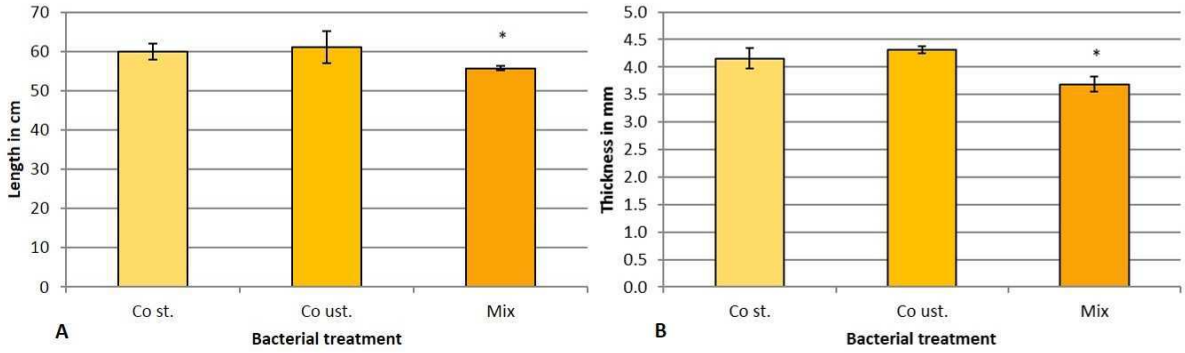

Figure 34: Evaluation of growth and vitality parameters of stressed maize plants. Average plant length (A) and stem thickness (B) at the end of the stress experiment.

Evaluation of biomass showed, that plant size correlates with FW and DW of maize plants (Figure 35). For both controls higher biomass was measured for FW (Figure 35A), as well as for DW (B). Only differences between Co ust. and Mix are statistically significant for both DW and FW (P<0.005).

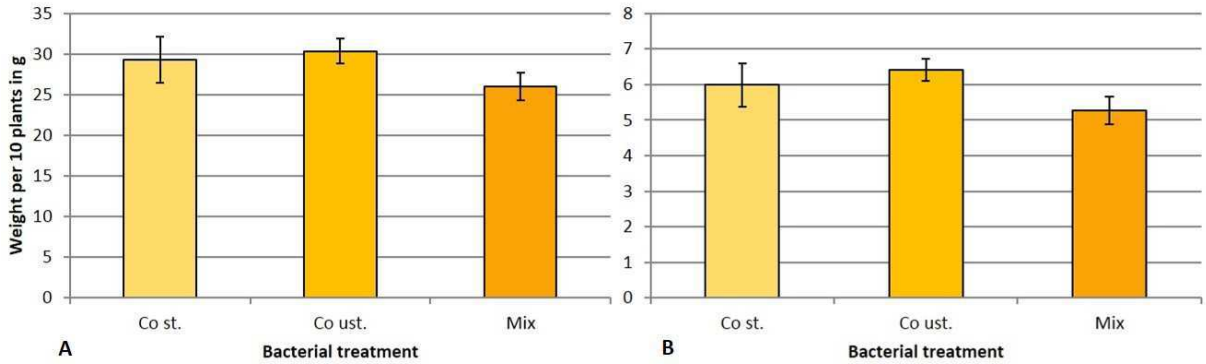

Figure 35: Biomass evaluation of maize plants after dehydration stress assay. A: fresh weight, **B**: dry weight. Note: scales are different for FW and DW.

A time line of the three different treated sorghum plants during various stages in the dehydration stress experiment can be found in Figure 36.

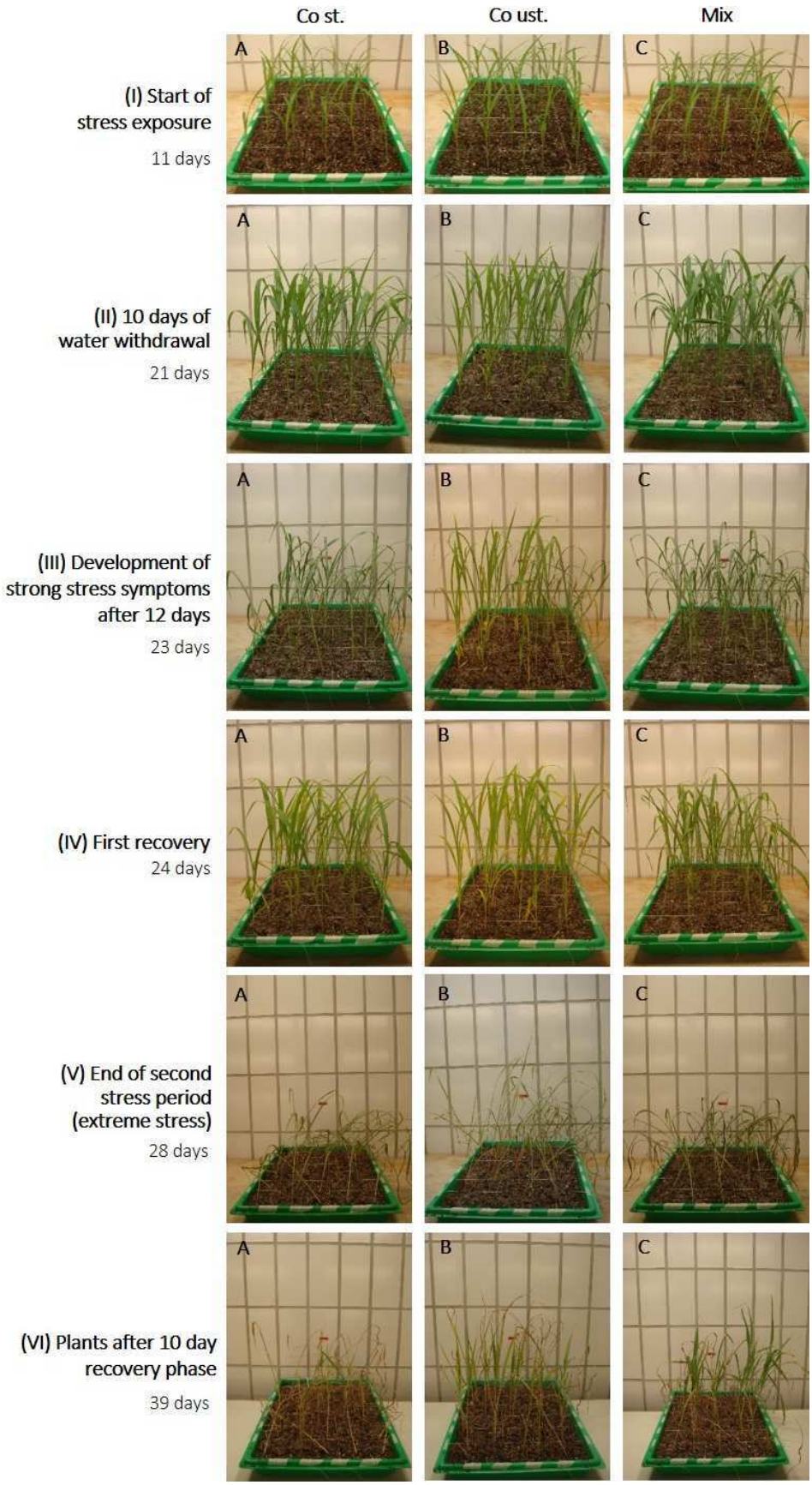

Figure 36: Time series of sorghum plants exposed to dehydration stress. Column **A**: sterile control, column **B**: non-sterile control, column **C**: Mix treatment. Rows **I** to **VI** show different phases during the experiment. Days show time point after sowing.

At early stages of the experiment, without stress after 11 days of sowing, no remarkable differences in growth were observed (Figure 36, I). As plants were exposed to light dehydration stress, appearance of stress symptoms were observed in different extent after 10 days of water withdrawal. Co st. and Mix plants showed symptoms of wilt with hanging leaves (II), whereas Co ust. leaves stood upright. Although, it was also observed that Co ust. leaves started to fade and lose the dark green color. Stress symptoms intensified with ongoing dehydration after 12 days without watering (Figure 36, III). Leaves started to enroll and appeared silvery-pale. Symptoms were worse for Co st. and Mix treated plants. Co ust. plants started to enroll leaves and turn into shades of light green and yellow. Regeneration from this state was possible for all three treatments (IV). Although, leaves of all treatments did not regain their dark green color and stayed light-green with shades of yellow. Further, consortium treated plants still had some enrolled leaves. After induction of extreme dehydration stress (V), plants of all trays looked hay-like and dead. In the next phase, it was observed if plants were able to survive this extreme condition and build up biomass again. After a longer regeneration phase of 10 days of regularly watering, several plants were found to have survived and build up new biomass (VI). Counting survived plants per tray, Co st. was the treatment with fewest plants survived with 21 dead and 11 living plants. From Mix treated plants 50% (16 plants) survived the extreme stress. Co ust. had the best survival rate with only 9 dead and 23 living plants. Although, it was noticeable that survived Mix plants built up more biomass and looked healthier and stronger than survived Co ust. plants (Figure 36, VI, B and C). Further, color of Mix leaves was a darker green than those of Co ust. leaves. As described for maize plants, at the end of the experiment several parameters were evaluated to observe differences between treatments in more detail.

The average number of leaves per plant did not differ significantly between treatments and was for all about 5 leaves per plant. Also, plant length was measured and values reached for controls from 41.8 cm for Co st. plants (SD ± 1.6) to 44.0 for Co ust. plants (SD ± 0.9) (Figure 37A). Mix plants were longest with an average length of 44.6 cm (SD ± 1.3). Differences between Co st. and Mix were statistically significant ($P<0.04$). Measurement of stem thickness showed significant differences between both controls and Mix plants ($P<0.03$, Figure 37B). On average, stems of Co st. plants were 1.88 mm (SD ± 0.15) thick, followed by Co ust. plants with 2.0 mm (SD ± 0.15) and Mix stems with a diameter of 2.22 mm (SD ± 0.05).

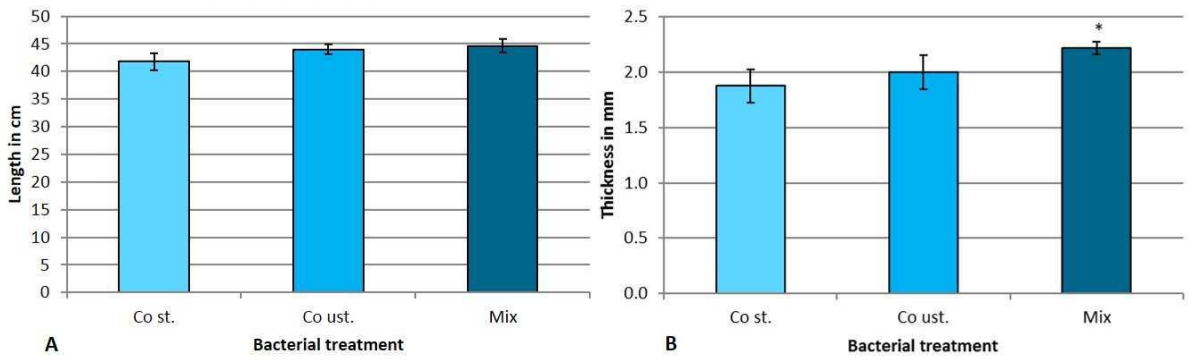

Figure 37: Evaluation of growth and vitality parameters of stressed sorghum plants. Average plant length (**A**) and stem thickness (**B**) at the end of dehydration stress experiment.

Biomass was investigated from all plants, including living and dead plants (Figure 38). Therefore, it should be noticed that even though dried out plants are included into the FW, it is here also seen as an indicator for survival and stress protection. FW of Co st. averaged 4.22 g per 10 plants (SD ± 0.49) followed by Co ust. with 5.03 (SD ± 0.68) and Mix with 8.09 g per 10 plants (SD ± 1.21). DW measurement resulted in similar tendency (Figure 38B). Biomass of control plants does not differ significant with 1.96 g (SD ± 0.17) for Co st. and 2.01 g (SD ± 0.11) for Co ust. plants. Mix plants had the highest amount of DW with 2.49 g per 10 plants (SD ± 0.28). As can be seen in both graphs, biomass of Mix plants is significantly increased in comparison to control plants with P<0.005 for FW and P<0.02 for DW calculation.

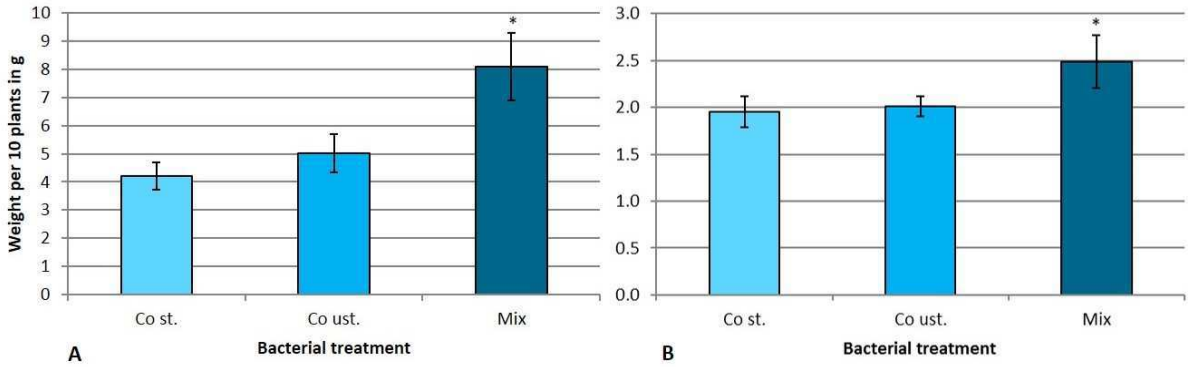

Figure 38: Biomass evaluation of sorghum plants after dehydration stress assay. A: fresh weight, **B**: dry weight. * significant difference (FW: P<0.005, DW: P<0.02)

Field trials and amplicon analysis of maize rhizosphere

For better understanding of how single strain application of *Stenotrophomonas rhizophila* P69 influences structure and diversity of soil and rhizosphere microbiomes, maize samples of different treated seeds have been investigated. Bacterial and fungal communities of two locations were studied, to get an idea of effects under different environmental conditions and climate or soil influences.

Influence on bacterial community

Between 10,022 and 202,027 sequences were obtained per sample via Illumina MiSeq sequencing, with a total count of 2,332,288 sequences. Read numbers were normalized to 10,022 per sample before further analysis. 3% genetic dissimilarity was set as a cut off level of divergence for operational taxonomic unit (OTU) clustering. Sequencing did not reach saturation, as can be seen by rarefaction curves of samples (Figure 39).

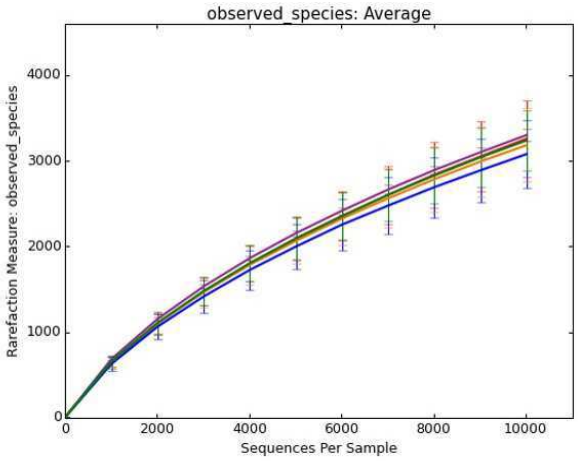

Figure 39: Rarefaction curves of bacterial sequences of maize rhizosphere and soil samples.

43.8 to 52.2% of the estimated OTUs were covered in the sequencing procedure. Detailed information about coverage and estimated number of OTUs, as well as α diversity is given in Table 5. As is can be seen via Shannon indices, treatment with P69 had no influence on α diversity.

Table 5: Species richness of bacterial communities of maize rhizosphere and soil samples from Illumina MiSeq sequences normalized to 10,022.

Origin	Habitat	Treatment	Shannon Index* (H')	Rarefaction (no. of OTUs)	Chao 1 (no. of OTUs)	Coverage (%)
Melk	Maize rhizosphere	STR-	9.5 (± 1.12)	2,272 (± 350.6)	5,924 (± 202.7)	46.8 (± 5.45)
		Co-	10.0 (± 0.43)	2,931 (± 412.9)	6,401 (± 15668)	46.8 (± 4.54)
		STR+	9.7 (± 0.67)	2,918 (± 210.2)	6,659 (± 253.9)	43.8 (± 1.71)
		Co+	9.7 (± 0.28)	2,279 (± 190.3)	5,892 (± 597.8)	47.3 (± 1.79)
	Soil	-	10.5 (± 0.05)	3,345 (± 73.4)	7,418 (± 209.3)	45.1 (± 0.5)
Mitterdorf	Maize rhizosphere	STR-	10.5 (± 0.16)	3,383 (± 145.2)	7,418 (± 570.5)	45.1 (± 2.24)
		Co-	10.5 (± 0.21)	3,582 (± 118.4)	8,149 (± 815.6)	44.2 (± 2.97)
		STR+	10.7 (± 0.1)	3,556 (± 69.0)	7,428 (± 358.8)	48.0 (± 1.73)
		Co+	10.7 (± 0.04)	3,585 (± 88.9)	7,571 (± 385.4)	47.4 (± 1.21)
	Soil	-	10.5 (± 0.1)	3,255 (± 37.3)	6,252 (± 373.5)	52.2 (± 2.8)

** the higher the number the more diversity*

Figure 40 shows the number and percentage of shared and individual OTUs of both locations. Shared OTUs amount to 43% and a total of 4,457.

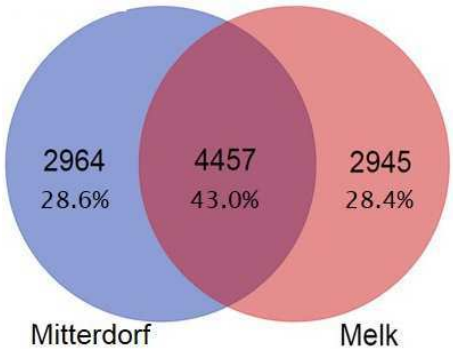

Figure 40: Venn diagram of shared and individual OTUs of Melk and Mitterdorf.

Principal coordinate analysis (PCoA) of all samples of the two locations showed, that samples from the same location were more similar to each other than same treated samples from different locations. Also, except for soil samples, different treatments do not cluster together and show high varieties within the same treatments (Figure 41).

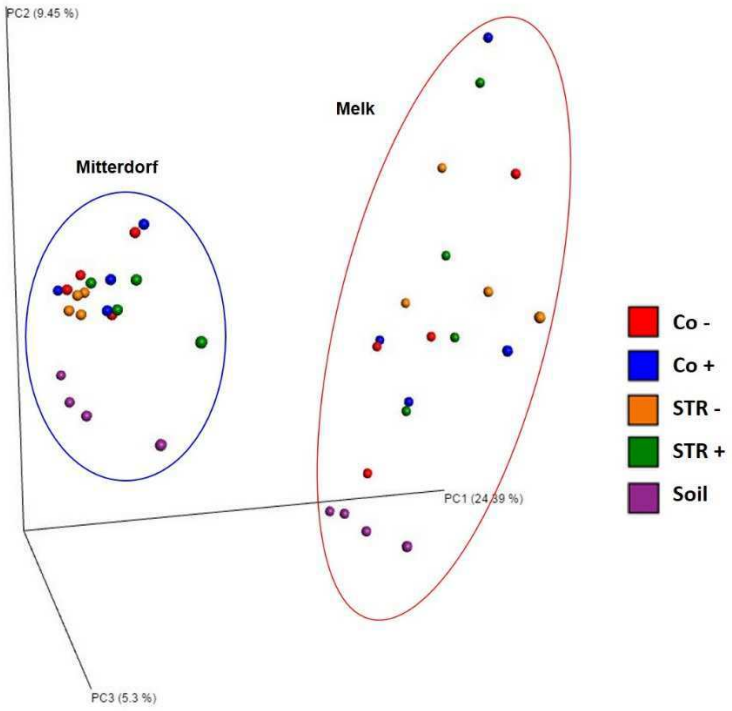

Figure 41: Principal coordinate analysis plot of bacteria in rhizosphere and soil samples from Mitterdorf and Melk. Co: rhizosphere of non-treated, NaCl primed maize plants, **STR:** rhizosphere of *S. rhizophila* P69 treated plants, **+/-:** with or without stripper coat.

Cluster formation of all samples from Mitterdorf and Melk is also shown in 2D PCoA in Figure 42. Due to these results, locations were analyzed separately in all further steps.

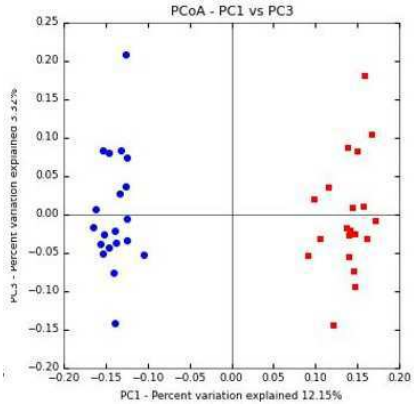

Figure 42: 2D PCoA of bacterial samples from Mitterdorf (blue) and Melk (red).

For comparison of shared and individual OTUs in the two sampling sites, replicates of the same sample were merged and OTUs were filtered for presence in at least 3 out of 4 replicates. Co+ had the least amount of OTUs in both locations with 2,522 OTUs in Melk and 3,154 OTUs in Mitterdorf. Rhizospheres of non-stripped maize (Co-) had a total number of 4,015 OTUs (Melk) and 4,850 OTUs (Mitterdorf), whereas 4,367 (Melk) and 4,255 (Mitterdorf) OTUs were identified in non-stripped P69 treated (STR-) rhizospheres. 4,778 and 4,003 OTUs were found in STR+ rhizospheres and 4,961 and 4,404 OTUs in soil of Melk and Mitterdorf samples. Analysis of OTUs showed, that all samples had individual OTUs (specialists), which were only present in one type of sample or only shared between controls or STR samples (Figure 43). Melk samples shared a core microbiome consistent of 1,838 OTUs and Mitterdorf samples share a core of 1,871 OTUs. A high number of OTUs, namely 878 for Melk and 687 for Mitterdorf, are only present in soil samples. Further, 27 OTUs in Melk and 61 OTUs in Mitterdorf are specialists for controls and shared only between Co- and Co+ samples. For STR treatments 171 (Melk) and 89 (Mitterdorf) OTUs were found to be specialists and shared between STR samples only. In further analysis, core microbiome and specialists need to be analyzed in detail to find out about abundant families and their possible tasks in the root- or soil system.

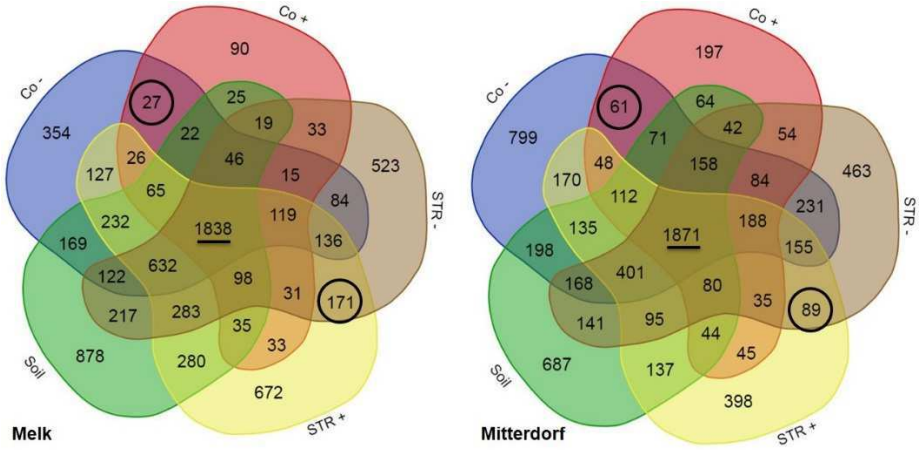

Figure 43: Venn diagram of shared and individual OTUs of different rhizospheres and soil of both locations. Core microbiome is underlined black. Black circles show shared specialists for control rhizospheres and P69 treatments, respectively. **Co:** rhizosphere of non-treated maize plants, **STR:** rhizosphere of *S. rhizophila* P69 treated plants, **+/-:** with or without stripper coat.

Taxonomic composition of samples from Melk and Mitterdorf was compared at phylum level. It must be noted, that taxonomic comparison is only possible in sense of relative abundance within samples, not absolute abundance in or between samples and locations. Figure 44 shows the most abundant phyla, each representing at least 1% of reads. All phyla that were less abundant than 1% are summarized in "Other". In both locations Proteobacteria represented the most abundant phylum, being relatively more dominant within Melk samples. Within locations this phylum is most abundant in STR- in Melk and in STR+ in Mitterdorf, relatively. Bacteroidetes and Verrucomicrobia showed to be more dominant within Mitterdorf samples. OTUs from candidate phylum WS3 (Wurtsmith aquifer Sequences-3), which were first described 1998 by Dojka *et al*., is also present in Melk samples, but abundance within samples were less than 1%. At first sight, phyla do not show strong differences comparing samples of one location.

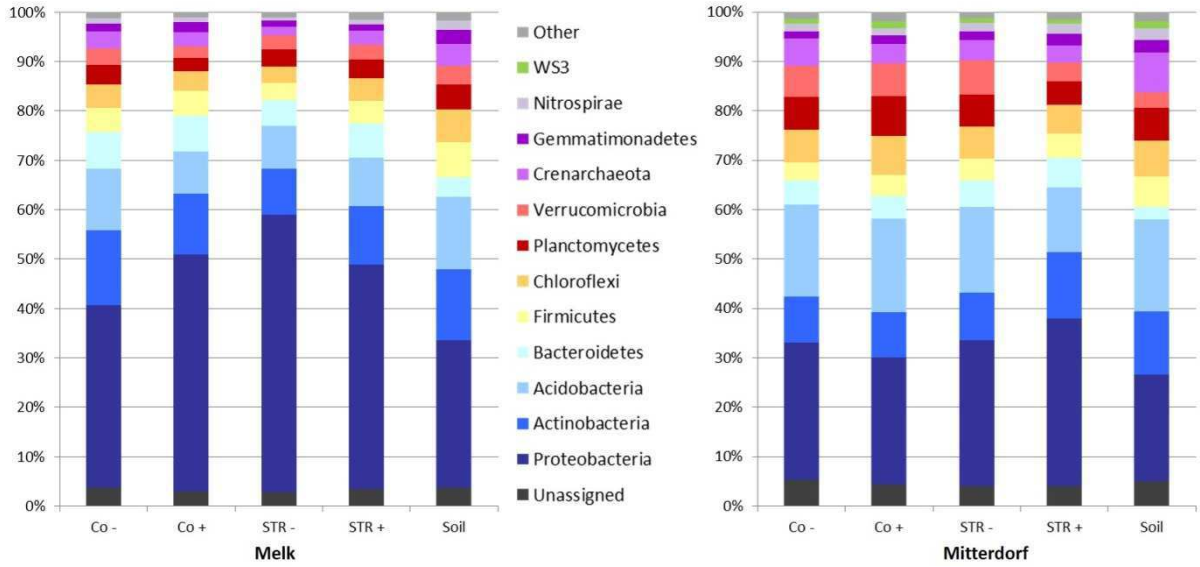

Figure 44: Relative composition of most abundant phyla of maize rhizosphere and soil samples in Melk and Mitterdorf. Co: rhizosphere of non-treated maize plants, **STR:** rhizosphere of *S. rhizophila* P69 treated plants, **+/-:** with or without stripper coat.

Observation in detail on order level showed that composition of orders within the class Gammaproteobacteria was more distinct than on phyla level (Figure 45). Within Melk samples, Enterobacteriales and Xanthomonadales represented the most abundant orders followed by Pseudomonadales. Whereas within Mitterdorf samples Xanthomonadales and Pseudomonadales were dominant within Gammaproteobacteria. Comparing samples within one location, Enterobacteriales were more dominant within STR treated samples in Melk compared to Co samples. In soil Enterobacteriales appeared as relatively low abundant order of all Gammaproteobacteria. Pseudomonadales were highly abundant in Melks Co samples and less dominant in STR and soil samples. For Xanthomonadales, to which *S. rhizophila* P69 belongs (family: Xanthomonadaceae, genus: *Stenotrophomonas*), no conclusive abundance pattern was observed. They were similar abundant within Co and STR + samples and represented a relatively small group in STR- samples. In both soils Xanthomonadales were the most abundant order. In Mitterdorf samples Xanthomonadales were slightly more dominant in rhizospheres of stripper coated treatments (Co+ and STR+). For Pseudomonadales it was observed

vice versa – this order was more dominant in rhizosphere samples of non-coated treatments (Co- and STR-) and least dominant in the soil. Other than in Melk, Enterobacteriales only made up a small part within Mitterdorf samples and were similarly dominant.

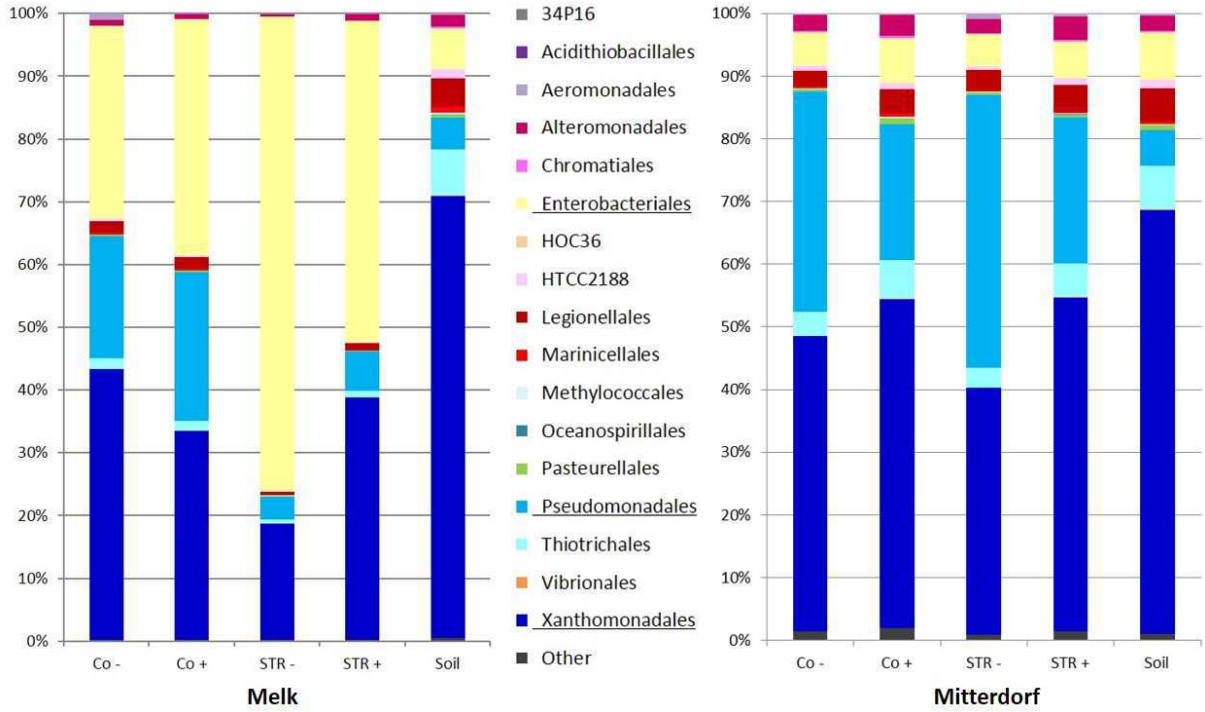

Figure 45: Taxonomic composition of orders within Gammaproteobacteria of Melk and Mitterdorf rhizosphere and soil samples. Co: rhizosphere of non-treated maize plants, **STR:** rhizosphere of *S. rhizophila* P69 treated plants, **+/-:** with or without stripper coat.

Genus *Stenotrophomonas* could be found in all samples in Mitterdorf and rhizosphere samples of Melk but was missing in soil from Melk under the described filtering steps. As it was not possible to detect the species level, *S. rhizophila* P69 could not be identified in STR treated rhizospheres. However, BLAST analysis of normalized read hits without filtering steps showed up to 7fold increased hits of genus *Stenotrophomonas* in STR rhizospheres in Mitterdorf with 48.7 (± 48.6, STR-) and 31.7 (± 31.6, STR+) compared to 33 hits (±.44.2, Co-). 4.4 (± 7.7, Co+) and 5.7 (± 5.0, soil) for control and soil samples. This tendency was not observed for Melk samples, although the number of hits for *Stenotrophomonas* in general was higher compared to Mitterdorf (soil: 6.9 ± 2.4, Co-: 218.7 ± 265, Co+: 303.9 ± 211.2, STR-: 200 ± 177.6, STR+: 307.1 ± 165.5 hits). This effect could be due to either a saturation of the genus in the rhizosphere or sowing delay of 3 weeks. In general, hit variations in replicates showed to be very high.

Influence on fungal community

For fungal communities, between 4,267 and 121,829 sequences were obtained per sample via Illumina MiSeq sequencing. Total count was 1,717,250 sequences. Read numbers were normalized to 4,260

per sample before further analysis. As mentioned for bacterial communities, 3% genetic dissimilarity was set as a cut off level of divergence for OTU clustering. Also, sequencing did not reach saturation (Figure 46).

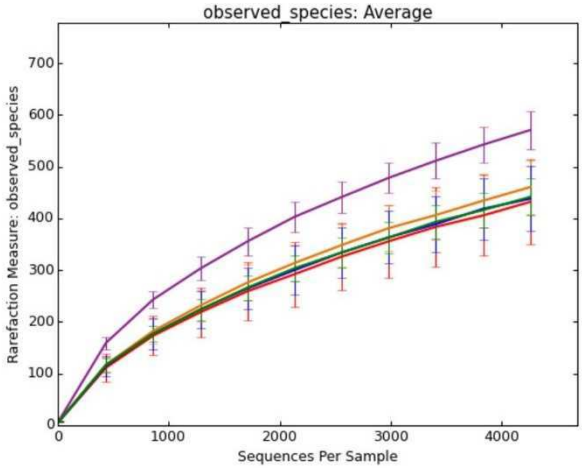

Figure 46: Rarefaction curves of fungal sequences of maize rhizosphere and soil samples.

As can be seen in Table 6, coverage lay between 43.1 and 57.1%. Values of Shannon indices showed, that a higher α diversity was found in soil samples than in rhizospheres. Therefore, P69 did not increase α diversity of fungal communities.

Table 6: Species richness of fungal communities of maize rhizosphere and soil samples from Illumina MiSeq sequences normalized to 4,260.

Origin	Habitat	Treatment	Shannon Index* (H')	Rarefaction (no. of OTUs)	Chao 1 (no. of OTUs)	Coverage (%)
Melk	Maize rhizosphere	STR-	6.0 (± 0.48)	465 (± 37.8)	944 (± 55.0)	49.3 (± 2.25)
		Co-	6.4 (± 0.21)	496 (± 16.9)	990 (± 42.5)	50.2 (± 1.89)
		STR+	5.9 (± 0.43)	446 (± 38.9)	904 (± 65.3)	49.3 (± 1.98)
		Co+	5.8 (± 0.62)	482 (± 58.9)	983 (± 98.2)	49.0 (± 2.10)
	Soil	-	6.9 (± 0.1)	551 (± 21.1)	1,055 (± 26.5)	52.2 (± 2.5)
Mitterdorf	Maize rhizosphere	STR-	5.0 (± 0.91)	411 (± 70.4)	780 (± 103.1)	52.4 (± 2.44)
		Co-	4.2 (± 0.82)	368 (± 68.8)	782 (± 107.8)	46.7 (± 2.46)
		STR+	5.2 (± 0.24)	437 (± 32.3)	825 (± 72.4)	43.1 (± 2.03)
		Co+	5.0 (± 0.51)	439 (± 30.4)	875 (± 56.0)	50.1 (± 0.98)
	Soil	-	7.1 (± 0.17)	591 (± 36.5)	1,037 (± 81.3)	57.1 (± 2.11)

** the higher the number the more diversity*

As already seen in bacterial communities, samples from the same location were more similar and clustered together in PCoA (Figure 47). Further, except for soil samples, all samples showed high varieties within replicates. Therefore, locations were analyzed separately in further investigations.

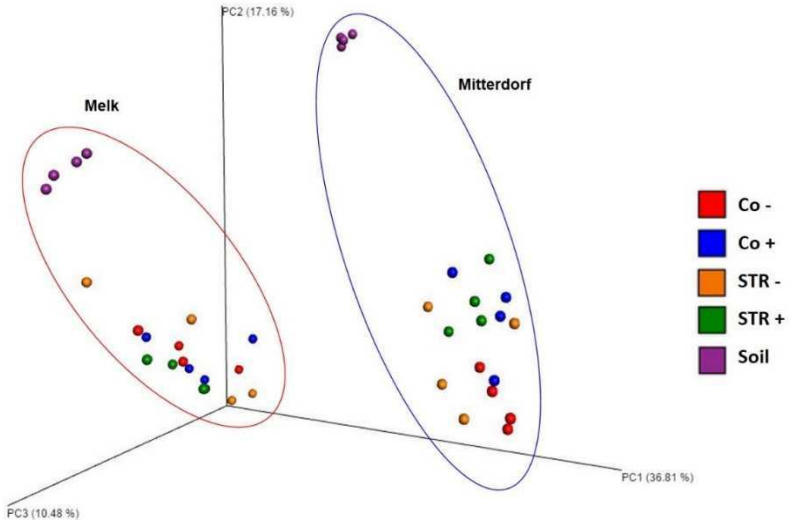

Figure 47: PCoA plot of fungal rhizosphere and soil samples from Melk and Mitterdorf. Co: rhizosphere of non-treated maize plants, **STR:** rhizosphere of S. rhizophila P69 treated plants, **+/-:** with or without stripper coat.

For comparison of shared and individual OTUs as well as for taxonomic comparisons, replicates of the same sample were merged and OTUs were filtered for presence in at least 3 out of 4 replicates. Total numbers of OTUs were lower for fungal communities than they were for bacteria. In Melk, 525 OTUs were found in soil, followed by 690 (-) and 710 (+) OTUs for STR rhizospheres. Highest numbers of OTUs were found in Co samples with 863 OTUs for Co- and 1,025 for Co+. In Mitterdorf numbers were similar, except that in Co+ only 789 OTUs were identified. Highest number was found in Co- with 911 OTUs followed by STR+ with 818, STR- with 632 and soil with 510 OTUs. All samples had an individual amount of specialists that were only present in their sample type (Figure 48).

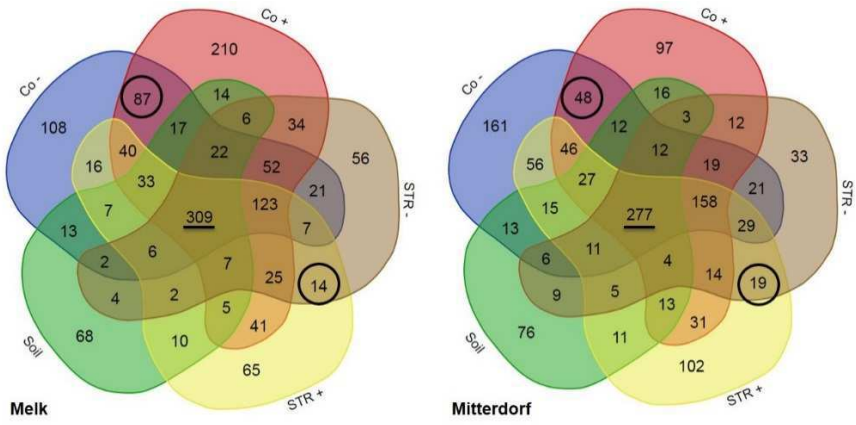

Figure 48: Venn diagram of shared and individual fungal OTUs of rhizosphere and soil samples from Melk and Mitterdorf. Core microbiome is underlined black. Black circles show shared specialists for control rhizospheres and P69 treatments. **Co:** rhizosphere of non-treated maize plants, **STR:** rhizosphere of *S. rhizophila* P69 treated plants, **+/-:** with or without stripper coat.

For Melk, core microbiome consists of 309 OTUs, whereas for Mitterdorf 277 OTUs were shared between all samples. In soil only 68 or 76 OTUs were found, respectively. Shared specialists for controls were represented by 87 OTUs in Melk and 48 in Mitterdorf, whereas shared specialists of STR rhizospheres were smaller in number with 14 and 19 OTUs, respectively. As mentioned for bacterial analysis, core microbiome and specialists need to be analyzed in detail to find out about abundant families and their possible tasks in the root or soil system.

Fungal communities were compared at phylum level (Figure 49). Again, the taxonomic comparison was only possible in sense of relative abundance within samples, not absolute abundance in or between samples and locations. As only six to seven phyla were detected, all phyla present in at least 3 out of 4 replicates are shown. No threshold level for abundance was set. Most abundant phyla were Ascomycota, Basidomycota, Chytridiomycota and Zygomycota. Allthough these four phyla were most abundant in both locations, Ascomycota were more dominant within Melk samples and Chytridiomycota in Mitterdorf samples. Also, by comparing both soils, relative abundances and distribution varied with Zygomycota being more dominant in Mitterdorf and Basidomycota in Melk. Interestingly, the kingdom Protista belonging Cercozoa, which naturally occur in water and soil (Bass and Cavalier-Smith, 2004), were detected only in soil samples of Mitterdorf and only in both control rhizospheres (+ and -) in Melk. Glomeromycota were only present in Melk samples (Co- and STR+). Further, candidates of the phylum Rozellomycota were found in control rhizospheres and soil in Melk and both control rhizospheres and STR+ in Mitterdorf. Phyla without BLAST hit, which occurred only in Melk samples (Co-, Co+ and STR-) are summarized in Other.

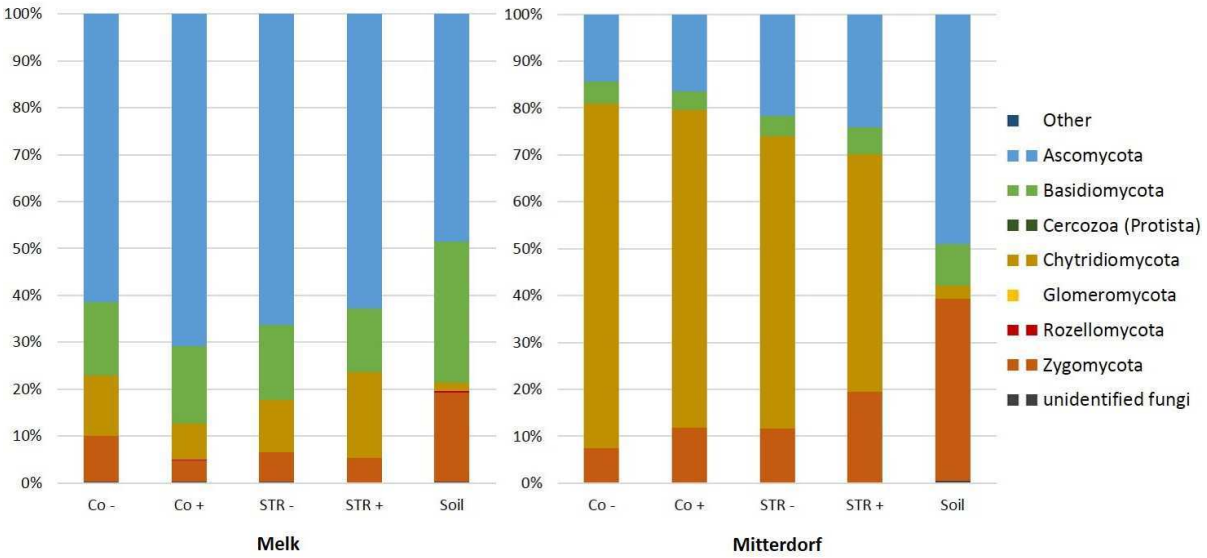

Figure 49: Relative composition of most abundant fungal phyla of maize rhizosphere and soil samples in Melk and Mitterdorf. Co: rhizosphere of non-treated maize plants, **STR:** rhizosphere of *S. rhizophila* P69 treated plants, **+/-:** with or without stripper coat.

For detailed analysis, selected phyla, like Ascomycota and Basidomycota were compared for differences.

The classes of the phylum Ascomycota are shown in Figure 50. In both locations, Sordariomycetes were the most abundant class, followed by Dothideomycetes. In Melk samples, relative abundance of Sordariomycetes was lower in soil samples, whereas it was similar or more abundant compared to rhizosphere samples in Mitterdorf. For Dothideomycetes, relative abundance was more dominant in STR- in Mitterdorf and Co- in Melk. In Mitterdorf, low abundant Lecanoromycetes were only found in STR+ samples, whereas in Melk they were present in all samples except for STR-. Pezizomycetes were observed to be more dominant within stripper coated samples. Although, this effect only occurred in Mitterdorf, whereas this class was similar dominant in rhizospheres and more abundant in soil in Melk samples. Concerning Saccharomycetes, classes from this phylum were less abundant in STR rhizospheres in Melk and STR+ and Co- rhizospheres in Mitterdorf and no OTU belonging to the class of Saccharomycetes was found in STR (Mitterdorf).

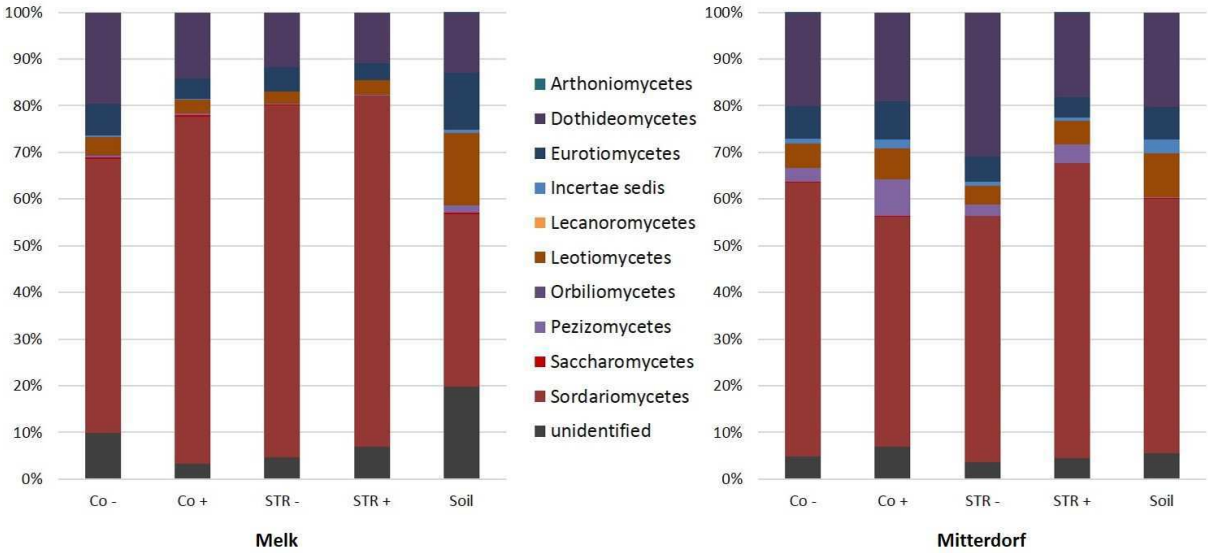

Figure 50: Taxonomic composition of Ascomycota in rhizospheres and soil samples from Melk and Mitterdorf. Co: rhizosphere of non-treated maize plants, **STR:** rhizosphere of *S. rhizophila* P69 treated plants, **+/-:** with or without stripper coat.

Comparison of Basidomycota showed, that as previously observed for total fungal community and for Ascomycota, strong differences in relative distributions of classes can be observed between locations (Figure 51). For Melk samples, Co-, Co+, STR+ and soil showed a very similar distribution concerning the most dominant classes - Tremellomycetes, Wallemiomycetes and Agaricomycetes. In STR- samples Agaricomycetes were more dominant and Tremellomycetes less compared to the other samples. In STR+ rhizospheres Agaricomycetes is slightly more abundant than in Co rhizospheres. It is striking, that relative abundance of Ustilaginomycetes is very low in Co+ samples, missing in Co- and highest in soil samples. In Mitterdorf samples, Wallemiomycetes are more dominant than in Melk. Comparing other classes of Basidomycota, Co+, STR+ and soil as well as Co- and STR+ shared similar dominance of Agaricomycetes and Tremellomycetes. Ustilaginomycetes were only detected in Co- samples.

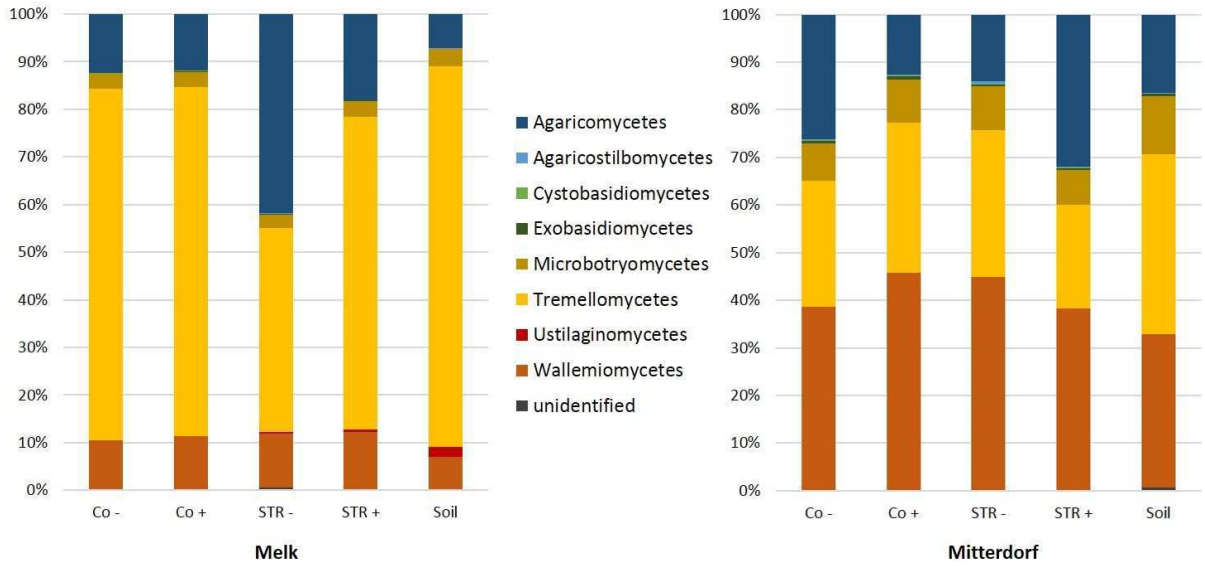

Figure 51: Taxonomic composition of Basidomycota of soil and rhizosphere sampels from Melk and Mitterdorf. Co: rhizosphere of non-treated maize plants, **STR:** rhizosphere of *S. rhizophila* P69 treated plants, **+/-:** with or without stripper coat.

Further, taxa which were found to be increased or decreased in experiments with P69 in tomato rhizospheres using SSCP analysis performed by Schmidt *et al.* (2012) were also checked for differences between samples. The genus *Cladosporium* was found in all samples in Melk and Mitterdorf with similar relative abundance (between 1.8 and 2% in Melk and 1.4 and 2% in Mitterdorf). Genus *Mortierella* was highly abundant in soil samples in both locations, being lower in Melk (18.6% and 38.9% in Mitterdorf). Also in rhizosphere samples, abundances were lower in Melk compared to samples from Mitterdorf. *Mortierella* showed to be relatively higher abundant in coated samples from Mitterdorf (STR+: 19.4%, Co+: 11.6%,) compared to non-coated (STR-:11.5%, Co-: 7.3%). Abundance in STR samples and Co+ were lower (STR+:5.3%, STR-: 6.2%, Co+: 4.6%) compared to Co- showing highest abundance of this genus (9.7%) in Melk. Concerning *Candida* spp., no OTUs were found in Mitterdorf samples and in Melk *Candida* spp. was only found in Co- rhizospheres.

4 Discussion

In this study, assays were performed to obtain knowledge about plant-consortia interactions with focus on diverse beneficials and their contribution to a better plant health. Four main objectives were set for gaining important insights and could be summarized: (i) strain characterization led to important insights on microbial tools for consortium assembly, (ii) advantages of consortia in plant cultivation could be shown, especially for oilseed rape, (iii) first stress protecting effects of a consortium were observed for sorghum plants during dehydration tests and (iv) seed application of SPA *Stenotrophomonas rhizophila* P69 for maize field trials was had no influence on bacterial or fungal α diversity in rhizosphere communities.

One of the four main objectives of this study, was the characterization of strains and consortium assembly. Characterization assays led to more insight on beneficial tools for consortium assembly. In microbe-microbe interaction tests different patterns from enhanced swarming to inhibition or killing of other bacterial strains, were observed. For the present, all reactions observed were considered to fulfil a positive and beneficial task in a consortium assembly. A bacterial strain inhibiting or killing another strain might have positive influences on other strains, e.g. enforcing cooperation between strains to facilitate competitive strategies (Hibbing *et al.*, 2010). Other strains, as shown for *Chryseobacterium wanjuense* ZM2-1-1 and *Pseudomonas poae* RE*1-1-14, promote swarming activity, which thereby might help in plant colonization (Venieraki *et al.*, 2016). The shown interactions only represent an interaction between two strains and not the entire consortium or with other groups like protozoa, nematodes, viruses and more. These interactions were considered to be much more complex and therefore need to be investigated in future *in vitro* and *ad planta* experiments.

Further, two of the strains tested, *Serratia* sp. SF1-3-1 and *Pseudomonas mediterranea* RM2-1-3, were able to perform quorum sensing via the production of N-Acyl homoserine lactones (AHLs). AHLs are involved in complex gene transcription regulation systems, which can have different effects dependent on the producing bacterial species. For example, in *Serratia plymuthica* HRO-C48 AHLs are known to be crucial for biocontrol activity of *Verticilium* wilt on oilseed rape and to be involved in negative regulation of the phytohormone indole-3-acetic acid, but they have no influence on biofilm formation or root colonization of oilseed rape (Müller *et al.*, 2009), whereas in *Pseudomonas aeruginosa* AHLs also influence biofilm formation (Miller and Bassler, 2001) or colonization of wheat rhizosphere in *Pseudomonas fluorescens* 2P24 (Wei and Zhang, 2006). Furthermore, it has to be kept in mind that AHLs can be degraded by enzymes of competing bacteria or production can be prevented by pathogens (Lugtenberg and Kamilova, 2009). Therefore, the complex effects of AHLs on the consortium strains as well as the host plants and other quorum sensing systems remain worthy of further investigation in future experiments, although degradation by local field microbiomes might be hard to evaluate.

All of the strains tested were able to swim and five of them to swarm. As motility, especially swarming is mentioned to be a huge advantage for plant colonization and surviving in ecological niches, these strains harbor another beneficial tool for possible consortium advantages. They should be tested for the

genetic basis behind their ability and for further potential positive effects for other consortium strains like the transport of cargo bacteria (Venieraki *et al.*, 2016). To test the ability of biofilm formation, which is an important feature for root colonization, solid surface assays could be performed in future experiments (Haggag and Timmusk, 2008). As rhizosphere competence is also an essential feature for the successful application of BCAs and SPAs and plays a key role in plant growth promotion, the ability to colonize roots is not only a necessary but crucial feature of consortium strains. As shown in CLSM and FISH experiments, the chosen bacterial strains showed to be rhizosphere competent and were able to colonize the roots of oilseed rape in sterile systems.

A beneficial tool for protection against biotic stress like fungal pathogens is the production of volatile organic compounds (VOCs). All tested consortia strains had a high antagonistic potential against at least two out of four fungal phytopathogens tested. Inhibitive effects of VOCs of *Stenotrophomonas rhizophila* P69 and *Bacillus subtilis* B2g on *Rhizoctonia solani* have already been reported by Kai *et al.* (2007) by the production of several species-specific volatiles, of which not all could be identified yet. However, the inhibition is strongly dependent on the growth medium. Bitas *et al.* (2013) reviewed, that microbial VOCs can not only act as antimicrobial compounds, but are also involved in many processes, like interspecies regulations, activating induced systemic resistance in plants or triggering plant growth promotion and plant stress protection. Also, swarming motility is an ability which can be influenced by VOCs (Venieraki *et al.*, 2016), which could be, besides secretion of diffusible compounds, a possible explanation for increased swarming in *P. poae* RE*1-1-14 if grown together with *C. wanjuense* ZM2-1-1. It is also mentioned, that VOCs not only have positive effects on plants. Kai *et al*. (2009) reported an inhibiting effect of VOCs of *S. rhizophila* P69 and *B. subtilis* B2g on growth of *Arabidopsis thaliana* and the moss *Physcomitrella patens*. This effect was not seen in sterile environments for P69 treated sorghum plants in this study. In experiments with maize, results varied dependent on the growth conditions, e.g. sterile filter or germination pouch. Influence of strains if plants are grown in soil needs to be investigated in more detail. To enlighten the mechanisms and effects behind the VOCs produced by potential consortia strains, future steps should include identification of VOCs under different growth conditions and evaluation of direct effects on host seeds and plants. Also further plant specific pathogens, as well as testing of the antimicrobial effect *ad planta* should be included in future study.

Survival under drought or desiccation conditions is one of the favored goals for SPAs. For biological seed treatments appropriate fermentation and formulation technologies need to be applied to secure survival and fitness of bacteria during the commercial seed pill production and afterwards to be active in fields. Different techniques like lyophilisation, embedding in powder formulations or encapsulation can enhance survival rates (Schoebitz *et al*., 2013). In this study, a direct seed priming was performed for single and consortia strain applications. Desiccation assays showed very promising results with five of the tested bacteria surviving at least 70 days. The gram-positive bacterium *Bacillus* sp. ZP2-1-3 even survived after 280 days of desiccation and storage at room temperature, which could be explained by spore formation. The integrations into commercial seed pill production still need to be performed.

For plant performances indirect biocontrol mechanisms can be used by applying endophytic microorganisms. Results of pre-trials (data not shown) showed that *B. subtilis* B2g and *S. rhizophila* e-p17 can also be found as endophytes in leaves of maize plants. For SPA *S. rhizophila* P69 the ability to grow endophytically in tomato plants was already known (Schmidt *et al.*, 2012). Future experiments should also explore the role of the present consortium strains as endophytes, their preferred colonization zones (leaves, roots, fruits, stem) as well as their effect on the plant and the consumer (if present in fruits). For the last fact potentially negative effects on human health should be already avoided during consortia composition by exclusion of risk group 2 and higher risk groups. A positive effect on health could be supported by the composition of strain inclusion possible stimulation the immune system or support the nutrition as supposed for the lettuce inhabitants (Berg *et al*., 2014).

Observing positive impacts of a high diversity consortium on plant health was one of the main objectives and could be confirmed in this study. As microbial poorness in diversity or imbalances is known to often support the outbreaks of pathogens, microbial diversity should be maintained in humans, food and environmental systems (Berg *et al*., 2014). In oilseed rape pre-trials, the most diverse consortium (Mix 6) showed best germination and leaf formation compared to single strain applications. Also, consortium treated sorghum plants showed increased dry weight compared to single strains or controls in filter cultivation. Garbeva *et al.* (2004) reviewed, that the extent of microbial diversity in soil is reported to stand in close relationship with sustainability of ecosystems and quality of soils and plants. Further, in microbial diverse soils pathogens can be suppressed by microbiota, as found for suppressive soils (Mendes *et al*., 2011). Therefore, the use of a high diverse consortium not only seems plausible but is striven for (Berg *et al*., 2013).

However, beneficial effects are obviously influenced by growth conditions and environment. In pouches, which provide more space for root development, P69 heavily increased the root fresh weight of maize plants and consortium treatment resulted in significant increase in biomass of green parts and roots, whereas in germination filters this effect was not observed. Fluctuations also occurred in other growth experiments, which indicates, that there might be unknown environmental factors influencing the experiments and therefore are worth further investigations. However, these results showed a possible beneficial impact of the consortium on plant growth of maize. Increased root growth, as shown for consortium and P69 treatment, might enable a better nutrient and water supply, and therefore could provide a head start for growth and plant health (Egamberdiyeva, 2007), although of course this positive effect needs to be proven for soil cultivation.

Many studies showed beneficial effects of microbial consortia or single strain applications in sterile systems under greenhouse or laboratory conditions (Kannan *et al*., 2009, Wang *et al.*, 2012, Jain *et al*., 2012, Rybakova *et al*., 2015). The question arises, if those beneficial effects can also be proven for natural and non-sterile environments. According to Lugtenberg and Kamilova (2009), these effects often lack in field trials. Rybakova *et al*. (2015) observed positive effects of plant growth promotion in *P. polymyxa* Sb3-1 primed oilseed rape seedlings in sterile soil, whereas in non-sterile soil this effect was

missing. They concluded, that observed effects are linked to a shift in the microbiome of the soil and the presence of microbes in non-sterile soil. These findings emphasize the importance of rhizosphere competence, which is known to be a key feature of BCAs and SPAs (Scher *et al*., 1984, Lugtenberg and Kamilova, 2009), and non-sterile soil as growing substrate.

As this study is only part of a long project and is just at the beginning, many questions and tasks remain open for future experiments. However, first positive effects of consortia on host plants are observed in this study under laboratory and greenhouse conditions. In the next steps, these plant growth promoting effects have to be proven in additional experiments in non-sterile soil and field trials, facing complex interactions with soil and animal microbiomes in nature. Also, the influence and interactions of the respective seed microbiomes on and with consortia strains are important questions and will be part of future investigations with non-sterilized seeds.

As consortia in this study should not only promote plant health under normal conditions, but protect plants under environmental stresses, another main goal of the study was to evaluate the stress protecting abilities of consortia for future field applications. Testing the protective abilities of different consortia and host plants in greenhouse, first promising results were obtained, but also puzzling questions worth further investigation arose. In dehydration stress tests, maize plants showed heavy dehydration symptoms like decolored, hanging leaves of which the tips were turning brown after repeated dehydration stress over a period of 3.5 weeks. Consortium plants showed to be smaller and maybe due to decreased surface and transpiration had no hanging leaves. However, with ongoing plant growth and stress, consortium plants colored purplish. A purple coloration of maize can indicate a deficiency in phosphorus (Agrios, 2005). It also goes hand in hand with a reduced plant growth. Emerson Nafziger from the University of Illinois stated that, if the plant has more sugar than it needs, the purple pigment is formed in the leaves and, as low presence of phosphorus inhibits the movement of sugar, the color stays in the leaves. He also mentioned that cool and dry soil can also cause the inhibition of sugar movement (Nafziger, 2013). As it is known that phosphorus belongs to the list of macro nutrients for microorganisms, it could be theorized that the addition of consortium strains resulted in a higher need for phosphorus and thereby caused the deficiency symptoms. A possible solution to phosphorus deficiency could be the addition of a further, phosphate solubilizing strain to the consortium. Amongst the characterized strains from this study, several bacteria are capable of phosphate solubilization (Zachow *et al*., 2013). However, greenhouse potting systems only provide limited nutrients and space. Therefore, to confirm the deficiency of phosphorus and to evaluate the impact of consortium strains without nutrition deficiency, further experiments with controlled fertilization could be performed. Nevertheless, field trials are amongst the next steps necessary to evaluate growth promoting and stress protecting effects under natural conditions and influences. Understanding of consortium influencing environmental factors is also crucial for further optimization. However, experiments with sorghum plants show first stress protecting effects against dehydration stress by consortium seed treatment.

As climatologists warn, a rise in average temperatures will influence soil moisture (Battisti and Naylor, 2009). This might also cause a shift in the soil microbiome and thereby might directly influence plants and interact differently with SPAs. It is therefore crucial to understand the mechanisms and borders behind stress protecting effects to compile useful SPA consortia. Also further stress tests should be performed to create a holistic picture of the stress protecting capabilities of the consortium. Dehydration stress therefore could also be generated by controlled salt solution watering. Several studies, including this study, show beneficial effects of SPAs or BCAs seed treatment using sterilized seeds (Jain *et al*., 2012, Couillerot *et al.*, 2013, Rybakova *et al*., 2015). Also, studies often lack the comparison to non-sterilized control seeds. Yet the seed itself harbors its own microbiome, which is an important reservoir for features essentially needed for rhizosphere functionality (Zachow personal communication). The compatibility of applied consortia or single strains with the whole seed microbiome is a very important key feature for successful plant protection and will be a focus of future experiments. As mentioned before, the plant itself can recruit microbiota e.g. via root exudates (Badri and Vivanco, 2009). Hence, the long-term goal for successful and sustainable agriculture must be the support of a harmonic teamwork of plant microbiome, soil microbiome and supporting SPA consortia. To enable this, Mendes *et al*. (2014) suggested a selection based on functional microbiome cores related to the metabolisms of nitrogen, iron, phosphorus and potassium, which are related to benefits to the plant, such as growth promotion and nutrition from bulk soil to the rhizosphere.

To enable this harmonic symbioses of microbiota with the plant, impact of single strain seed treatments and later on consortium treatment on the rhizosphere, as well as the impact of soil and environmental conditions needs to be evaluated and thereby represent the fourth aim of this study. Evaluations can be performed by the analyses of the community structure of plant-associated microbiota with amplicon sequencing. In Melk and Mitterdorf, the rhizosphere samples from the same location were more similar to each other in OTU based cluster analysis than to same treated rhizospheres from different locations. Individual analysis showed similar high diversity of bacterial and fungal species within rhizosphere and soil samples, with the fungal community being less diverse in general. Therefore, it is concluded that the SPA P69 treatment did not increase the diversity within bacterial or fungal communities compared to soil or control rhizospheres. The field site, plant growth state and microenvironment had a crucial impact on the communities of the rhizospheres and the similarities of samples as found for lettuce by Scherwinski *et al.* (2008).

Analyzing bacterial communities in more detail on respective of the Gammaproteobacteria, three genera differed most in abundance: Enterobacteriales, which showed to be more abundant in Melk, especially in STR-/+ samples, Pseudomonadales and Xanthomonadales. The last two genera were more dominant in Mitterdorf rhizospheres, with Pseudomonadales being relatively more abundant within uncoated and Xanthomonadales being more dominant within stripper coated samples. As it was impossible to fully detect species level, *S. rhizophila* P69 could not be identified for sure in any rhizospheres. It is not clear, if and to what abundance the strain really was present after a certain period of time. Although, for

Mitterdorf a tendency of increased hits for *Stenotrophomonas* was observed in STR rhizospheres compared to other samples, which could be a hint of presence of SPA P69. A clear identification of the strain, e.g. with special designed probes, is a puzzle that is already been worked on by a smart mind at the Institute of Environmental Biotechnology in Graz. However, Schmidt *et al.* (2012) reported beside a positive effect on plant growth, that they were not able to find *S. rhizophila* P69 in profiles of Gammaproteobacteria in soil using single strand conformation polymorphism profiling. Several studies reported no or only a short term changes in microbial rhizosphere communities (Scherwinski *et al.*, 2008, Berg and Zachow, 2011, Yergeau *et al*., 2015). However, Schmidt *et al.* (2012) observed noteworthy changes in fungal communities of tomato rhizospheres caused by inoculation with *S. rhizophila* P69. They reported major disappearance of bands from the genus *Mortierella* and a band closely related to *Candida subhashii*, if plants were treated with P69. Further *Cladosporium*-relatives were present in P69 treatments. In this study, the genus *Mortierella* was present in all rhizosphere and soil samples. Also, no evidence for enhancement of *Cladosporium* in P69 rhizospheres was observed. *Candida* spp. was not found in any sample in both locations with exception of Co- in Melk. Therefore, same observations as described by Schmidt *et al*. (2012) for tomato rhizosphere were not found in rhizospheres of maize. However, the influence of the plant species and the soil type are crucial factors in shaping of the rhizosphere microbiome (Berg and Smalla, 2009). Keeping this in mind, different influences on the rhizosphere microbiome seem plausible. As the data presented is only an observation of part of the microbial community, further data mining and detailed analysis has to be performed to decipher possible alterations in the communities. Thereby, the focus should also be on evaluation of specialist families and genera in Co and STR rhizospheres. This might permit explanation of potential relationships between microbes and their functions for the plant and environment.

5 Conclusion and outlook

This study shows first positive impacts of application of consortium strains on maize, sorghum and oilseed rape and reveals topics worth further investigations. Consortium strains were proven to harbor a variety of beneficial tools for plant colonization and protection against biotic and abiotic stresses. All consortia were able to colonize roots in sterile systems and were found to increase germination, biomass or leaf rates of host plants. Also first positive effects in stress protections could be documented in greenhouse trials. In future steps, goals will be the testing of positive biocontrol and stress protecting effects during commercial seed pill production and in field trials, followed by eventual optimization steps of consortia to fulfil all requirements for the respective host plants. Also, influence of consortium use on rhizosphere microbiomes via amplicon sequencing should be part of future experiments.

It will also be necessary to learn more about microbe-microbe and plant-microbiome interactions. Increased understanding of requirements for all partners involved and identification of influencing environmental and pedo-climatic factors will help to understand the needs of host plants, environment and SPAs. Understanding and deciphering this “code” of microbe-plant interactions and the mechanisms behind mediated stress protecting effects may offer us a key to the solution of how to face diminishing crop yield and plant loss in extreme climate situations in the future. As we learn to completely understand the complex network between species and environmental factors involved in plant cultivation, in future times we might be able to provide personalized treatments in respect to host plants, soil type and nutrient composition, microbiomes and other influencing environmental factors.

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