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"Investigation of rice-diazotrophic associations under gnotobiotic conditions and evaluation of Gold-FISH for the detection of microorganisms on root surfaces"

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Abstract

Microscopy is central to biological research and indispensable since the discovery of microorganisms by Leeuwenhoek in the 17th century. The aim of this master thesis was to gain insights into the association of diazotrophs with rice plants. Diazotrophs are microorganisms that are capable of fixing atmospheric nitrogen (N_2) and transform it into more available forms such as ammonia (NH_3). In wetland rice, it is assumed that roots enter associations with diazotrophs, a non-symbiotic association that is beneficial for the rice plant through microbial supply with nitrogen (N). Our understanding of the underlying mechanisms involving epi- and endophytic colonization of rice roots is still limited.

With the present master thesis, I aimed to isolate diazotrophs from the surface of rice roots and associated paddy-soil, to subsequently re-associate the isolate with rice plants under gnotobiotic conditions. This reductionist model system would serve for the development and evaluation of various microscopic tools, that were finally used to visualize microorganisms on the rhizoplane of rice roots under natural conditions. One of those tools is Gold-Fluorescence *in-situ* hybridization (Gold-FISH). I have evaluated the applicability and specificity of the method for the detection of microorganisms via fluorescence microscopy and scanning electron microscopy (SEM). Through correlation of fluorescence microscopy images and SEM images it was shown, that the hybridization of probes, the binding of tyramides, and the binding of nanogold carrying streptavidin conjugates is specific to the target organism. Furthermore, I successfully applied Gold-FISH for the visualization of microorganisms on the surface of rice root. Gold-FISH can be used for future research in combination with the detection of stable isotopes via nanoscale secondary ion mass spectrometry (NanoSIMS) After SEM, the sample to be investigated is not subjected to any treatment which would alter the structure of the sample (e.g. washing, drying). This greatly facilitates the possibility to produce correlating images via two differing imaging techniques). My successful efforts to apply Gold-FISH for the detection of microorganisms on the surface of rice roots via SEM now for the first time allow for the detection of $^{15}N_2$ in single microbial cells on the rhizoplane of rice roots via a Gold-FISH-NanoSIMS approach.

Zusammenfassung

Mikroskopie ist von höchster Bedeutung für biologische Forschung und ist seit der Entdeckung der Mikroorganismen durch Leeuwenhoek im 17 Jahrhundert nicht mehr wegzudenken. Die vorliegende Arbeit hatte vorwiegend zum Ziel über Mikroskopie Einsichten hinsichtlich der Assoziation zwischen Prokaryoten und Reispflanzen in der Rhizosphäre der Pflanze zu gewinnen. In einer solchen Assoziation ist Diazotrophie von Wichtigkeit. Diazotrophe sind Mikroorganismen, welche Stickstoff aus der Atmosphäre (N_2) fixieren können um dieses zu Ammoniak (NH_3) zu konvertieren. Pflanzen hingegen sind dazu nicht befähigt. In Bezug auf Reis wird davon ausgegangen, dass Diazotrophe eine Assoziation mit den Wurzeln der Reispflanze eingehen. Für die Reispflanze ist dies vorteilhaft, da sie durch Mikroorganismen mit Stickstoff (N) versorgt wird. Unser Verständnis über die Mechanismen, denen eine epi- und endophytische Besiedlung von Reiswurzeln zu Grunde liegt, ist jedoch nach wie vor begrenzt. Das Ziel der vorliegenden Master Arbeit war es Diazotrophe von der Oberfläche von Reiswurzeln, sowie aus dem umliegenden Boden zu isolieren, um anschließend das Isolat mit Reispflanzen unter gnotobiotischen Bedingungen in Assoziation zu setzen. Dieses reduktionistische Modellsystem diente es zur Entwicklung verschiedener mikroskopischer Techniken, die schließlich zur Visualisierung von Mikroorganismen auf der Oberfläche von natürlich gewachsenen Reiswurzeln genutzt wurden. Eine dieser Techniken ist Gold-Fluoreszenz *in-situ* Hybridisierung (Gold-FISH). Ich habe die Anwendbarkeit und Spezifität der Methode mittels Fluoreszenzmikroskopie und Rasterelektronenmikroskopie (REM) evaluiert. Durch die Korrelation von fluoreszenzmikroskopischen Bildern und REM-Bildern wurde gezeigt, dass die Hybridisierung von Sonden, die Bindung von Tyramiden, sowie die Bindung des Nanogold Streptavidin-Konjugats spezifisch für den Zielorganismus ist. Des Weiteren habe ich erfolgreich mit Gold-FISH Mikroorganismen auf der Oberfläche der Reiswurzel visualisiert. Gold-FISH kann für zukünftige Forschung in Kombination mit der Detektion von stabilen Isotopen über Nano Sekundärionen-Massenspektrometrie (NanoSIMS) verwendet werden. Nach REM wird die zu untersuchende Probe keiner Behandlung unterworfen, welche die Struktur der Probe verändern würde (zB Waschen, Trocknen). Dies erleichtert die Möglichkeit korrelierende Bilder über zwei unterschiedliche bildgebende Verfahren zu erzeugen. Meine erfolgreichen Bemühungen zur Anwendung von Gold-FISH für den Nachweis von Mikroorganismen auf der Oberfläche von Reiswurzeln über SEM, erlauben nun zum ersten Mal

den Nachweis von $^{15}N_2$ in einzelnen mikrobiellen Zellen auf der Oberfläche von Reiswurzeln via Gold-FISH-NanoSIMS.

Introduction

N2-Fixation – an important process within the microbial N-cycle

Every living organism requires the incorporation of nitrogen (N). On the ecosystem-level, organisms compete for N as a substrate, and frequently the availability of this element can limit the overall productivity of many marine and terrestrial ecosystems (Vitousek and Horwarth, 1991). Diazotrophs can withdraw chemically inert di-nitrogen (N_2) from the atmosphere and transform it into bioavailable N compounds. Therefore, they could be key to the functioning of ecosystems, as they have the potential to regulate their productivity (Vitousek and Horwarth., 1991). N is a basic building block of life. It is the fifth most abundant element in our solar system (Canfield *et al*., 2010) and constitutes a fundamental role for synthesis of nucleic acids and proteins on earth (Sterner *et al*., 2002). Microorganisms are primarily responsible for converting N throughout its multiple oxidation states and chemical forms (Francis *et al*., 2007). N is at its most reduced state when assimilated within organisms. Once nitrogenous substances are excreted due to an active process or cell death, many compounds become available to other heterotrophic organisms (Arrigo, 2005). N compounds can be hydrolysed to ammonium (NH_4^+), which can subsequently be oxidised sequentially to nitrate (NO_3^-) via nitrite (NO_2^-) aerobically. This process, termed "nitrification", is mediated by nitrifiers, a functional group that consist of chemolitoautotrophic bacteria or archaea (Daims *et al*., 2016). Denitrification is a primarily anaerobic process carried out by heterotrophic members of all three domains, which are responsible for converting NO_3^- back to its chemically inert form of triple-bonded N_2 gas (Zumft *et al*., 1997). The subsequent reduction of N_2 into bioavailable N compounds is yet another key process that can be carried out by microorganisms. This process is termed "biological nitrogen fixation" (BNF) and is mediated by the so called diazotrophs, microbial members of both, bacteria and archaea (Franche *et al*., 2009).

Phylogeny and function of the nitrogenase enzyme

N fixation is, owing to the stability of triple bonded N_2, a very energy-demanding process. The nitrogenase enzyme is widespread among prokaryotic lineages and is generally believed to be ancient (Young, 1992). The enzyme complex conducts the transformation of atmospheric N_2

to ammonia ($N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$; Dixon and Kahn, 2004). It consists of two distinct proteins: the dinitrogenase and the dinitrogenase reductase. The *nif* gene cluster is required for the synthesis and regulation of the nitrogenase. *NifH* is a gene sequence from this cluster that is essential for all N-fixing diazotrophs, as it encodes for the subunits of the dinitrogenase protein. The *nifH* gene and protein sequence have the property of being highly conserved within each diazotrophic species (Zehr *et al*., 2003). Typically, 16S rRNA gene sequences are being targeted via sequencing approaches to obtain information about the microbial diversity inhabiting an environmental sample. Alternatively, 2 studies in 1995 have shown the potential of *nifH*-targeting primers to investigate the microbial community with the genetic potential to fix N_2 (Zehr *et al*., 1995; Ueda *et al*., 1995). After decades of collecting genetic information of diazotrophs, five major clusters were defined based upon phylogenetic characterization of *nifH* sequences. Cluster I encoding the conventional bacterial FeMo nitrogenases, abundant in the lineages of *Proteobacteria, Cyanobacteria, Firmicutes* and *Actinobacteria*. Cluster II is a smaller cluster containing sequences from alternative FeV and FeFe nitrogenases and from some *Archaea*. Cluster III mainly consists of *nifH* sequences from anaerobes, abundant in *Spirochaetes*, sulphate-reducing bacteria, green Sulphur bacteria, *Clostridia*, methanogens and acetogens (Zehr *et al.*, 2003). Clusters IV and V consist of *nifH* paralogues, which were considered not to be involved in N fixation (Young *et al.*, 2005). However, recently researchers isolated an *Endomicrobium* that fixes N with a Group IV nitrogenase (Zheng *et al.*, 2016).

Prospects of BNF for rice agriculture

Plants cannot assimilate all forms of N and therefore primary production is very often limited by the availability of N compounds. It is widely known that plants can form symbiotic relationships with N-fixing microorganisms to improve their productivity (Hunt *et al.*, 1988). Many legumes for instance can form root nodules to establish a symbiotic environment with N-fixing *Rhizobia*, in which the plant receives bio-available N in return for Carbon (Lowdig *et al.*, 2003). The most important crop plant for human nutrition is rice (*Oryza sativa* L.), which belongs the family of grasses (*Poaceae*) and does not form root-nodules. It was reported that diazotrophs can transfer up to 70% of the fixed N_2 to the plant (Chalk *et al.*, 2017). To sustain the global demands for rice, it was estimated that we are using up to 100 kg artificially produced N-fertilizer per hectar rice paddy-soil (Ladha *et al.*, 2016). Those artificial fertilizers

derive from the Haber Bosch process - an industrial method for the synthesis of NH_3 out of N_2 and H_2 that has set the basis of today's agricultural practice (Galloway *et al.*, 1995). In total, the anthropogenic N sources contribute twice as much to terrestrial systems than terrestrial BNF, and account for 45% of the total bioavailable N fixed per year on Earth (Canfield *et al.*, 2010). However, the massive application of NH_3 has also negative consequences, such as eutrophication of fresh water and coastal zones, or an increase of greenhouse gases (Gruber *et al.*, 2008). In order to reduce the amount of fertilizers used, it is of interest to understand the full potential of rice-associated diazotrophs in supplying the plant with N. Therefore, it is necessary to identify diazotrophs and analyze their interaction in association with rice plants to better understand the process of N fixation and the N transfer from microbe to plants.

Cultivation of diazotrophs associated with rice

The *nif* regulon was studied extensively in *Klebsiella pneumoniae*, a heterotrophic *γ-Proteoba*cterium with a facultative anaerobic lifestyle (Menzel *et al.*, 1997; Ahrens *et al.*, 1998). *Klebsiella* species are one of many endophytic or free-living plant-growth-promoting bacteria (PGPB; Jha *et al.*, 2007). It was shown that the organism can form biofilms on the rhizosphere (Liu *et al.*, 2011). The researchers indicate that the formation of biofilms in association with plants is believed to be of importance for growth-promotion and disease resistance. The ability of specific microorganisms to enhance plant-growth has motivated many researches to further isolate diazotrophic organisms and characterize their potential. Nitrogen-free medium (NFB) described by Dobereiner *et al* in 1976 was used frequently for the isolation of diazotrophs from soil. To study the cultivable diversity between 2 different rice cultivars, Jha *et al., 2009,* have used three different variations of N-free semisolid media with different carbon sources and pH values for the enrichment and isolation of root-associated diazotrophs. Using 16S and 23S rRNA directed genus or species-specific oligonucleotide probes, they found a handful of members from *the α-, β-, or γ-Proteobacteria* to be dominant within their enrichments. In a recent study, researchers isolated diazotrophic species from rice roots, stems and leaves, using 10 different rice cultivars (Ji *et al.*, 2014). Their PCR based analysis confirmed the presence of *nifH* in 12 isolates, which belonged to *Paenibacillus sp.* and *Bacillus sp. (Firmicutes)*, *Microbacterium sp. (Actinobacteria)* and *Klebsiella sp. (γ-Proteobacteria).* Subsequently, they showed that plants treated with the isolated *Bacillus species* CB-R05 improved the most

regarding growth and resistance against fungal pathogens. To the best of my knowledge, the highest N-fixing diversity was cultivated from soil by Mirza *et al*. in 2012. By adding little changes to the previously described N-free medium, the researchers differentiated 794 diazotrophic isolates by combining solid and semisolid NFB under oxic or hypoxic growth conditions. The solid N-free medium and hypoxic conditions increased the observed diversity of diazotrophs by 62.6% in comparison to what the research team obtained by using a conventional semisolid medium-based method. Most of their isolates belonged to the phylum of *γ-Proteobacteria,* with *Pseudomonas* species as the dominant cultivated genus (Mirza *et al.*, 2012). All mentioned studies from this paragraph have the isolation of at least one member of the phylum of *γ-Proteobacteria* in common. *Enterobacter* is a genus within this phylum, that also comprises *Klebsiella sp*. for instance. This genus is polyphyletic based on the available 16S rRNA sequences and harbors many members of motile microbes, capable of fixing N_2 endophytically. Species belonging to this genus were isolated from the rhizosphere of wetland rice already in 1983 (Ladha *et al.*, 1983). In 2009, an Enterobacter species was isolated from surface-sterilized roots of the wild rice species *Oryza latifolia* and described as a novel N-fixing bacterium. For this organism, the name *Enterobacter oryzae* type strain Ola 51^T was proposed (Peng *et al*, 2009). Furthermore, a close relative of this organism was isolated from stems of sweet potato, first classified as a N fixing *Klebsiella oxytocoa* (Adachi *et al.*, 2002) and eleven years later renamed as *Enterobacter sacchari* (Zhu *et al.*, 2013). Recently, it was proposed to reclassify many members of the genus *Enterobacter* into new genera: *Lelliotia, Pluralibacter, Kosakonia* and *Cronobacter*. Among those, *Enterobacter oryzae and Enterobacter sacchari*, now *Kosakonia oryzae* and *Kosakonia sacchari* (Brady *et al.*, 2013), named with respect to their respective origin.

The in vivo distribution of diazotrophs associated with rice

Cultivation itself only provides a small glimpse into the microbial diversity of an environment, as it is not possible to isolate most microorganisms (Amann *et al.*, 1995). Being able to access isolated microorganisms does enable researchers to test for very specific physiological properties of an isolate, however, microbiologists are not able to cultivate the vast majority of environmental prokaryotes. This became apparent in e.g. the early study of Ueda *et al.* in 1995, in which most the detected *nifH* sequences in the rhizosphere belonged to uncultivated

prokaryotes. With the following paragraph I intend to illustrate the presence of microorganisms associated with rice that were detected in association with rice, not only via isolation, but also via modern techniques in microbial ecology (e.g. next generation sequencing (NGS), proteomic). A flooded rice paddy-soil has three major compartments: i) the anoxic bulk soil, ii) the rhizosphere, and iii) the endophytic niche, all characterized by different, specific physicochemical conditions (Liesack *et al.*, 2000; Lüdemann *et al.*, 2000). In each, diazotrophs can be encountered, affected by the type of soil, the growth stage of the plant, its root morphology, and its root exudates (Park *et al.*, 2005; Prakamhang *et al.*, 2009).

i) The anoxic bulk soil. In theory, the bulk soil is characterized as anoxic and not penetrated by roots. The typical (not necessarily diazotrophic) representatives in bulk soils are anaerobic members of the groups of *Verrucomicrobiae, Cytophaga-Flavobacterium-Bacteroides (CFB-cluster), Bacillus, Clostridiae* and *Actinobateriae* (Liesack *et al.*, 2000). The bulk soil harbors a high diversity of diazotrophs with representatives from each *nifH* clade (Teng *et al.*, 2009; Reed *et al.*, 2011). However, they are characterized as free-living and do not associate with plants (Norman and Friesen, 2016).

ii) The rhizosphere. The rhizosphere of rice plants is physicochemically similar to the endophytic environment as for the presence of O_2. The rice plant can introduce O_2 from the atmosphere into deeper layers of the soil via its roots due to the diffusive transport via aerenchyma (Colmer *et al.*, 2003). Just as in the upper soil layer, where oxygen depletes rapidly due to the oxidation of iron (II), the same process can be investigated in the rhizosphere, visible by a brownish accumulation of iron (III) (Schmidt *et al.*, 2011). Furthermore, it is generally supposed, that microbial population densities are higher in the rhizosphere than in bulk soil, which is due to the secretion of carbon by the plants into the rhizosphere (Berendsen *et al.*, 2012). However, it was observed that microbial rhizospheric communities are in general also less diverse (Costa *et al.*, 2006), indicating a selection of specific microorganisms by plants. Researchers have often tried to physically separate particles attached to roots for subsequent analysis of root associated microorganisms. Recently scientists have isolated 11 unique bacterial diazotrophs from the rhizosphere of rice roots, that all belong to either *γ-Proteobacteria* or *Firmicutes*, to subsequently analyze their PGPB abilities (Sarathambal *et al.*, 2015). Ueda *et al.* have shown in 1995, that the diazotrophic community in the rhizosphere mainly consists of *Proteobacteria* and uncharacterized representatives. Furthermore, it was

shown that *nifH* gene pool in the rhizosphere of rice plants is highly variable, influenced by environmental parameters, especially by N-fertilization (Tan *et al.,* 2003). Knief *et al.* have analyzed the diversity of nitrogenase proteins in the rhizosphere of rice plants in 2012. They showed that most NifH protein subunits were related to protein sequences known from *Azospirillum sp., Bradyrhizobium sp.* and *Magnetospirillum sp.,* which suggests that those species would be the active diazotrophs in the rhizosphere. The colonization of the root surface was best studied in *Azospirillae species*, a genus belonging to the phylum of *α-Proteobacteriae*. These bacteria are motile, aerobic chemoorganotrophs (Hauwaerts *et al.,* 2002).

iii) The endophytic niche. Endophytes are microorganisms with the ability to colonize the interior of plants and grow within their tissue. It has often been reported that diazotrophic endophytes have the potential to improve nutrition, growth and health of the plants, as for example *Burkholderia cepacia* and *Herbaspirillum seropedica* can increase the shoot length beyond 75%, without causing disease symptoms (Divan Baldani *et al.,* 2000). *Azospirillum sp. B510* can induce resistance in rice against diseases caused by *Magnaporthe oryzae*, a fungus, and *Xanthomonas oryzae*, a bacterium (Yasuda *et al.,* 2009). The most studied diazotrophic endophyte is *Azoarcus* BH72, originally discovered as an endophyte in Kallar grass in Punjab (Reinhold *et al.,* 1987; Hurek *et al.,* 1994). The genome of the organism was sequenced (Krause *et al.,* 2006), a gfp cassette was constructed for a transcriptional fusion with the *nifH* gene (Egener *et al.,* 1998) to study its diazotrophic activity while in association with rice, and a *nifH::tdTomato* fusion mutant of *Azoarcus* sp. BH72 was established to study its gene expression in the same association (Sarkar 2016). Recently *Azoarcus olearius* DQS-4T was isolated from an oil-contaminated soil. Its genome is highly similar to *Azoarcus* sp. BH72 (98.98%) and contains several genes related to plant growth promotion and endophytic colonization (Faoro *et al.,* 2016). Recent metagenomic analysis of rice-root endophytic microbial communities has revealed that *Bacillus* species are dominant genera in terms of abundance (Sengupta *et al.,* 2017). Furthermore, it was shown that *Stenetrophomonas sp*. is a dominant genus within rice roots before flooding of paddy-soils, whereas after flooding members of the *β-Proteobacteria* emerge as the dominant endophytic phylum (Ferrando *et al.,* 2015). It was suggested that the endophytic niche would allow for more efficient N fixation, as the habitat offers areas of reduced oxygen concentration (Faoro *et al.,* 2016) besides oxygen-saturated areas (e.g. aerenchmya) (Colmer *et al.,* 2002). Over the past decades many

reviews were published concerning endophytes, but the focus mostly relied on isolation and identification of the respective microorganisms, rather than on the verification of endophytic BNF itself (Chalk, 2016). One of the first studies to investigate endophytic BNF was conducted by Ito *et al.* in 1980. The researchers observed incorporations of ^{15}N in the basal node and in inner leaf sheaths. In 2001, Elbeltagy *et al.* isolated a rice endophytic *Herbaspirillum sp.*, they verified via microscopic observations that the organism colonizes intracellular spaces. Afterwards, the researchers inoculated rice seedling with *Herbaspirillum sp.* and observed significant incorporations of ^{15}N$_2$ in the sample, which can be attributed to the diazotrophic activity of their isolate.

Detection of microorganisms in the environment *via* Fluorescence *in-situ* Hybridization

The gold standard for the assessment of microbial diversity in environmental samples is via sequencing of 16s rRNA genes. FISH is an elegant method that can be used in combination with sequencing-approaches, to localize and quantify non-cultivated microorganisms in their natural environment. Generally, FISH provides phylogenetic identification of single cells with the possibility to localize cells and quantify their abundance in a specific environment via fluorescence microscopy (Amann *et al.*, 1995). The method was applied successfully for the visualization of root associated microorganisms (Gilbert *et al.*, 1998; Eller & Frenzel 2001). However, the autofluorescence of the root itself as well as soil particles often impede the detection of rather weak signals originating from standard FISH procedures (Briones *et al.*, 2002). To enhance signal intensities, catalyzed reporter deposition (CARD) − FISH was developed and employed for the detection of diverse marine (Pernthaler *et al.*, 2002) and terrestrial (Eickhorst *et al.*, 2008) samples. The increased signal intensity observed with CARD-FISH is based on the enzymatic deposition of fluorescently labelled tyramines after the binding of oligonucleotide probes labelled with horse radish peroxidases (Pernthaler *et al.*, 2002). Recently, the use of CARD-FISH was reported to be suited for the visualization of bacteria and archaea on the surface of rice roots (Schmidt *et al.*, 2014). The researchers analyzed the distribution of methanogenic archaea and methanotrophic bacteria along the rhizoplane of rice roots. CARD-FISH is a powerful tool to detect specific microorganisms in complex environmental samples, but the method alone does not provide information concerning their physiologies (Wagner *et al.*, 2003). Archaea have already been detected on rice roots in 1998

through 16S rRNA sequencing in combination with FISH (Großkopf *et al.*, 1998). Their existence in association with the rice root is intriguing, as it was recently proposed that methanotrophic archaea might prepossess a key-role in N_2 transformation in unfertilized paddy-soils (Minamisawa *et al.*, 2016). CARD-FISH was used in combination with a metaproteomic analysis to show that CH_4 oxidation and N_2 fixation occurs simultaneously in rice roots (Bao *et al.*, 2014). *Methylocystaceae* species were identified as N-fixing methanotrophs and detected as endophytes within rice roots.

FISH in combination with Scanning Electron Microscopy (SEM) for the detection of microorganisms in complex environments

The detection of targets via FISH techniques is limited by the resolution and sensitivity of light microscopes. Visualizing microbial cells via fluorescence microscopy is generally not problematic as single cells are still large enough. However, objects smaller than 0.2 µm in the surrounding of the targets, which actually form the cells microenvironment, can hardly be represented via light microscopy (Schmidt *et al.*, 2017). Oligonucleotide probes that are commonly being used for FISH can be combined with gold markers. Due to the high atomic density of the gold particle, the marker can be detected via electromagnetic radiation (e.g. at SEM) (Eickhorst and Schmidt, 2016). The first application of gold particles for the detection of microorganisms from complex environments was conducted by Spring *et al.* in 1998, who combined a fluorescent and a nanogold label with polynucleotide probes. A modification of CARD-FISH was recently developed, allowing for the combined use of fluorescence microscopy and SEM for the detection of specific microorganisms in-situ (Gold-FISH, Schmidt *et al.*, 2012). Very similar to CARD-FISH, Gold-FISH employs HRP-labelled probes, that serve for a subsequent amplification of tyramides. However, for Gold-FISH the tyramides are biotinylated and serve for one further hybridization of streptavidin-conjugates to the target. The conjugates ultimately depict the marker molecules, as they carry the gold particle and a fluorescent dye. Gold-FISH can be used for the detection of specific targets with low rRNA content in complex environments via fluorescence microscopy, but also via SEM, at a resolution and magnification beyond light microscopy (Schmidt *et al.*, 2012). The analysis via SEM gives important additional insights concerning complexity of the habitat (Oburger and Schmidt, 2016). More important however, the possibility to conduct either one or both types of observation (fluorescence microscopy or SEM) has important implications for further downstream analyses. Fluorescence

microscopy requires the embedding of a specimen in mounting medium. For subsequent analyses, the sample might need to be de-mounted from the microscopic slide and washed. In contrast, an analysis via SEM requires dry samples, placed on a conductive layer of carbon (see methodological section, preparation of samples for fluorescence microscopy and SEM). For the detection and identification of diazotrophs on the surface of rice roots, a method to trace stable isotopes in combination with FISH would be extremely powerful (e.g. FISH-NanoSIMS). It will be discussed later in detail, that the possibility to identify microorganisms on dry samples via SEM is very beneficial for a subsequent detection of stable isotopes in a correlative approach. To date, Gold-FISH was applied to marine and terrestrial samples, as well as on the surface of plant roots, with obtained signal intensities that are comparable to those obtained through a CARD-FISH procedure (Schmidt *et al.*, 2012).

Methods to detect diazotrophic activity in paddy-soils

The acetylene reduction assay (ARA) is a method described by Koch and Evans in 1966, that is still being used since it is cheap and applicable in the field. It relies on the fact, that the nitrogenase also reduces acetylene to ethylene (Waughman 1971). For instance, the N-fixing activity of isolated *Azotobacter spp.* from different rice fields was evaluated using ARA (Sahoo *et al.*, 2014). Furthermore, ARA was used to monitor N fixation for five years in rice fields. The researchers reported partial inhibition of N fixation activity in fertilized plots (Valiente *et al.*, 1997). The use of ARA in paddy-fields for both, the surface and the water-saturated soil, is however questionable concerning the quantification of N fixation activity. The conversion rates of ARA to N_2 fixation might be biased, because of the slow diffusion rate of acetylene and ethylene in water-saturated soils. Furthermore, it was reported that not every nitrogenase is capable of reducing acetylene instead of N_2, as reported in the case of *Streptomyces thermoautrophicus* (Gadkari *et al.*, 1992).

N fixation can be quantified via $^{15}N_2$ tracer assays. Isotope Ratio Mass Spectrometry (IRMS) is an analytical tool that allows for the detection of incorporated ^{15}N from the $^{15}N_2$ gas within complex environmental samples (Montoya *et al.*, 1996). The spectrometric technique is highly sensitive, it provides unequivocal evidence for N_2 fixation, and gives reliable quantitative information concerning fate of biologically fixed N (Dabundo *et al.*, 2014). In an early study by Yoshida and Yoneyama in 1980, the BNF rate in the rice rhizosphere was analyzed via a $^{15}N_2$

tracer assay. The researchers observed a translocation of up to 25% of the atmospheric $^{15}N_2$ into the plant biomass. To the best of my knowledge, this study and an already mentioned study by Ito *et al.*, 1980, were the first to analyze BNF in rice via $^{15}N_2$ tracer assays. In 2013, Bei *et al.* developed a growth chamber for rice plants, in which the plants could be exposed to a $^{15}N_2$ comprising atmosphere for 70 days. Their results indicate that rice plants favor heterotrophic N-fixers over autotrophic. To date, nanoscale Secondary Ion Mass Spectrometry (NanoSIMS) combines the highest spatial and chemical resolution for chemical imaging (Wagner, 2009). The method employs a primary ion beam to investigate the chemical composition of an organism. The thereby created secondary ions can be analyzed via a quadrupole mass-spectrometer, which separates ion and ion clusters according to their mass to charge ratio. The obtained results are maps of isotope compositions at resolutions up to 30 nm, combined with a mass-differentiation of up to 7 elements simultaneously in modern dynamic SIMS techniques (Wagner, 2009). In 2007, Lechene *et al.* were the first to demonstrate incorporations of $^{15}N_2$ using NanoSIMS, in a pure culture of *Teredinabacter turnerae*, a marine *γ–Proteobacterium*. The diazotrophic activity of a single cell originating from soil was first measured by Eichorst *et al.*, 2015. To the best of my knowledge however, there is no study yet that used NanoSIMS to visualize active diazotrophs on roots, in paddy-soils or other rice plant materials. The imaging of stable isotopes via NanoSIMS can be combined with FISH to link the identity of single cells with their function (Orphan *et al.*, 2001, Woebken *et al.*, 2012; Musat *et al.*, 2016) Such an approach would allow for the identification of associative diazotrophs on the rice root surface. However, it is notoriously difficult to detect microorganisms on a root surface via FISH and subsequently analyze their chemical composition via NanoSIMS in a correlative manner (Pett-Ridge and Firestone, 2017). For fluorescence microscopy, the root would need to be on a microscopic slide and embedded in an antifade mounting medium (e.g. Vectashield). For NanoSIMS however, the same specimen would need to be dry. Between both analyses the roots would need to be de-mounted from the microscopic slide, washed with ethanol and water to remove the mounting medium, and air-dried. This implies that cells would become lost due to the washing and the root would completely change its structure due to the drying procedure, which is problematic as both circumstances would heavily impair a correlative analysis via FISH-NanoSIMS on a root surface. Gold-FISH is a powerful alternative to FISH that could resolve this problem, because Gold-FISH-stained root samples would already be dry for the detection of specific microorganisms of

interest via SEM. Afterwards, no distorting preparations of the root-sample would be necessary for the analysis via NanoSIMS.

Rice under gnotobiotic conditions

A system is gnotobiotic ("gnostos" – known and "bios" – life) once every life-form within the system can be accounted for (Reyniers, 1959). Researchers often rely on reductionist models, as they facilitate the insight into e.g. the behavior of an organism during controlled stress situations, due to the minimized complexity. Such models can be very powerful as they allow us to test very specific research questions.

Rice plants can be grown without the influence of any microorganism, cultivated under aseptic conditions. It was reported that the chemical treatment with sodium hypochlorite (NaOCl) in combination with H_2O or NH_4^+ leads to the formation of toxic HOCl or chloramines (Miché and Balandreau, 2001). Those toxins are responsible for the efficient removal of bacteria from the surface of *Oryza sativa* roots (Reinhold-Hurek *et al.*, 2015). Furthermore, NaOCl can be applied for disinfection of rice seedlings without reducing their ability to germinate (Abdul-Baki *et al.*, 1974). Subsequently, likewise under sterile conditions, they can germinate and flourish while being provided enough nutrients. In 1981, Maudinas and co-workers described rice plants growing in a sterilized liquid medium without any excess N, performing a complete growth cycle in the presence of only two free living diazotrophs – *Azotobacter vinelandii* and *Rhodopseudomonas capsulate,* which were used as an inoculum. In a recent study, two different rice varieties were inoculated with growth-promoting microorganisms under gnotobiotic conditions, using either the FCN- medium (a plant nutrient solution) or sterile soil extract (Shrivastava, 2015). The results of the study show that the inoculation with an *Agrobacterium sp*. led to an increase in the plants height, fresh weight and chlorophyll-a content in comparison to rice plants associated with other diazotrophs or a mixture of defined microorganisms. In a study from Chi *et al.* in 2005, the colonization patterns of *gfp* tagged R*hizobia* on rice roots were studied under gnotobiotic conditions. It was shown that rhizobia can penetrate the root surface, preferentially at lateral root emergences, and subsequently ascend along the stem base into leaves, where they develop the highest endophytic population densities (Chi *et al.*, 2005). With *Azoarcus* BH72, the model organism for endophytic colonization, it was shown that *Azoarcus* BH72 transcribes most of its genes for plant

colonization if exposed to low oxygen concentrations. In addition, the researchers visualized the diazotrophic activity of BH72 during association with the rice plant, based on a *nif* fluorescence reporter gene fusion (Sarkar and Reinhold-Hurek, 2014). Furthermore, it was shown for *Enterobacter cloacae* to improve fresh weight, root length, shoot length, and N content of rice plants under gnotobiotic conditions (Shankar *et al.*, 2011). However, *E. cloacae* remained in the genus *Enterobacter* and was not re-classified into a novel genus as described previously (e.g. *E. sacchari* to *K. sacchari*) (Brady *et al.*, 2013). To the best of my knowledge, there is no gnotobiotic study conducted with a species from the genus *Kosakonia* and rice. Furthermore, root exudates are essential compounds that affect the microbial community in soils, and that have the property to stimulate and enable beneficial associations between plants and microorganisms (Bacilio-Jimenez *et al.*, 2003). Under gnotobiotic conditions, the researchers observed that rice plants exude significantly more sugars and amino acids when microbes are in the system, as when no microbial partners are present. Variations in root exudates were observed in response to different microorganisms. The highest amounts of plant growth promoting substances, total sugars, reducing sugars and amino N was measured in rice plants inoculated with a microbial consortium, in which diazotrophic organisms have also been involved (Raja *et al.*, 2006). In general, rice plants grown under gnotobiotic conditions depict a great model system to unravel underlying mechanisms behind microbial colonization as well as the mutualistic consequences of plant-associated diazotrophy.

Motivation and aims of the present thesis

The rhizosphere is a hot-spot of microbial activity in soil (Berendsen *et al.*, 2012). Investigating the associations between plants and diverse microorganisms in the rhizosphere is seminal to enhance our understanding of nutrient cycling in soil environments. With the present study, I have focused on the imaging of one particular rhizospheric association, namely the partnership between diazotrophs and rice plants. Rice is the most important crop plant in terms of human nutrition (Ladha *et al.*, 2016), and diazotrophs have the potential to transform N_2 into plant-accessible nutrition. The identification of diazotrophs in the rice rhizosphere, together with investigating the fate of shared nutrients is fundamental to understand the mechanisms underlying the formation and sustainment of this association.

The first aim of the present thesis was to isolate diazotrophs from a paddy-soil environment. The isolate would then serve for the second aim - the development of a gnotobiotic system. Therefore, surface-sterilized rice seeds would be inoculated with previously isolated diazotrophs. In consequence, the rice plant would then grow with a diazotrophic associate under gnotobiotic conditions. This reductionist model system would then serve for my third aim - the development and evaluation of various microscopic tools, that were finally used to visualize microorganisms on the rhizoplane of rice roots under natural conditions. One of those tools was Gold-FISH, a method that allows for the combined use of fluorescence microscopy and SEM for the detection of microorganisms *in-situ* (Schmidt *et al.*, 2012). Gold-FISH offers the potential to detect specific microorganisms via SEM at a resolution and magnification beyond light microscopy, providing important insights concerning the complexity of the habitat (Oburger and Schmidt, 2016). Furthermore, Gold-FISH in combination with NanoSIMS is a powerful alternative to FISH-NanoSIMS, as the possibility to detect microorganisms via SEM drastically facilitates the correlation of SEM images and NanoSIMS images (Pett-Ridge and Firestone, 2017).

Material and Methods

Experimental Setup

Cultivation of wetland rice IR64 in paddy-soil

Rice seeds (IR64, Indica Rice semi-dwarf) were provided by the International Rice Research Center (IRRI, Philippines). Two different rice field soils were obtained, one from Italy (Crea, Vercelli) and one from Sri Lanka (RRDI, Bathalagoda). The soils were kept in separate reservoirs in the greenhouse at the University of Vienna (see Supplementary Figure 1). Prior to planting IR64 rice seeds, old roots and stones were removed from the soils by hand. Soils were puddled after submerging, which helps to break up soil aggregates and facilitates growth of rice roots. Before planting the IR64 seeds, they were surface-sterilized by washing in EtOH and H_2O, and subsequently by immersion in 20 ml H_2O containing 1ml sodium hypochlorite (NaOCl solution, reagent grade, Sigma Aldrich GmbH, Steinheim, Germany; final conc.: 1%) for 10 minutes. Afterwards, seeds were pre-germinated on a moist tissue in autoclaved glass dishes, enclosed in aluminium foil. After 7 days of growth the seedlings were transferred into either the Italy or the Sri Lanka soil.

Isolation of diazotrophic microorganisms from soil and plant material

N-fixing microorganisms were isolated on NFB. This medium was prepared as described previously by Dobereiner *et al*., 1976, with changes concerning additional carbon sources as described by Mirza *et al*., 2012 (0.25 M pyruvate, 0.25M glucose and 0.25M sodium carbonate were added in addition to 0.25 M malate). For preparation of solid N-free media plates, NFB medium was solidified with 2% Gellan Gum (Gelrite, Carl Roth GmbH, Karlsruhe, Germany) or 2% Agar (Sigma Aldrich Chemie GmbH, Steinhelm, Germany). Environmental samples were spread on N-free plates in two different approaches. First, bulk soil samples were taken from puddled and flooded Sri Lanka bulk soil. In this soil, no rice plants were growing at the moment of sampling. Several samples were taken at different depths and approx. 8 g fresh weight of each sample was diluted in 40 ml 1 × PBS. After shaking thoroughly by hand, samples were diluted in a serial dilution with 1 × PBS. A 100 µl inoculum was taken from the serial dilutions 10^{-3} to 10^{-7} and spread on a solid N-free plate and incubated under hypoxic conditions (1% O_2)

(Hypoxic chamber, Coy Laboratory Products Inc, Grass Lake, MA, USA). Second, roots harvested from Italy-soil-grown rice plants were washed in H_2O by shaking thoroughly by hand, to separate excess root-attached soil (rhizosphere) material. Subsequently, the roots were transferred into 40ml 1 × PBS. After shaking thoroughly by hand, the roots were discarded and 1 ml of the remaining liquid was diluted in a serial dilution with 1 × PBS. A 100 µl inoculum was taken from the serial dilutions 10^{-3} to 10^{-7} and spread on a solid N-free plate and incubated under hypoxic conditions. In a third approach, de-husked IR64 seeds were placed onto N-free plates without prior surface-sterilization. All plates that were used for the spreading of an inoculum or placing of seeds were solidified with Gellan Gum. They were monitored for the growth of microbial colonies by eye. After approximately 2 to 3 weeks, single colonies detected in any of the three approaches were transferred onto fresh N-free plates solidified with Agar. Colonies were transferred 3 to 4 times onto fresh plates before they were inoculated in the 5 ml wells of a 12-well-plate filled with 4 ml liquid NFB. Growth within the wells was identified by eye, based on the turbidity of the liquid medium. Cultures that grew up to this point were transferred into sidearm flasks containing 25 ml NFB, which allow for growth characterization via OD_{600} measurements (Optical density, measurement of the absorbance of sample at a wavelength of 600 nm) without disturbing the system. Each transfer and OD_{600} measurement was conducted under hypoxic conditions in the chamber. The setup for the isolation of diazotrophic organisms is presented in detail in Figure 1.

Figure 1: Isolation of rice-associated and free living N-fixing organisms from soil. A) Schematic illustration of transfer-steps of diazotrophic organisms from environmental sample spread on solid NFB plate (upper left) to isolates cultured in liquid NFB in sidearm-flasks (lower right). B) Two solid NFB plates are shown. In the lower one rhizospheric samples were spread upon, and the NFB was solidified with Gellan Gum. From this plate colonies were picked and transferred to NFB plates that were divided into eight sections as illustrated with the upper plate. Each colony was spread into one eighth, and the upper on was solidified with Agar (Note the difference in transparency). C) Bulk soil dilution 10^{-3} was spread upon a NFB plate solidified with Gellan Gum. D) Hypoxic chamber (E). In order to grow isolates in liquid medium, six out of twelve wells were filled with 4 ml NFB medium.

Storage and maintenance of diazotrophic isolates and other strains

For DNA extraction, samples were prepared as following: once turbidity was visible in respective wells of the 12-well plate, fresh NFB was prepared and incubated in the hypoxic tent overnight. Afterwards, 100 µl were transferred from the old well into a new one and 2 ml of the inoculated medium were transferred into a 2 ml Eppendorf tube. The rest of the old 12-well plate was discarded. The 2 ml sample was centrifuged (13000 rpm, 20°C, 15 min), re-suspended in 2 ml 1 × PBS via vortexing, and centrifuged again with the same settings. The resulting pellet was stored at -20°C if DNA was soon to be extracted, otherwise at -80°C. *K. sacchari* was maintained in culture in NFB medium (solid and liquid) under hypoxic conditions. In addition, a cryostock of the *K. sacchari* culture was prepared by adding 500 µl of an active *K. sacchari* culture (sampled at the moment of exponential growth phase, after approximately 3-4 days, growth in NFB medium at 1% O_2) to a 50% glycerol stock in a 2 ml screw top tube and

was stored at -80°C. Furthermore, *Bacillus subtilis* was obtained from a glycerol stock of the DOME culture collection. The organism was grown in 25 ml Lysogeny Broth (LB; Bertani, 1951), at 21% oxygen and at room temperature. For fixation of *B. subtilis*, 2 ml samples of *B. subtilis* were taken after approximately 12 h of growth, samples were centrifuged (13000 rpm, 20°C, 15 min), re-suspended in 2 ml 1 × PBS via vortexing, and centrifuged again with the same settings. The resulting pellet was fixed with EtOH and stored in 1 x PBS/ethanol (50/50, vol/vol) at -20°C.

Cultivation of gnotobiotic plants in open test tubes

Yoshida solution was prepared as described by Yoshida *et al.*, 1976, without adding N compounds (NaH_2PO_4 * 2 H_2O 0.26 mol L^{-1}, K_2SO_4 0.41 mol L^{-1}, $CaCl_2$ 0.80 mol L^{-1}, $MgSO_4$ *7 H_2O 1.32 mol L^{-1}, $MnCl_2$ *4 H_2O 0.0076 mol L^{-1}, Mo_7O_{24} * 4 H_2O 0.0001 mol L^{-1}, H_3BO_3 0.0151 mol L^{-1}, $ZnSO_4$ * 7 H_2O 0.0001 mol L^{-1}, $CuSO_4$ * 5 H_2O 0.0001 mol L^{-1}, $FeCl_3$ * 6 H_2O 0.0285 mol L^{-1}). Solidified with autoclaved Gellan Gum (1.5% final conc.), it was used as a soil-substitute for plant growth. Seventy ml of the hot Gellan Gum and Yoshida solution mixture (approximately 70°C) were poured into individual tubes (test tube, 25 mm, 300 mm, VWR) under sterile conditions. The mixture solidified after approximately 30 min. Rice seeds were surface-sterilized as described above and pre-germinated on a tissue moistened with autoclaved H_2O in a petri dish for 4 days. In parallel, *K. sacchari* grew on NFB under hypoxic conditions until the optical density measure reached a value of OD_{600}=0.2 (exponential phase, typically after 3 to 4 days). Seedlings were inserted into the solidified Yoshida solution in test tubes with tweezers under sterile conditions. For association with *K. sacchari*, seedlings were dipped into 10 ml of the culture medium for 15 min prior to planting, or 200 µl of the culture was injected into the gel in close proximity to the seedling. Afterwards, liquid Yoshida solution was poured over the gel and the tube was sealed with aluminium foil and brought into the greenhouse. In order to irrigate the plants during the incubation in the greenhouse, Yoshida solution was added through a syringe. After perforation of the aluminium foil, a new layer of foil was added to seal the injection hole. Once the plant reached the height of the tube, a small hole was cut into the foil with a scalpel and the leaf was pulled out with tweezers. From this moment on the Yoshida Solution was added through this hole along the plants stem with a syringe, but without perforating the aluminium foil anymore. The setup is visualized in Supplementary Figure 2.

Cultivation of gnotobiotic plants in enclosed test tubes

All glassware was washed in 18% HCl, rinsed with H_2O, autoclaved and muffled at 500°C before use. All work was furthermore conducted within a laminar flow (Thermo Fisher Scientific, Waltham, Massachusetts). Yoshida solution without N was mixed with autoclaved Gellan Gum (1.5% final conc.) and 25 ml of the mixture was poured into respective tubes. Once solidified, autoclaved cotton wool was used to seal the tubes from extrinsic contamination. Prior to planting rice seedlings (IR64) within the tube, the seeds were surface-sterilized and afterwards pre-germinated in separate wells of a 12-well plate, each amended with 1 ml autoclaved H_2O. Seedlings that showed signs of contamination (e.g. bacterial, fungal growth) were discarded. In parallel, *K. sacchari* was cultivated in 7 ml NFB medium under hypoxic conditions in separate wells of a 6-well plate. Once the medium inoculated with *K. sacchari* showed significant turbidity (approx. 3 to 4 days growth without N at 1%O_2), seedlings were inserted into the gel with tweezers under sterile conditions. For inoculation of seedlings with *K. sacchari*, the amount of four wells was pooled (28 ml), centrifuged (Eppendorf Austria GmbH, centrifuge 5804, 8000g, 15 min) and re-suspended in 2 ml 1 × PBS. A 500 µl aliquot of this inoculum containing 1.3×10^7 cells were pipetted onto the root of each seedling that were still in their wells. After 30 min of incubation, the seedlings were planted using the following procedure: Steel tweezers long enough to reach the gel within the tube were flamed and a small hole was melted into the gel. Afterwards, the same tweezers were used to transfer the seedling into the medium. Then, approximately 500 µl, N-free Yoshida solution was added and the tube was sealed with cotton wool again. Afterwards, the tubes containing inoculated rice seedlings were brought into the greenhouse. Once the Yoshida solution on the surface depleted, the tubes were opened under sterile conditions to allow for gas exchange with the surrounding atmosphere, 1 ml of fresh Yoshida solution was added, and the system was sealed again with autoclaved cotton wool. Negative controls consisted of surface sterilized seeds planted at the same described conditions, but without inoculation with *K. sacchari*. In addition, nutrient rich LB was inoculated with a surface-sterilized seed and water used for pouring seeds after seed sterilization. If neither of the inoculants led to contaminations after 4 days, the gnotobiotic experiment was continued. Furthermore, 4 different media (Yoshida solution + Glucose with or without N, SM medium (Sussmann, 1966), and LB) were incubated under hypoxic conditions and inoculated with root material sampled from 3-week-old gnotobiotic negative controls

(seedling planted into tube without *K. sacchari*). This analysis was performed to monitor the sterility of the negative controls at the end of the experiment. In Figure 2, the enclosed setup for the establishment of a gnotobiotic system for the growth of rice in re-association with previously isolated diazotrophs is described in detail.
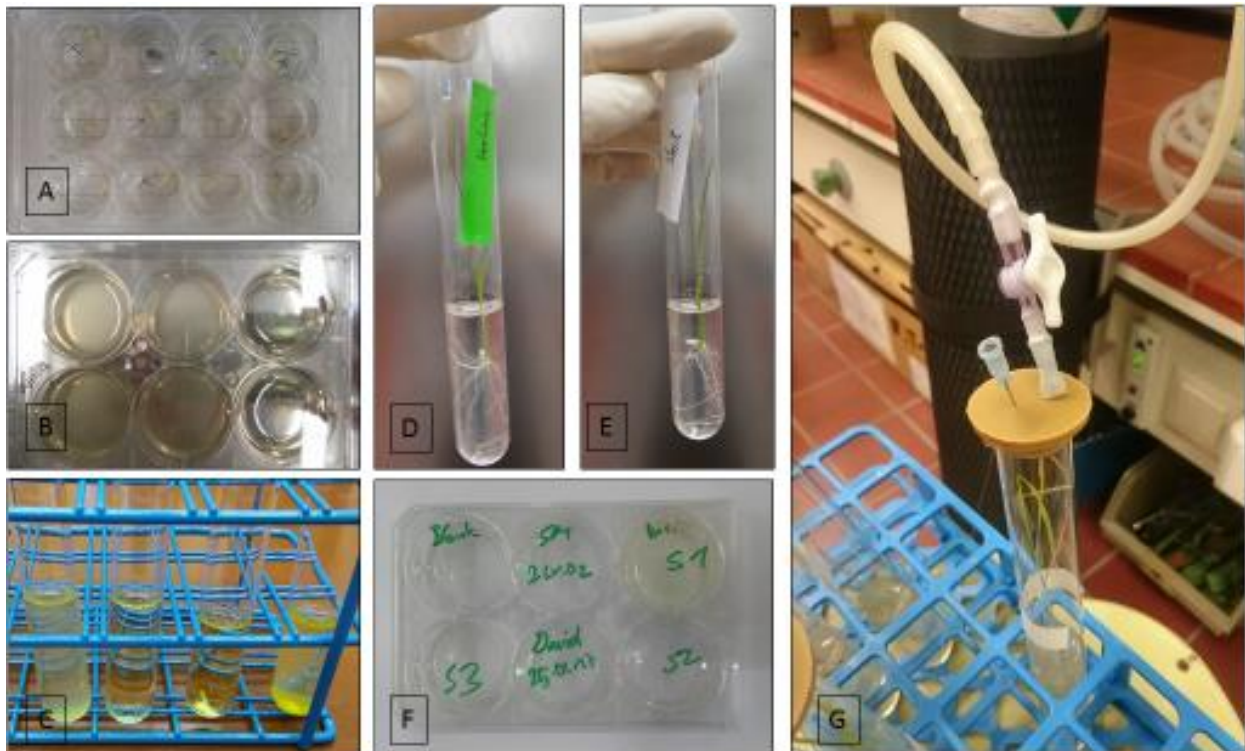

Figure 2: Establishment of gnotobiotic model system for the growth of rice (IR64) in association with *K. sacchari*. A) Rice seeds after four days of growth under sterile conditions. Wells marked with an "x" were used for inoculation into the gnotobiotic system. B) Growth of K. sacchari in 8ml NFB after 4 days of growth (left). From this six-well plate two were used as negative blank controls (right). C) From left to right: LB inoculated with i) 1 ml of water suspension taken from the last washing step of seeds (=last step in surface sterilization procedure of seeds); ii) 1 ml of sterilized water used for pouring the seeds; iii) a surface-sterilized seed; and iv) a non-surface-sterilized seed. D) Example of a rice plant under gnotobiotic conditions in association with K. sacchari E) a germ-free example. F) One out of 4 media controls is illustrated (SM medium) that was used for the inoculation root material from 3-week-old gnotobiotic negative controls (S1, S2 and S3, seedlings planted into enclosed tubes without K. sacchari). The well in the upper-left corner served as a blank. S1 has led to a contamination in the medium while S2 and S3 have not G) Preparation of the incubation of gnotobiotic rice plant with $^{15}N_2$. Enclosed tubes were sealed with a sterile rubber stopper; picture was taken before the enclosed system was flushed with helium.

Sampling of gnotobiotic rice plants

Samples from our gnotobiotic model were taken as following: The cotton wool was taken off in the greenhouse. After discarding the Yoshida solution on the surface, the plant was pulled out of the gel with tweezers and placed on a surface-cleaned glass plate. Plant material (roots or aboveground material) of interest was cut with a scalpel. Plant material was divided into

samples for DNA extraction (stored in 15ml tubes at -20°C and -80°C) and microscopic analysis. Samples for microscopic analysis were transferred into 5 ml 1 × PBS in 15 ml tubes directly, so they would not dry out. The correct amount of PFA (2% end-concentration) was added afterwards in the laboratory (fixation for 3 hours at 4°C) After two washing steps in 1 × PBS the samples were stored in 1 × PBS:EtOH (40:60,vol:vol) at -20°C.

Incubation of gnotobiotic rice plants with $^{15}N_2$ in enclosed tubes

Enclosed tubes of gnotobiotic rice plants were opened under sterile conditions, excess Yoshida solution was replaced with autoclaved H_2O_{MQ} to exclude that microorganisms reside in the liquid on the surface, which might hamper the diffusion of $^{15}N_2$ into the rhizosphere. Afterwards, the system was sealed again with previously autoclaved rubber stoppers that were glued to the tube (Figure 2G). The air volume constituted approximately 64 ml, which was flushed with Helium for 2 minutes. Afterwards, 38.5 ml of the helium atmosphere was removed from each tube and replaced with 13.4 ml O2 (21%), 25ml of $^{15}N_2$ (39.5%) and 1 ml of CO2 (0.2%; Percentage values refer to the volume-fraction of the respective gas in the tube atmosphere). Plants were incubated for 72 hours in the greenhouse. Afterwards, the tube was opened, the plant was pulled out of the gel by hand and placed on a clean glass surface. Plants were divided into aboveground and root material. A portion of each sample type was PFA-fixed and stored in 1 × PBS:EtOH (40:60,vol:vol) at -20°C. These remaining samples were stored in 2 ml tubes at -20°C overnight. The next day the samples were dried in a 60°C incubator for 48 hours in 2 mL tubes. These samples were processed by milling (MM400, Retsch, Haan, Germany, 2 × 1 min, 30m/s) with a 5 mm and a 7 mm steel ball. Afterwards, 200 µL of MQ water were added to rinse the steel balls before removal to recover as much sample as possible. That 200 µl rinsing-product was added to the samples. Samples were dried at 60°C for 72 hours. In supplementary Table 1 it is shown how much dry weight of each sample was transferred into tin cups, that was subsequently analysed by Isotope ratio mass spectrometry.

Molecular methods

DNA extraction and quantification

DNA was extracted from diazotrophic cultures (2 ml) by chemical lysis with a DNA extraction kit (Blood and Tissue, Qiagen, Hilden, Germany) following the manufacturers' protocol for pure culture DNA extraction. The protocol was performed either manually (5805 Eppendorf, Vienna, Austria) or in assistance of a robot (Qiacube, Qiagen Vertriebs GmbH, Vienna, Austria). DNA extracts were eluted in 30 µl nuclease free water (ThermoFISHER Scientific, Waltham, MA, USA), the DNA concentration was quantified via NanoDrop (ThermoFISHER Scientific, Waltham, MA, USA) and the samples were stored at -20°C. The soil extraction kit (Power soil DNA, Qiagen, Hilden, Germany) was used for extraction of DNA from rice roots sampled from enclosed gnotobiotic tubes. DNA was extracted from these samples by following the companies' protocol. Prior to DNA extraction, the root samples were homogenized by milling with a 5 mm and a 7 mm steel ball. DNA extracts were eluted in 30 µl nuclease free water, the DNA concentration was quantified via NanoDrop, and the sample was stored at -20°C.

Sample preparation for Sanger sequencing

The primer pairs listed in Table 1 were used to generate 16S rRNA gene and *nifH* gene amplicons from DNA isolated from cultures growing under hypoxic conditions in NFB. The correct size of the PCR products was checked via gel electrophoresis and amplicons were purified using the Quiaquick PCR purification kit (Quiagen, Hilden, Germany) or the ZR-96 DNA clean-up Kit™ (Zymo research, Irvine, CA, USA). Afterwards, the PCR products were eluted in 30 µl nuclease free water and stored at -20°C. Prior to sequencing, the amplicon concentration was measured via NanoDrop. To meet the requirements of the sequencing company, amplicons were diluted in nuclease free water to a concentration of 22.5 ng per 100 bp in 15 µl. The PCR products were mixed with the corresponding forward primer and sent for Sanger sequencing (Microsynth, Vienna, Austria). The quality of sequences obtained was analyzed with Chromatogram Explorer Lite 4.0.0 (Heracle BioSoft, Pitesti, Romania). The nucleotide basic local alignment search tool (BLASTn; NCBI, MD, USA) was used to identify isolated microorganisms according to their 16S rRNA and *nifH* gene sequences, respectively.

Table 1: List of primers used for generation of amplicons via PCR

Name	Purpose	Sequence (5'->3')	Reference
Ueda19F	*nifH*, forward primer	GCIWTYTAYGGIAARGGIGG	Ueda *et al*. (1995)
R6	*nifH*, reverse primer	GCCATCATYTCICCIGA	Marusina *et al*. (2001)
IGK3	nifH, forward primer	GCIWTHTAYGGIAARGGIGGIATHGGIAA	Ando *et al* (2005)
DVV	nifH, reverse primer	ATIGCRAAICCICCRCAIACIACRTC	Ando *et al* (2005))
8F	16S rRNA, forward primer	AGAGTTTGATCCTGGCTCAG	Lane *et al* (1991)
1492R	16S rRNA, reverse primer	GGTTACCTTGTTACGACTT	Lane *et al* (1991)

Quantification of nucleic acids

To quantify the amount of nucleic acids and to evaluate the level of other contaminating substances (e.g. phenol, proteins, EDTA, hydrocarbons) a NanoDrop Spectrophotometer ND-1000 (ThermoFISHER Scientific, Waltham, MA, USA) was used. The nucleic acid concentration was measured in 1 µl PCR product at OD_{260nm}. A refraction (R) ratio of $R_{260nm}/R_{280nm} \geq 1.8$ could indicate an impurity of the extract, due to contaminations through e.g. proteins.

Agarose gel electrophoresis

PCR products were separated based on their size on a 1% agarose solution gel (1% LE Agarose in TBE Buffer; Biozym Scientific GmbH, Heissisch Oldendorf, Germany). Gels were pre-stained with GelRed (Biotium, Inc, Fremont, CA, USA). In reference to Gene ruler 1kb DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA) or the 100bp DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA), the correct length of the amplicon was controlled in order to confirm its successful amplification. Gel images were taken with a GelDOC XR+ System (Bio-rad Laboratories, Copenhagen, Denmark) connected to the Image Lab™ software.

Amplified ribosomal DNA restriction analysis (ARDRA)

DNA was extracted from sterile grown rice plants, rice plants colonized with *K. sacchari* and a *K. sacchari* pure culture with the protocols described above. From those DNA samples, 16S rRNA amplicons were generated using the corresponding primers listed in Table 1 and the correct size of the amplicons was controlled via gel-electrophoresis. Afterwards, Taq1 (Digestion enzyme; ThermoFISHER Scientific, Waltham, MA, USA) was used to digest all 16S rRNA amplicons. Digestions were conducted at the specific temperature optimum at 69°C for 12 hours. Ten µl of the digested product were loaded on a 3% agarose gel, which was running

at 50V for 5 hours. The gel was post-stained with GelRed. In reference to the 1kb and the 100bp DNA ladder the lengths of the fragments resulting from digestion were identified. Gel images were taken to compare the fragment lengths and restriction patterns between each sample type.

Visualization techniques

Sample preparation for in-situ hybridization and microscopy

Three different sample types (pure cultures, soil-grown rice roots, gnotobiotic rice roots) were used for *in-situ* hybridization and microscopic analysis and prepared as follows: The pure cultures used for evaluation of visualization techniques were *K. sacchari* (grown in NFB and sampled as described previously) and *B. subtilis. K. sacchari* samples were fixed with 4% PFA for 30 minutes at room temperature (RT). *B. subtilis* was grown in LB, after 12 hours of growth samples were taken as described previously for diazotrophic cultivates, and fixed with EtOH$_{abs}$ for 30 minutes at RT. All root samples were fixed with 2% PFA for 3h at 4°C as described above.

For fixed pure-culture samples, to be analyzed via DNA-based staining and fluorescence *in-situ* hybridization (FISH), 5 to 10 µl of the sample was pipetted onto wells of Teflon coated slides and dried at 46°C for 15 min. For CARD-FISH and Gold-FISH, fixed pure cultures were filtered (Vacuum/Pressure pump, Pall Laboratory, USA) either separately (5 µl *K. sacchari*; 2 µl *B. subtilis*) or as a mixture (3 µl *K. sacchari* + 1 µl *B. subtilis*) onto filters (Whatman Nuclepore Track-Etched Membranes, 25 mm, 0.2 µm). For the analysis of fixed root samples via all molecular and microscopic methods, root pieces having approximately 1 cm length were cut. Drying steps were omitted to preserve the morphological structure of the roots.

DNA-based staining

To stain the DNA in a sample, 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI; Vector Laboratories) and SYBR-Green I staining dye (Lumiprobe, Germany) were used. Therefore, fixed samples (e.g. roots, pure cultures, filter pieces) were immersed in DAPI (1000×) or SYBR green (10×) solutions in H$_2$O and incubated in the dark for 10 min. Afterwards, the liquid was removed via pipetting and the sample was washed in excess H$_2$O for 3 minutes. After removal of the water the sample was mounted for microscopy. All samples were placed on a microscopic slide and covered with VectaShield H-1000 (Vector Laboratories, USA) to prevent fading of the

fluorescence signal. Pure cultures were air dried before mounting. For the mounting of root and seed samples, pieces of adhesive carbon pads (Christine Gröpl Electron Microscopy, Tulln, Austria) were placed onto the microscopic slide. The pads served as spacers between the slide and the cover slip, ensuring a parallel placement of the cover slip and preventing destruction of the plant tissue (Richter-Heitmann *et al.*, 2016). Carbon pads were not necessary for the mounting of specimens other than roots and seeds.

Fluorescence in-situ hybridization

FISH analysis was performed according to a standard FISH-protocol (Amann et al., 1995). The probes used in combination with respective formamide concentrations are listed in Table 2. For the FISH analysis of gnotobiotic rice roots, the protocol was adjusted as follows: To preserve the root structure, steps for drying and dehydrating the sample were omitted and soft tweezers were used. Roots were incubated in 400 µl hybridization buffer containing 1.5 µl probe working solution (50 ng µL^{-1}) for *in-situ* hybridization for 2 hours at 46°C. Subsequently, the roots were washed in pre-warmed washing buffer. The samples were embedded in Vectashield H-1000 and prepared for microscopy as described previously.

Table 2: List of probes used for *in-situ* hybridization with corresponding formamide (FA) concentration. Probes for CARD-FISH and Gold-FISH are labelled with horse radish peroxidases (HRP).

Method	Dye	Probe	Target	Reference	FA (%)
FISH	Fluos / Atto 565	EUB338	Most bacteria	Amann *et al.* (1990)	35
FISH	Fluos / Atto 566	EUB338 II	*Planctomycetales*	Daims *et al.* (1999)	35
FISH	Fluos / Atto 567	EUB338 III	*Verrucomicrobiales*	Daims *et al.* (1999)	35
FISH	Fluos / Atto 568	NONEUB	none	Wallner *et al.* (1993)	35
CARD-FISH / Gold FISH	Oregon Green 488 / Alexa 488	EUB338	Most bacteria	Amann *et al.* (1990)	35
CARD-FISH / Gold FISH	Oregon Green 488 / Alexa 488	EUB338 II	*Planctomycetales*	Daims *et al.* (1999)	35
CARD-FISH / Gold FISH	Oregon Green 488 / Alexa 488	EUB338 III	*Verrucomicrobiales*	Daims *et al.* (1999)	35
CARD-FISH / Gold FISH	Oregon Green 488 / Alexa 488	NONEUB	none	Wallner *et al.* (1993)	35
CARD-FISH / Gold FISH	Oregon Green 488 / Alexa 488	LGC354A	*Firmicutes*	Meier *et al.* (1999)	35
CARD-FISH / Gold FISH	Oregon Green 488 / Alexa 488	GAM42A	*γ-Proteobacteria*	Manz *et al.* (1992)	35

CARD-FISH

Pure cultures were fixed and filtered as described previously. The filter pieces were coated with 0.05% low melting point agarose (ThermoFISHER Scientific, Waltham, MA, USA) in order to prevent losing cells during washing procedures in particular. For cell wall permeabilization, the filter pieces were incubated with Lysozyme solution (10 mg ml^{-1}; 60 min, 37°C in 1 ml H$_2$O$_{MQ}$ with 0.05 M EDTA and 0.1 M Tris-HCl). The CARD-FISH analysis was performed according to the protocol of Pernthaler *et al.*, 2002. The protocol was modified slightly for the visualization of microorganisms on the surface of roots. As already mentioned, an important difference was to omit dehydration and drying steps to preserve the root structure and to use soft tweezers. Furthermore, after permeabilization of cell walls with Lysozyme (10 mg ml^{-1}; 60 min, 37°C in 1 ml H$_2$O$_{MQ}$ with 0.05 M EDTA and 0.1 M Tris-HCl), Achromopeptidase was used for additional permeabilization (60 U ml^{-1}, 30 min, 37°C in 1 ml H$_2$O$_{MQ}$ with 0.1 M NaCl and 0.01 M Tris-HCl). Endogenous peroxidases were inactivated by incubation of filters and roots in 0.15% hydrogen peroxide (H$_2$O$_2$) in MQ. for 30 minutes at 25°C. *In-situ* hybridization was performed with roots or filters incubated in 400 µl hybridization buffer containing 1.5 µl probe working solution (50 ng µl^{-1}). The used probes, labelled with horseradish peroxidase (HRP), are listed in Table 2. For CARD, tyramides labelled with either Oregon Green® 488 (ThermoFISHER Scientific, Waltham, MA, USA) or Alexa Fluor™ 488 (ThermoFISHER Scientific, Waltham, MA, USA) were used. The deposition of tyramides was conducted as described by Pernthaler *et al.*, 2002, for both filter pieces and roots. Afterwards, the specimens were washed in H$_2$O$_{MQ}$ and TXP (Triton X in 1 × PBS; final concentration: 0.05%), filter pieces were submerged in EtOH$_{abs}$ and air-dried afterwards, root pieces were shortly placed on a tissue to remove excess water. Finally, mounted onto microscopic slides as described above.

Gold-FISH

The original protocol for Gold-FISH was developed by Schmidt *et al.* in 2012. Fixed rice roots as well as *B. subtilis* and *K. sacchari* cells immobilized on filters were used for Gold-FISH analysis. Filter pieces were coated with 0.05% low melting point agarose. For cell wall permeabilization, they were incubated in Lysozyme solution (10 mg ml^{-1}; 60 min, 37°C in 1 ml H$_2$O$_{MQ}$ with 0.05 M EDTA and 0.1 M Tris-HCl) and roots were treated with Achromopeptidase (60U ml^{-1}, 30 min,

37°C in 1 ml H_2O_{MQ} with 0.1 M NaCl and 0.01 M Tris-HCl) in addition. Endogenous peroxidases were inactivated by incubation of filters and roots in 10 ml methanol (conc. 15%) for 30 minutes at 25°C. Afterwards, endogenous biotin and streptavidin were deactivated in all sample types using a blocking-kit (Vector Laboratories Inc, CA, USA). Hybridizations with HRP-labelled probes (listed in Table 2) were performed with all sample types in 400 μl hybridization buffer containing 1.5 μl probe working solution (50 ng $μl^{-1}$) for at least 3 hours at 46°C (instead of 3 hours at 37°C, as in the original protocol). Subsequently biotinylated tyramides were used for catalyzed reporter deposition (CARD) at 46°C for 20 minutes.

For the binding of fluoro-nanogold-streptavidin conjugates, samples were incubated overnight (maximum of 12 hours) (instead of 3 hours, as in the original protocol) in 400 μl 1 × PBS-BSA (1% albumin fraction V, Roth, Karlsruhe, Germany) containing 0.25% of a streptavidin conjugate covalently labelled with a 1.4 nm nanogold particle and 2-3 Alexa Fluor 488 (AF 488) fluorophores (0.08mg mL^{-1}, Alexa Fluor® 488 FluoroNanogoldTM-Streptavidin, Nanoprobes, NY, USA). After several washing steps in H_2O and 1 × PBS-gelatine-Tween-20 (PGT; 1 × PBS containing 0.1% Gelatin and 0.1% Tween-20), samples were either prepared for microscopy as described previously, or for autometallographic enhancement of nanogold particles using a gold developer kit (2113 GoldEnhance EM 2113, Nanoprobes, NY, USA). Therefore, the enhancer and activator solution were mixed at a ratio of 1:3 in a glass petri dish. After 5 minutes of incubation, one drop of each, the initiator and buffer solution, was added. Roots or/and filter pieces were immediately added to the mixture, and incubated for 8 minutes, turned around with tweezers (soft tweezers for roots) and incubated for another 4 minutes (instead of 10 min without turning the specimen, as in the original protocol). Subsequently, one washing step in H_2O_{MQ} and one in 1% $Na_2S_2O_3$ in H_2O_{MQ} were conducted in 15 ml tubes while rotating. Finally, rice roots were air dried on self-adhesive carbon pads, while filter pieces were air dried in a petri dish.

Microscopy

Pure cultures, filter pieces, and roots were mounted onto Teflon coated or standard microscopy slides as described above. For microscopic analysis, two different microscopes were used. A Zeiss Axioplan2 Imaging (connected to a HXP120 power station and an AxioVision Rel.4.6 software, with a Axio Cam Hrc camera) and a Leica TCS SP8 Confocal Microscope, with

a Leica Super K Lasers and a Leica Ctr6500 Power source, connected to a Leica LAS X software platform. The used filter cubes and laser channels are listed in Table 3. Furthermore, prior to SEM, filter-samples were investigated using the Laser Micro Dissection Microscope (Leica CTR 6500, Leica Microsystems, Wetzlar, Germany) to mark regions of interest. Therefore, filter pieces were placed on a microscopic slide and covered with a cover slip to minimize the 3D-structure of filter-piece.

Table 3: Filter cubes and laser channels

Filter Cube - use for	Microscope	Excitation (nm)	Emission (nm)
DAPI	Zeiss Axioplan 2	405	470 (UV)
Fluos/Alexa488/Oregon green and Cy3/Atto565	Zeiss Axioplan 2	485/20 + 213/12	490 (green) and 554 (red)
Dapi	TCS SP8	340 - 380	470 UV
Fluos/Alexa488/Oregon green	TCS SP8	546/12	490 (green)
Cy3/Atto565	TCS SP8	490/20	554 (red)

Scanning electron microscopy and EDAX

Air-dried specimens (filter pieces and rice roots) were attached onto stubs (Micro to Nano, EK Haarlem, Netherlands) with self-adhesive carbon pads. The samples were coated with an electron conductive layer of carbon, which has a thickness of approximately 5 nm (Leica MED20). Images were taken at the CIUS department (Core Facility Cell Imaging and Ultrastructure Research, CIUS, University of Vienna) using a Scanning Electron Microscope (Jeol IT300 LAB6LV, Japan). Images were obtained using detectors for secondary electrons (SE) and backscattered electrons (BSE), for SE and BSE images, respectively. Images were taken under high-vacuum and 20k mV (voltage applied on the cathode). Furthermore, Energy dispersive X-ray spectroscopy (EDAX Ametec, Software TEAM) was applied to analyze the chemical composition within an area of interest.

Results

Isolation of free-living and plant-associated N-fixing microorganisms

Isolation. Serial dilutions of paddy-soil and rice-rhizosphere samples ($10^{-3} - 10^{-7}$) were plated directly onto solid NFB to isolate free-living and root-associated N_2-fixing microorganisms. In addition, seed-associated diazotrophs were isolated through placing seeds of genotype IR64 on solid NFB plates. Samples were processed under hypoxic conditions as described in the methodological section and in Figure 1. My initial idea for the isolation of diazotrophs was to identify microorganisms after the final transfer into fresh liquid NFB medium, as I was only interested for the isolation of diazotrophs that are able to grow in liquid NFB. Only those would provide enough biomass, which I needed for subsequent experiments (e.g. development of gnotobiotic model). Based on the turbidity in liquid NFB I selected for organisms that shall be identified via Sanger sequencing of 16S rRNA and *nifH* genes. However, for most of the organisms it was not possible to obtain the corresponding *nifH* gene sequence, and thus the experiment was repeated to re-sequence PCR-amplified 16S rRNA genes and *nifH* genes. In this second round of sequencing, we exclusively identified *K. sacchari*, *Paenibacillus panacisoli and Paenibacillus massiliensis* in each well, indicating a cross-contamination of the two organisms into other wells of the 12-well plate. It is likely that the content from one well spilled over to another, as they were filled with too much NFB in this particular experiment. However, the cultures were not completely lost, some of them were still growing on the original NFB plates. For a third round of Sanger sequencing, these cultivates were sampled for DNA extraction from the plate directly, their 16S rRNA gene sequence was amplified via PCR, and the amplicons were subsequently Sanger sequenced. In Table 4, all the isolated organisms are listed, together with the organisms that were solely observed once in liquid NFB, but afterwards got lost due to contamination. The organisms marked with '+' in Table 4 refer to the organisms that were growing on NFB plates, identified in the third round of sequencing, as described above. Those isolates were transferred to fresh NFB plates after identification via Sanger sequencing of the 16S rRNA sequence and BLASTn. However, I did not try to transfer them into liquid NFB afterwards. Furthermore, the potential of these isolates to fix atmospheric N was evaluated via PCR with *nifH*-specific primers. It was again not possible to detect *nifH* genes in those cultivates via PCR. In total, I have isolated 8 different organisms on solid NFB (marked in Table 4 with '+')

and 3 different organisms in liquid NFB (marked in Table 4 with '*'). For the latter, it was possible to detect nifH gene sequences in each organism via PCR. Those organisms were prepared as a glycerol stock and stored at -80°C.

Table 4: Summary of diazotrophs identified on solid or in liquid NFB. Identification via 16s rRNA gene sequencing and BLASTn analysis

Organism‡	Similarity to 1st blast hit / E-value	Origin	Number of times observed	Phylum	Order	Maintenance of culture
Terrabacter sp. (+)	99%; 0.0	rhizosphere	2	not classified	not classified	NFB plate (ID: R3.2.011)
Terrabacter lapilli (+)	99%; 0.0	rhizosphere	3	Actinobacteria	Actinomycetales	NFB plate (ID: R3.2.042)
Bacillus arbutinivorans	99%; 0.0	rhizosphere	1	Firmicutes	Bacillales	lost due to contamination
Paenibacillus panacisoli (+)	98%; 0.0	rhizosphere	1	Firmicutes	Bacillales	NFB plate (ID: R3.1.043)
Paenibacillus massiliensis (+)	99%; 0.0	rhizosphere	8	Firmicutes	Bacillales	NFB plate (ID: R3.1.035)
Bacillus sp. (*)	100%; 0.0	rhizosphere	3	Firmicutes	Bacillales	Cryostock (ID: CRY 1)
Bacillus megaterium (+)	99%; 0.0	rhizosphere	1	Firmicutes	Bacillales	NFB plate (ID: R3.2.021)
Bacillus cereus	99%; 0.0	rhizosphere	1	Firmicutes	Bacillales	lost due to contamination
Bacillus thuringiensis (+)	99%; 0.0	rhizosphere	1	Firmicutes	Bacillales	NFB plate (ID: R3.1.039)
Streptomyces sp.	99%; 0.0	rhizosphere	1	Actinobacteria	Actinomycetales	lost due to contamination
Bacterium T64	99%; 0.0	seed	1	Actinobacteria	Actinomycetales	lost due to contamination
Curtobacterium sp. (+)	99%; 0.0	seed	1	Actinobacteria	Actinomycetales	NFB plate (ID: S3.1)
Panibacillus xylanilyticus	99%; 0.0	seed	1	Firmicutes	Bacillales	lost due to contamination
Paenibacillus sp. (*)	99%; 0.0	seed	1	Firmicutes	Bacillales	Cryostock (ID: CRY 3)
Rhodococcus sp.	99%; 0.0	bulk soil	1	Actinobacteria	Actinomycetales	lost due to contamination
Kosakonia sacchari*	99%; 0.0	bulk soil	1	Gamma-Proteobacteria	Enterobacteriales	Cryostock (ID: CRY 2)
Microbacterium sp. (+)	99%; 0.0	bulk soil	1	Actinobacteria	Actinomycetales	NFB plate (ID: 4)
Bacillus sp.	99%; 0.0	bulk soil	2	Firmicutes	Bacillales	lost due to contamination

‡: Organisms were identified via Sanger sequencing and BLASTn. The organisms listed in the first column refer to the first hit found via BLASTn analysis; It was possible to grow the organism in liquid NFB for isolates marked with (*). Organisms marked with (+) grew on solid NFB.

Kosakonia sacchari sp. This species was isolated from a bulk-soil sample of our Sri Lanka soil. The organism belongs to the phylum of γ–*Proteobacteria*, and the genus *Kosakonia*. The N-fixing ability of our isolate was verified via PCR amplification of the *nifH* gene. Our isolate is 99% similar to *K. sacchari* strain SP1[T] on 16S rRNA level, and its *nifH* gene is 94% similar to *K. sacchari* strain R4-724 (analysed via BLASTn). Figure 3 depicts the rod-formed morphology visualized via scanning electron microscopy, and furthermore in Figure 4, it is shown that under N-free, hypoxic conditions the organism reaches its exponential phase between three and four days.
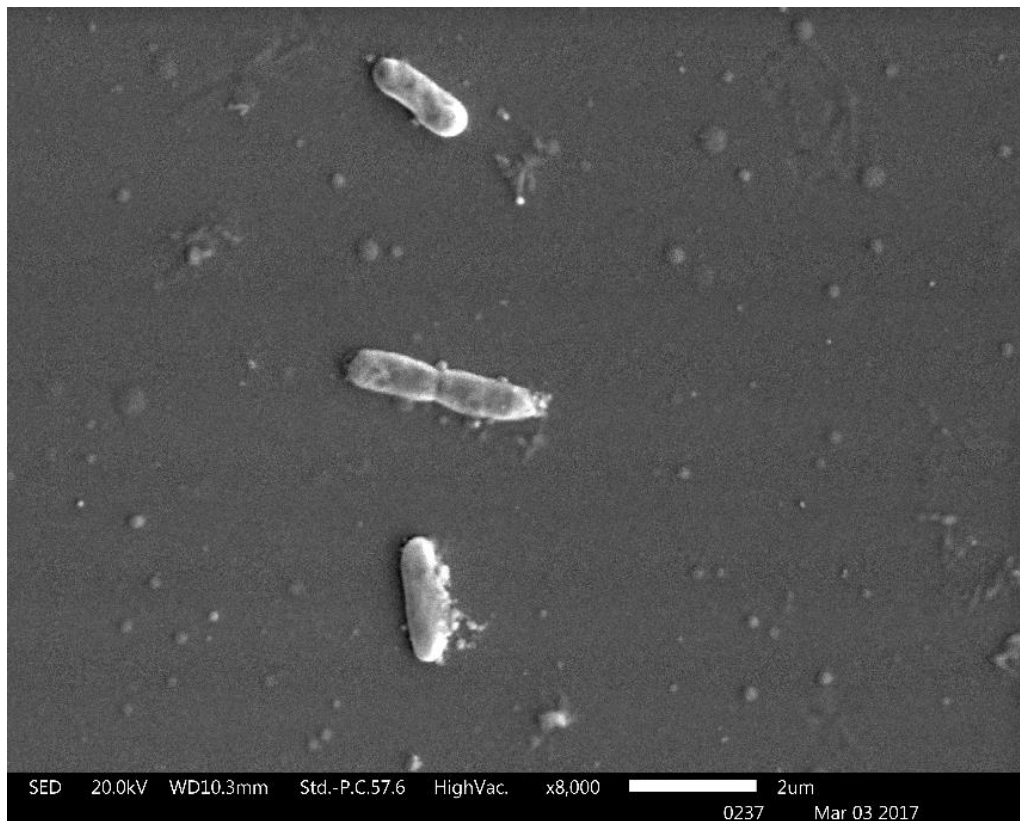

Figure 3: SEM image of *K. sacchari* cells. Four rods of approximately 2 µm in length are visualized.
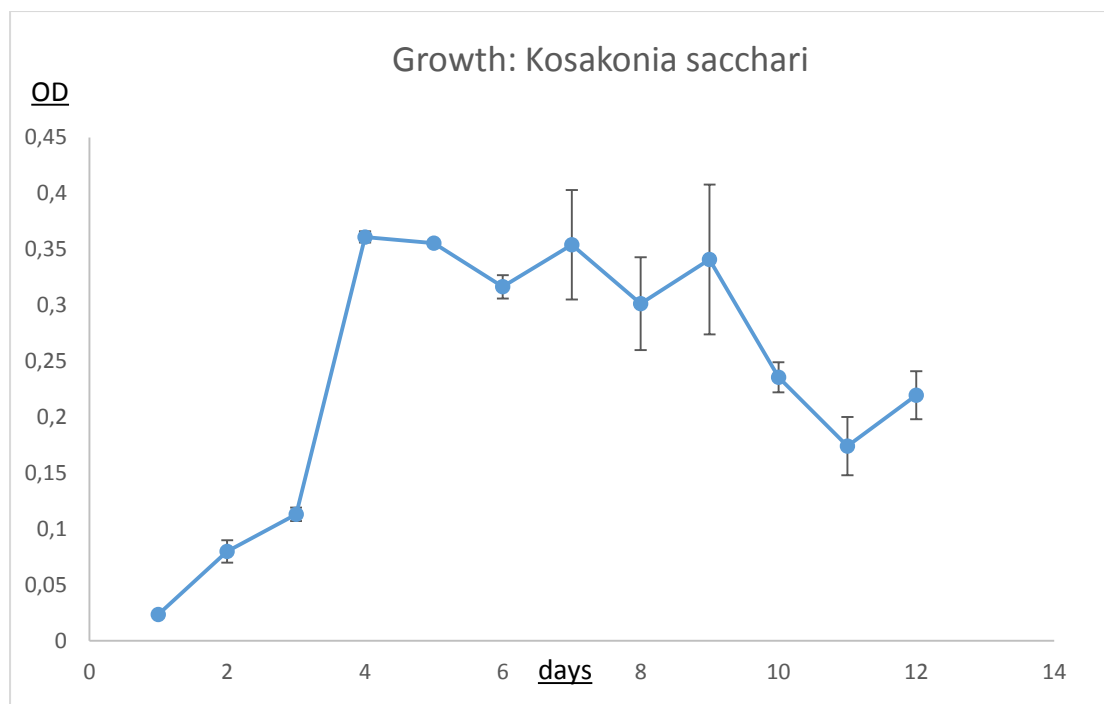
Figure 4: Growth curve of *K. sacchari* grown in NFB. The mean and standard deviation of three parallel cultures are depicted.

Cultivation of rice under gnotobiotic conditions

Approaches for the development of a gnotobiotic model system. Different attempts were conducted for the development of a model system that allows for cultivation of rice plants in association with N-fixing microorganisms (Table 5). In all experimental approaches the rice plants were able to sustain growth for three weeks. Furthermore, the rice roots were branching in all treatments and they developed morphologies similar to the soil-grown plants (see supplementary Figure 3). Plants inoculated with cells of *K. sacchari* showed a formation of a dense biofilm in all samples, indicating that the organism can colonize the rice rhizoplane. Our first approach (open atmosphere "OA") consisted of sterile plants (OA-sterile) and plants in association with *K. sacchari* (OA-kosa). The second approach (contained atmosphere "CA") and its repetitions consisted of three different treatments: i) sterile plants (CA-sterile), ii) plants in association with *K. sacchari* (CA2-kosa) and iii) "wild" plants without surface sterilization of seeds (CA3-wild). Triplicates of each treatment were obtained weekly.

Table 5: Summary of all approaches to develop a gnotobiotic model systems for the cultivation of rice plants in association with diazotrophic microorganisms.

Experimental approach	Samples obtained	Plant growth and root branching	Association with K. sacchari	Occurance of contaminants
open atmosphere (OA)	40 (all at once)	natural morphology	not well investigated	in every sample (after 7 days of growth)
contained atmosphere (1CA)	27 (9, weekly)	natural morphology	positive	in 1/9 sterile samples (after 21 days of growth)
contained atmosphere (2CA)	27 (9, weekly)	natural morphology	positive	none (after 21 days of growth)
contained atmosphere (3CA)	27 (9, weekly)	natural morphology	positive	in 2/9 sterile samples (after 21 days of growth)

Maintaining sterile growth of rice plants. It was a key challenge to enable sterile growth conditions of the rice plants. Surface sterilized seeds were planted into a gel amended with nutrients within tubes in two different approaches as described above. To achieve sterile growth conditions, it was necessary to surface-sterilize rice seeds prior to planting. The surface sterilization procedure was evaluated as presented in Figure 5. Here, microorganisms were visible on the surface of natural rice seeds that were not surface-sterilized after staining with CARD-FISH. In contrast, no microorganisms were detected by CARD-FISH on the surface of rice seeds that underwent a prior surface sterilization treatment with NaOCl. Despite surface-sterilizing seeds, it was not possible to achieve sterile growth conditions within our first approach (OA), where the tubes were open at the top and therefore exposed to the greenhouse atmosphere. The experimental setup of this approach is shown in supplementary Figure 2, together with the contaminations that were detected in this approach via fluorescence microscopy. However, it was possible to achieve sterile growth conditions within our second approach in contained tubes (1-3CA-sterile). This experimental setup included tubes sealed from the outside using cotton wool as shown in Figure 2. Sterile samples from all contained approaches remained sterile in the first two weeks. Contaminations occurred as listed in Table 5, but only in some replicates obtained from the third week. In Figure 6 H.1 such a contamination of a sample obtained at the third week is visualized. To further evaluate the sterility of rice plants under contained growth conditions, we repeated the experiment two times as summarized in Table 5. In the second attempt (2CA-sterile), no microorganisms were detectable via microscopy on the surface of sterile growing rice plants, even after three weeks. In the third attempt (3CA-sterile), four different growth-media were prepared as controls to additionally test for sterility (as described in the methodological section). Those media were incubated under oxic and hypoxic conditions and inoculated with sterile-grown roots that were

sampled after the third week. SybrGreen staining and epifluorescence microscopy showed contaminations in two out of three sterile replicates of the third week (3CA-sterile). The contaminated replicates simultaneously led to turbidity within the SM and LB under oxic as well as under hypoxic conditions. No contaminations were observed in the Yoshida solution with or without N. One replicate, which was defined as sterile via microscopy, did not cause such turbidity in any medium control.
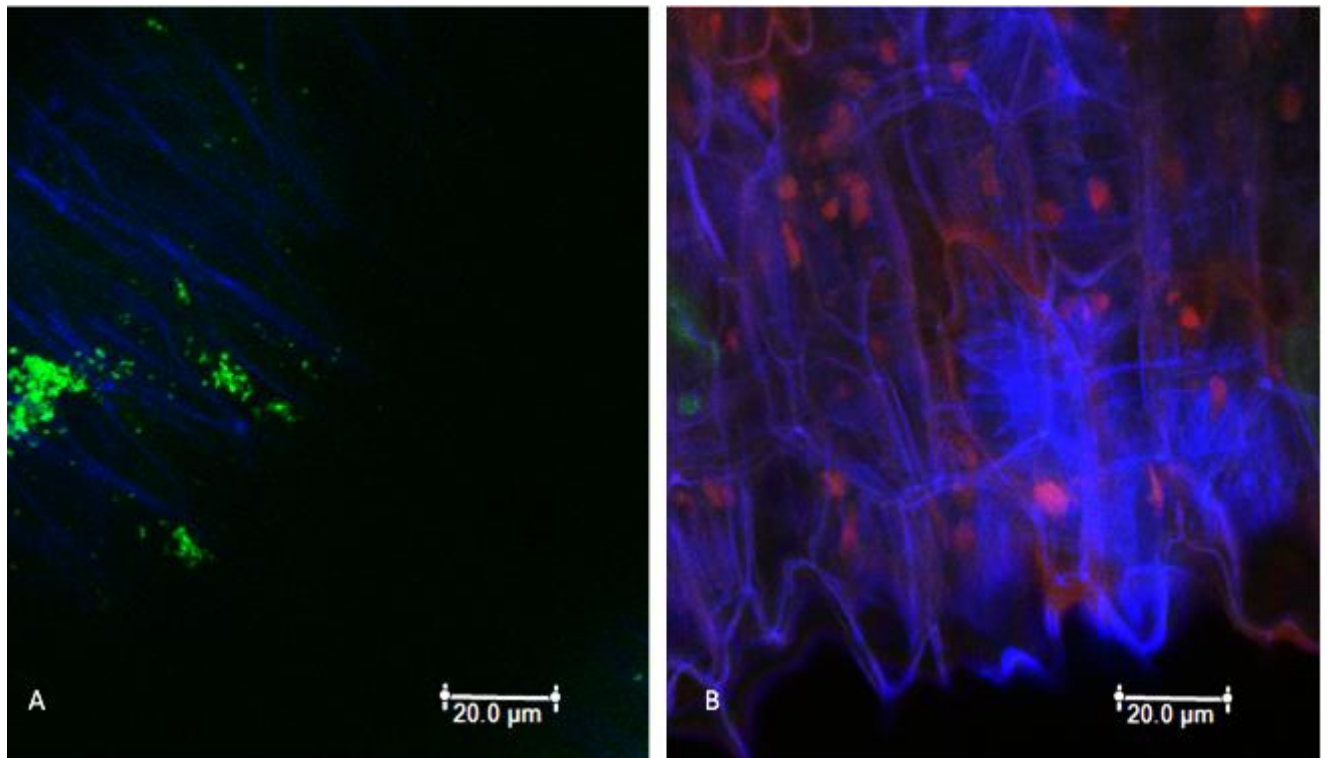

Figure 5: Analysis of untreated seeds (A) and surface sterilized seeds (B) via CARD-FISH and CLSM. Both specimens were targeted with EUBI-III probes. Images were obtained at excitation of 488 nm (Fluos channel) and at 360 nm (UV channel). The UV-channel was used to image the seed, as its' surface shows strong autofluorescence in UV light (blue). An overlay of images was created afterwards. In A) bacteria were detected, visualized in green (Oregon Green® 488). In B) no bacteria could be detected.
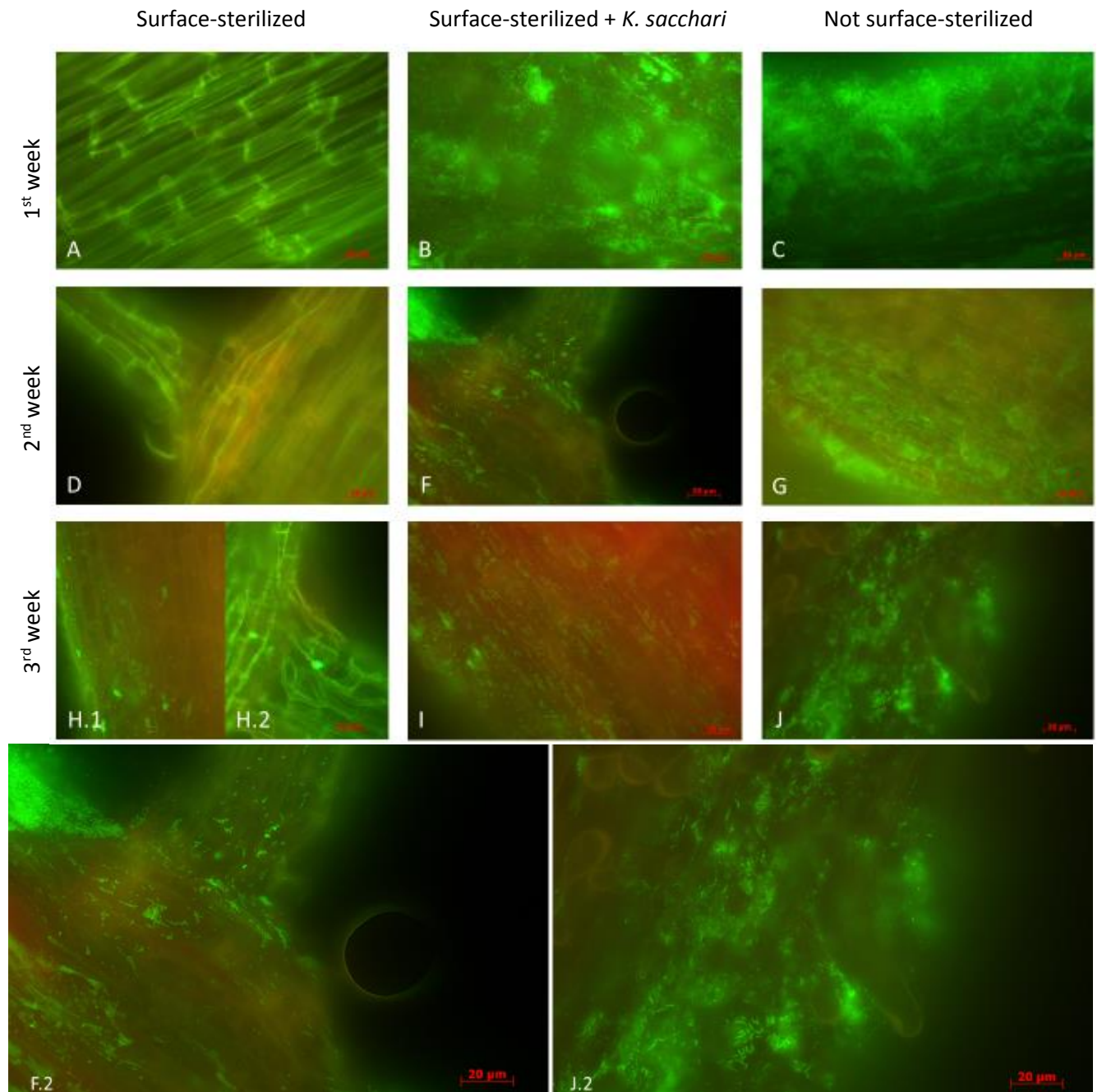
	Surface-sterilized	Surface-sterilized + *K. sacchari*	Not surface-sterilized
1st week	A	B	C
2nd week	D	F	G
3rd week	H.1 H.2	I	J
	F.2	J.2	

Figure 6: Analysis of SYBR green (absorption λ_{max} = 494 nm, emission λ_{max} = 521 nm, green) stained microorganisms on gnotobiotic rice roots by fluorescence microscopy. The Zeiss Axioplan2 microscope was used in combination with a double excitation filter cube (Table 3). The root tissue shows strong autofluorescence at the excitation 590 nm (red emission) after 2 weeks of growth in the gnotobiotic system. Each picture is representative for three replicates. Three different sample types are shown. In A, D, H.1 and H.2 sterile grown rice roots are visualized, in B, F and I rice roots incubated with *K. sacchari* are shown, and C, G and J shows non surface sterilized rice seeds grown under the same conditions as the previous two sample types. From each sample type, triplicates were sampled weekly. A, B and C are samples taken after one week of growth. D, F, and G are samples taken after two weeks of growth and H.1, H.2, I and J show samples taken after three weeks. H.1) A

contamination was detected in one out of three replicates of samples grown under aseptic conditions. B, F, I) *K. sacchari* colonizes rice roots already after one week of growth. Furthermore, only one cell morphology can be observed in these samples, whereas many different morphologies are visualized in samples C, G and J. This can be better in comparison of the enlarged pictures F.2 and J.2.

Growth of rice plants in association with K. sacchari

Plants inoculated with cells of *K. sacchari* showed a formation of a dense biofilm, indicating that the organism is capable of colonizing the rice rhizoplane. The visualized microorganisms all showed similar rod-shaped cell morphologies. In comparison, different cell morphologies were observed on the roots of rice plants cultivated under the same conditions but without surface-sterilization of seeds (Figure 6 F.2 versus J.2).

We furthermore analysed the patterns of colonization on the rhizoplane at an early stage of plant development in detail (Figure 7). High population densities of *K. sacchari* were observed close to root tips (Figure 7A). The tips themselves mostly remained un-colonized. The bacteria emerge in close proximity behind the tip (elongation zone) and extend along the root exodermis towards more mature regions (Figure 7.A). Although this is the case for most root tips, we made one single observation of *K. sacchari* surrounding the whole root tip (supplementary Figure 4). In mature regions, we observed a re-occurring establishment of colonies at lateral root junctions and in trenches between plant cells (Figure 7.B)
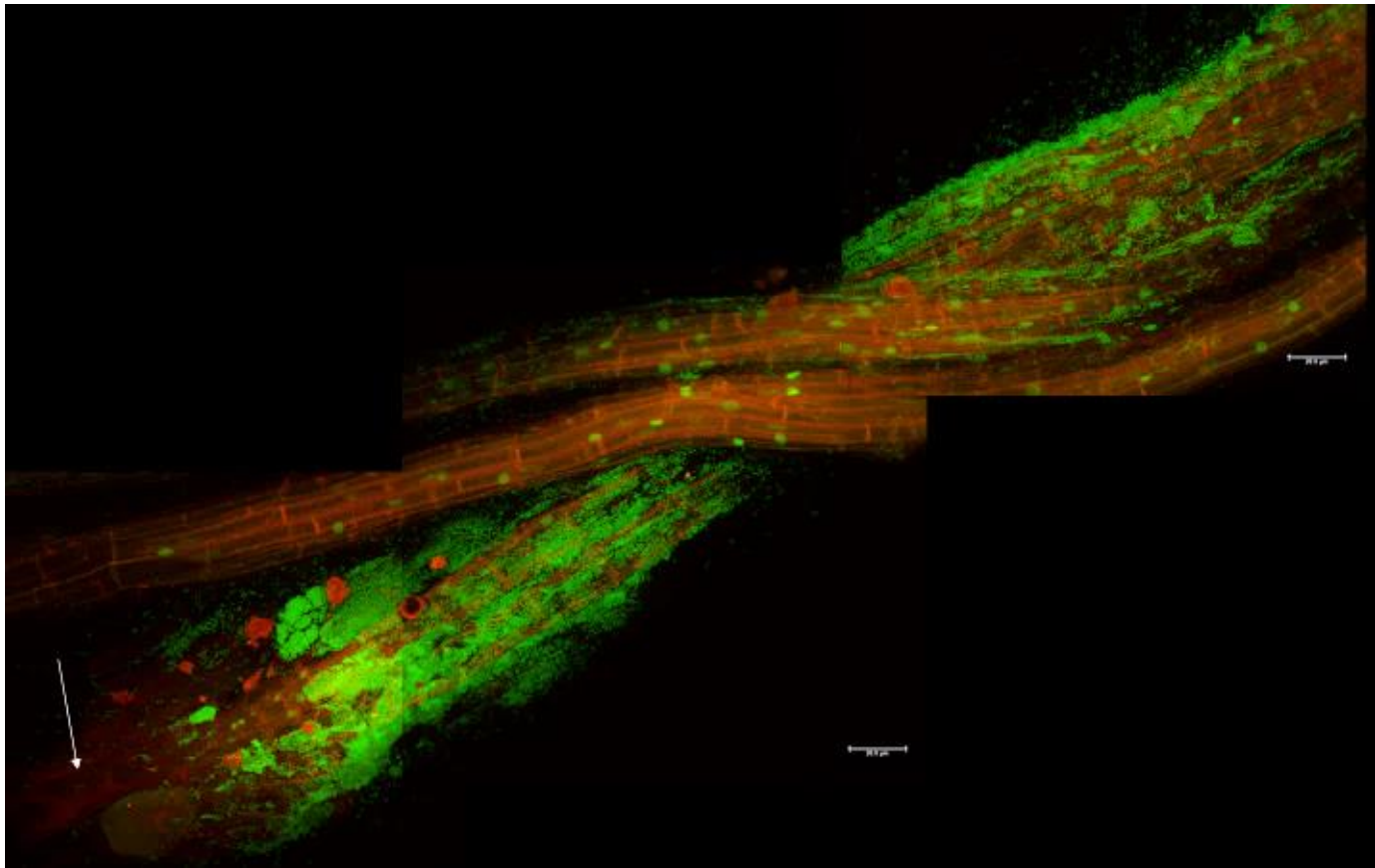
Figure 7.A: *K. sacchari* cells on the rhizoplane of gnotobiotic rice roots, visualized with SYBR green. The Cy3-channel was used to image the root tissue, as it shows strong autofluorescence at 590 nm excitation (red). White arrow marks the position of the root tip. Images were obtained with CLSM and subsequently compiled into the present mosaic.
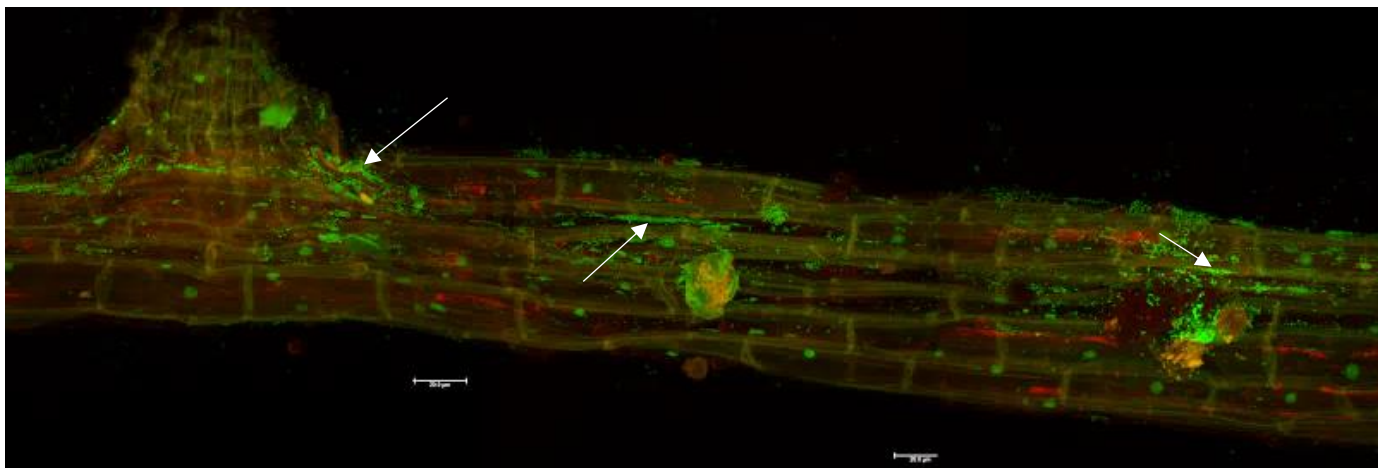

Figure 7.B: *K. sacchari* cells on the rhizoplane of gnotobiotic rice roots, visualized with SYBR green). The Cy3-channel was used to image the root tissue, as it shows strong autofluorescence at 590 nm excitation (red). White arrows mark establishment of colonies at lateral root junctions and in trenches between plant cells. Images were obtained with CLSM and subsequently compiled into the present mosaic.

The diazotrophic activity of K. sacchari in association with rice under gnotobiotic conditions. The incorporation of ^{15}N into gnotobiotically grown rice plants was analysed in plants grown in association with *K. sacchari (2CA-kosa)* as well as in plants where seeds were not surface sterilized prior to planting (2CA-wild). The plants (3 weeks old) were exposed to an atmosphere containing $^{15}N_2$ for 72 h. Plant material (above ground as well as below ground material) was dried and milled and analysed by IRMS. The root and aboveground plant material of both treatments (2CA-kosa and 2CA-wild) was enriched in ^{15}N (Table 6). The high values in aboveground plant material indicate a translocation of fixed ^{15}N from the root compartment upwards into the phyllosphere. Our findings show that our *K. sacchari* strain, previously isolated from a paddy-soil, is capable of associating with the rhizoplane of rice roots under gnotobiotic conditions (Figures 6 and 7), where it actively assimilates N_2 (Table 6). Furthermore, the enrichment of 15N in the "wild" sample indicate the presence of diazotrophs in the spermosphere, which the seed itself introduces into the model system.

Table 6: IRMS measurement of rice biomass after incubation with $^{15}N_2$ under gnotobiotic conditions in association with *K. sacchari* (2CA-kosa) and of plants originating from not surface sterilized (2CA-wild).

Sample type	Treatment	Incubation	$\delta^{15}N/^{14}N$ (‰)
Phyllosphere	2CA-wild	Natural abundance	-0.663
Phyllosphere	2CA-kosa	Natural abundance	4.657
Rhizosphere	2CA-wild	Natural abundance	6.581
Rhizosphere	2CA-kosa	Natural abundance	0.284
Phyllosphere	2CA-wild	15N incubation	7 252.400
Phyllosphere	2CA-kosa	15N incubation	6 943.841
Rhizosphere	2CA-wild	15N incubation	1 457.668
Rhizosphere	2CA-kosa	15N incubation	4 267.838

ARDRA of gnotobiotic rice roots. To verify that *K. sacchari* colonized rice roots in absence of any other microorganism in our gnotobiotic setup, DNA was isolated from gnotobiotic rice roots that grew in association with *K. sacchari* for two and three weeks as well as from a three-

week-old sterile root sample (2CA-sterile). From those samples, 16S rRNA genes were amplified via PCR and the amplicons were subsequently analysed via ARDRA. The sterile sample was digested into 7 distinct fragments that sum up to a total of approx. 3000bp. This corresponds to approximately twice the length of one amplicon, indicating a generation of two different 16S rRNA amplicons from the sterile sample. It is known that the primers I used (8F and 1492R, see Table 1 for sequence) can bind to plant DNA (e.g. DNA from mitochondria and amyloplastids). Therefore, I assume that plant derived DNA is responsible for the generation of amplicons from the sterile sample. The *K. sacchari* associated sample was digested into 4 fragments that sum up to a total of approx. 1500bp, which corresponds to only one 16S rRNA amplicon. The digestion patterns for root samples associated with *K. sacchari* clearly differ from a digested sterile sample. They furthermore, without any variation between the samples, match the pattern of a digested 16S amplicon that derived from a *K. sacchari* pure culture (Figure 8).
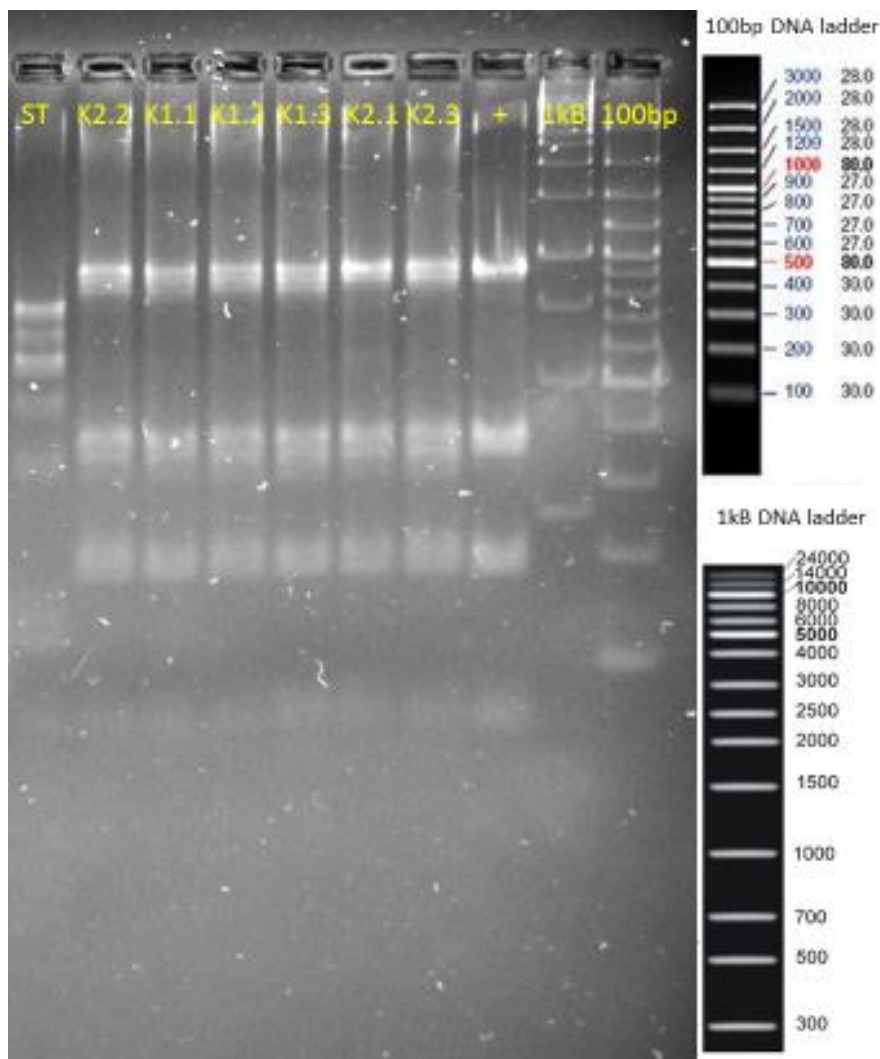
Figure 8: ARDRA of gnotobiotic rice: 16S rRNA amplicons were digested enzymatically with Taq I overnight. The digestion patterns for root samples associated with *K. sacchari* (K1.1 – K2.3) differ from the digested sterile sample (ST) and match the digestion pattern of the 16S rRNA from the K. sacchari pure culture (+). K1.1-K1.3 refers to rice plants in an enclosed gnotobiotic environment sampled after 2 weeks of association with K. sacchari. K2.1-K2.3 were sampled after 3 weeks. Two DNA ladders were used: The 1kb and the 100bp DNA ladder

Evaluation of tyramides for CARD-FISH and an alternative fluorophore for standard-FISH analysis in soil and root samples

CARD-FISH is a sensitive method for the in-situ identification of microorganisms in complex environments. The method employs fluorescently labelled tyramides, which results in an increase of signal intensities compared to FISH (Pernthaler *et al.*, 2002). The rice-root environment exhibits significant autofluorescence, which would hamper the detection of microorganisms (Briones *et al.*, 2002). It was shown that the enhanced signal intensities resulting from CARD-FISH are sufficient to overcome this obstacle (Schmidt *et al.*, 2014). Three different tyramides labeled with the fluorophores Alexa Fluor™ 488, Oregon Green® 488, and Atto 488 were synthesized and provided by Dr. Hannes Schmidt. Their applicability for the visualization of single-cell microorganisms associated to rhizoplanes via CARD-FISH was evaluated. The tyramides were applied in the signal amplification step in a CARD-FISH procedure with the HRP-labelled EUB I-III probe mix and *K. sacchari* immobilized on filter pieces. All images were taken via epifluorescence microscopy at the same exposure time using a short-pass/long-pass filter set (Table 3). In Figure 9, the signal intensities resulting from the applied tyramide solutions are presented. Weakest bacterial signals were obtained with tyramides labelled with the fluorophore Atto 488. Strong signals were obtained using the tyramides with Oregon Green and Alexa 488. Consequently, Oregon Green- and Alexa 488-labeled tyramides were used for subsequent CARD-FISH analysis of microbial cells associated with rice roots using the HRP-labelled probe mix EUB I-III (Figure 10B). Strong signals were obtained also on rice rhizoplane, indicating a good signal-to-noise ratio that can be obtained using these fluorophores. Furthermore, the applicability of standard FISH to visualize *K. sacchari* on gnotobiotic rice roots was evaluated using the Atto 565 fluorescence dye, targeting the cells with the probe mix EUB I-III (Figure 10A). Standard FISH using probes directly labelled with Atto565 seemed sufficient to detect single-cells on the highly auto fluorescent root

surface, due to the high fluorescence intensity of the Atto 565 dye along with low autofluorescence stemming from the root surface in the respective emission window (570 nm).
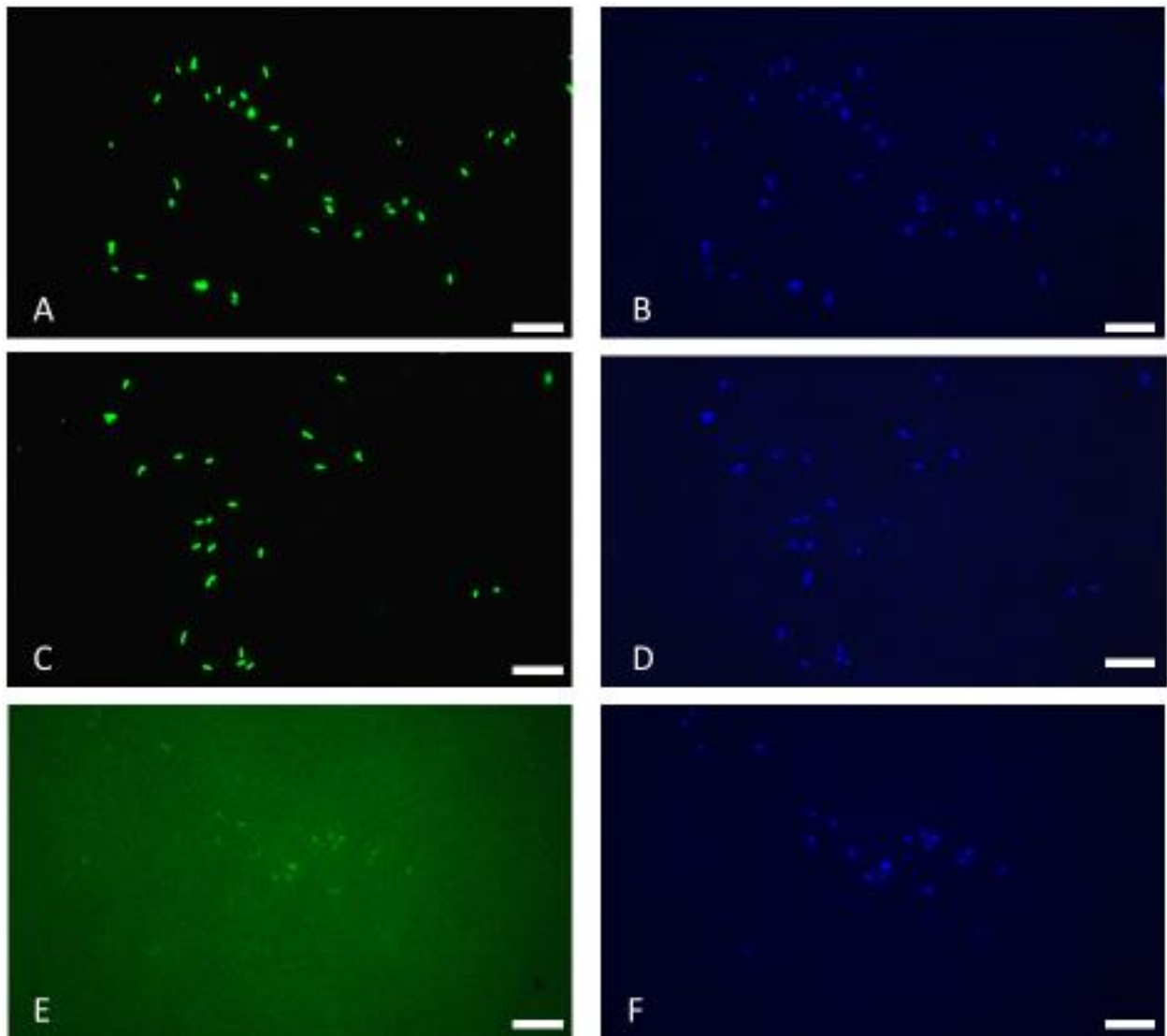

Figure 9: Comparison of tyramides for CARD-FISH analysis. White bar represents 20µm. All cells are *K. sacchari* cells, targeted with the probe mix EUB I-III. The signal amplification is based on tyramides having different fluorescent labels. A: Alexa Fluor™ 488, C: Oregon Green® 488, E: Atto 488. B, D, and F represent the corresponding DAPI images. Scale bars: 20 µm.
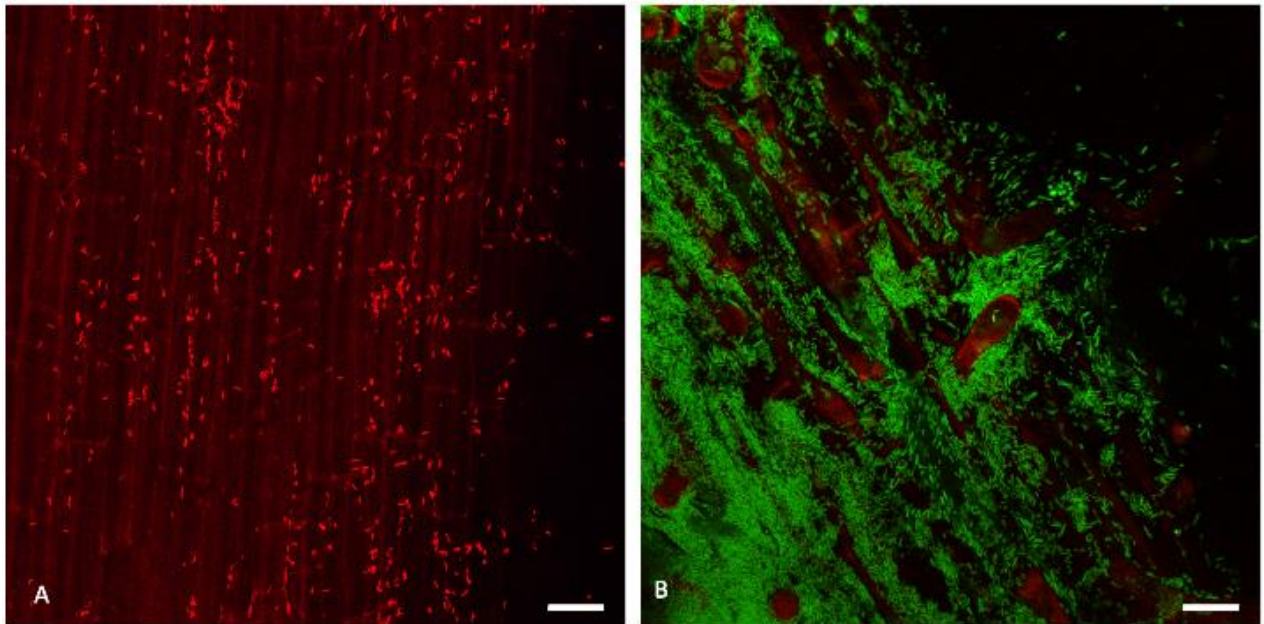
Figure 10: A) *K. sacchari* cells on the surface of gnotobiotic roots visualized with an Atto656 dye (excitation at 532 nm laser line, emission at 570 nm, red) in combination with an EUBmix probe. B) Oregon Green® 488 (excitation at 488 nm laser line, emission at 500) was used to detect bacteria on the surface of "wild" gnotobiotic rice roots via CARD-FISH. Scale bars: 20 µm.

Detection of single microbial cells via Gold-FISH.

Gold-FISH is a hybridization method that allows for the for detection of microorganisms via SEM *in-situ*. Because of this benefit, it was the aim within our group to establish Gold-FISH in combination with NanoSIMS as an alternative to FISH-NanoSIMS analysis. The applicability of the method to detect single cells was tested at first (Figure 11). It was possible to detect *K. sacchari* cells on filter pieces, as well as cells of unknown identity on the surface of rice roots.
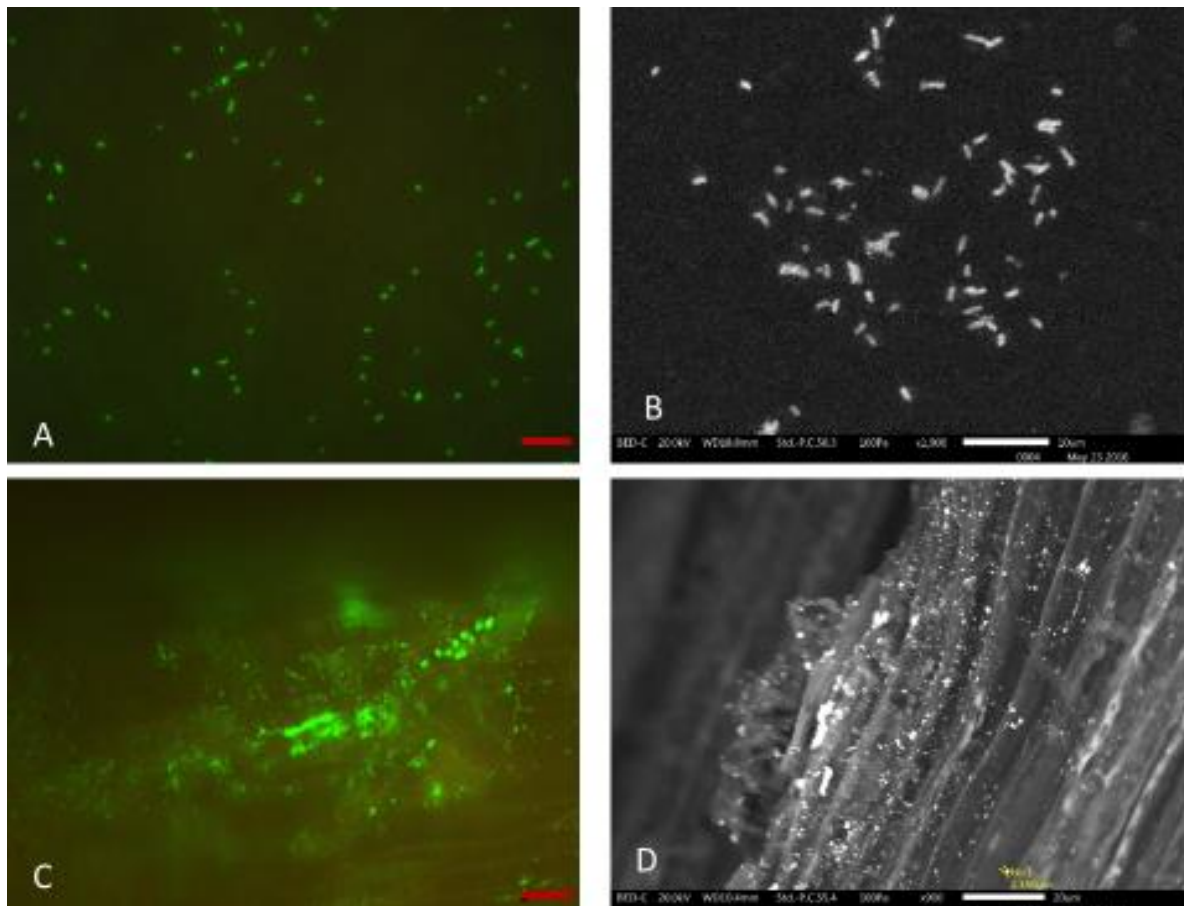
Figure 11: Example images of Gold-FISH applied to diverse sample type. A) and B): Application of Gold-FISH on a pure culture of *K. sacchari* hybridized with the probe GAM42a. A) Fluorescence microscopy of K. sacchari cells stained via gold-FISH, with the AF 488 fluoro-nanogold-streptavidin conjugate. B) Detection of *K. sacchari* via imaging of backscattered electrons after gold enhancement. C) and D): Gold-FISH applied to rice root samples. C) Application of Gold-FISH with probe EUB I-III on rice roots that developed from unsterilized seeds in sterile growth medium (1CA-wild). Fluorescence microscopy of bacteria detected via Gold-FISH, AF 488 fluoro-nanogold-streptavidin conjugate. D) Detection of bacteria on roots of soil-grown rice plants, targeting all bacteria with the probe EUB I-III, via imaging of backscattered electrons after gold enhancement. Red bar: 20µm.

Further experiments were conducted to evaluate the specificity in binding of streptavidin conjugates, biotinylated tyramides, and of the probe-hybridization to the correct target via epifluorescence microscopy, laser microdissection, scanning electron microscopy, and energy dispersive X-ray spectroscopy. The specificity of the method was evaluated by using a mixed culture of fixed *K. sacchari* and *B. subtilis* on filters. Laser microdissection was applied to mark specific regions on the filter, which enabled the detection of the same individual microbial cells with different microscopes. The LGC354A probe was used to target *B. subtilis*, while DAPI was used to visualize all microorganisms on filter pieces. In Figure 12 it is shown that both, the hybridization of the LGC354A probe and the binding of the streptavidin conjugate, are specific for the target organism, since the remaining *K. sacchari* cells are only visible due to the DAPI signal (Fig. 12A, B). After enhancement with gold particles, the same cells

were detected via scanning electron microscopy and backscattered electron detection (Fig. 12C, D). The deposition of nanogold particles correlated with the fluorescent signals observed previously, while *K. sacchari* cells were not detectable. In addition, the presence of gold particles within this region of interest was analyzed by energy dispersive X-ray spectroscopy (Fig. 12E). This analysis verified the correlation between fluorescent signals and deposition of gold particles.
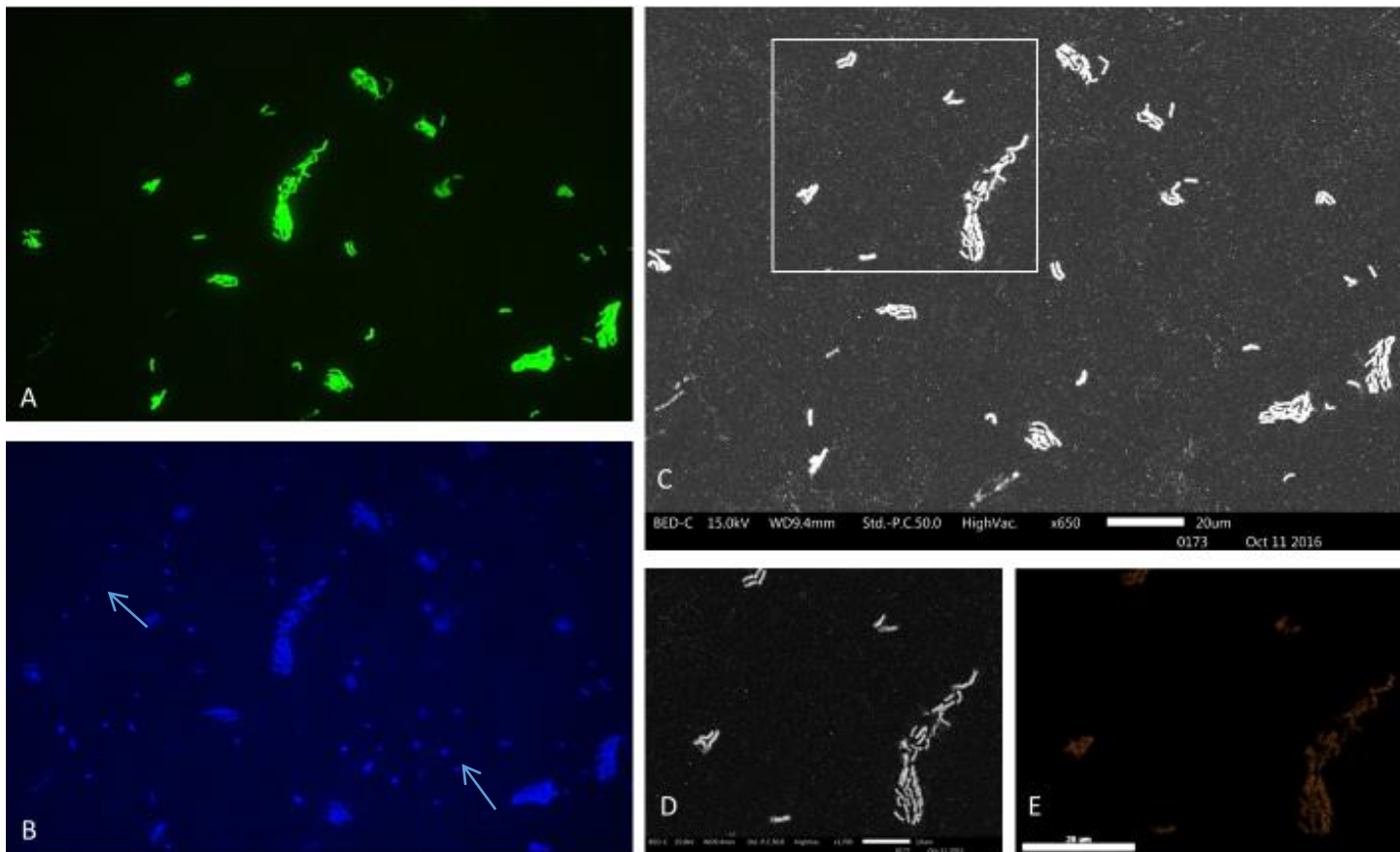

Figure 12: Analysis of Gold-FISH signals via correlative microscopy. A) Fluorescence microscopy of mixed culture of B. subtilis and K. sacchari, targeting Bacillus subtilis with the probe LGC354A, green signals obtained due to the fluorescing streptavidin conjugates covalently labelled with a 1.4 nm nanogold particle and 2-3 Alexa Fluor 488. B) Corresponding DAPI image visualizing both *K. sacchari* (arrows hint towards some) and Bacillus subtilis cells. C) SEM picture of the same area, correlative to the spot recorded via fluorescence imaging. White square indicates region shown in panel D). E) Measurement of gold particles via EDAX visualized in brown. The gold particles correlate to *B. subtilis* cells visualized in fluorescence microscopy and SEM.

Gold-FISH on rice-roots. I have modified the original Gold-FISH protocol (Schmidt *et al.*, 2012) in terms of longer incubation time that allows streptavidin nanogold particles to bind to biotinylated tyramides, as well as a slightly modified and prolonged protocol for metallographic enhancement (as mentioned in the Materials and Methods). After optimization of the Gold-FISH protocol on pure and mixed cultures the method was applied for the visualization of

microorganisms on the surface of rice roots. I successfully detected single cells on the rice root via scanning electron microscopy (Figure 13). I observed bright gold signals via backscattered imaging, which are less intense via standard secondary electron imaging (Figure 13A versus B). However, the visualization of morphologies and structures improves with secondary electron imaging, therefore it is recommended to use both detectors. Furthermore, I observed increased microbial densities near openings on the root surface (e.g. lateral root junctions, cracks) and in trenches between plant cells, which are colonization patterns (e.g. Figure 13A) that resemble our observations via fluorescence microscopy (e.g. Figure 11C). In Figure 13C microorganisms are visualized at the basis of a lateral root junction, which is an observation that was made previously, also via fluorescence microscopy (e.g. Figure 6 and 7). However, in gnotobiotic as well as environmental samples unspecific depositions of gold particles were observed throughout many experiments, and those often resided on very thin parts of the roots (e.g. root hairs or on the edges and endings of filter pieces (Figure 13F).
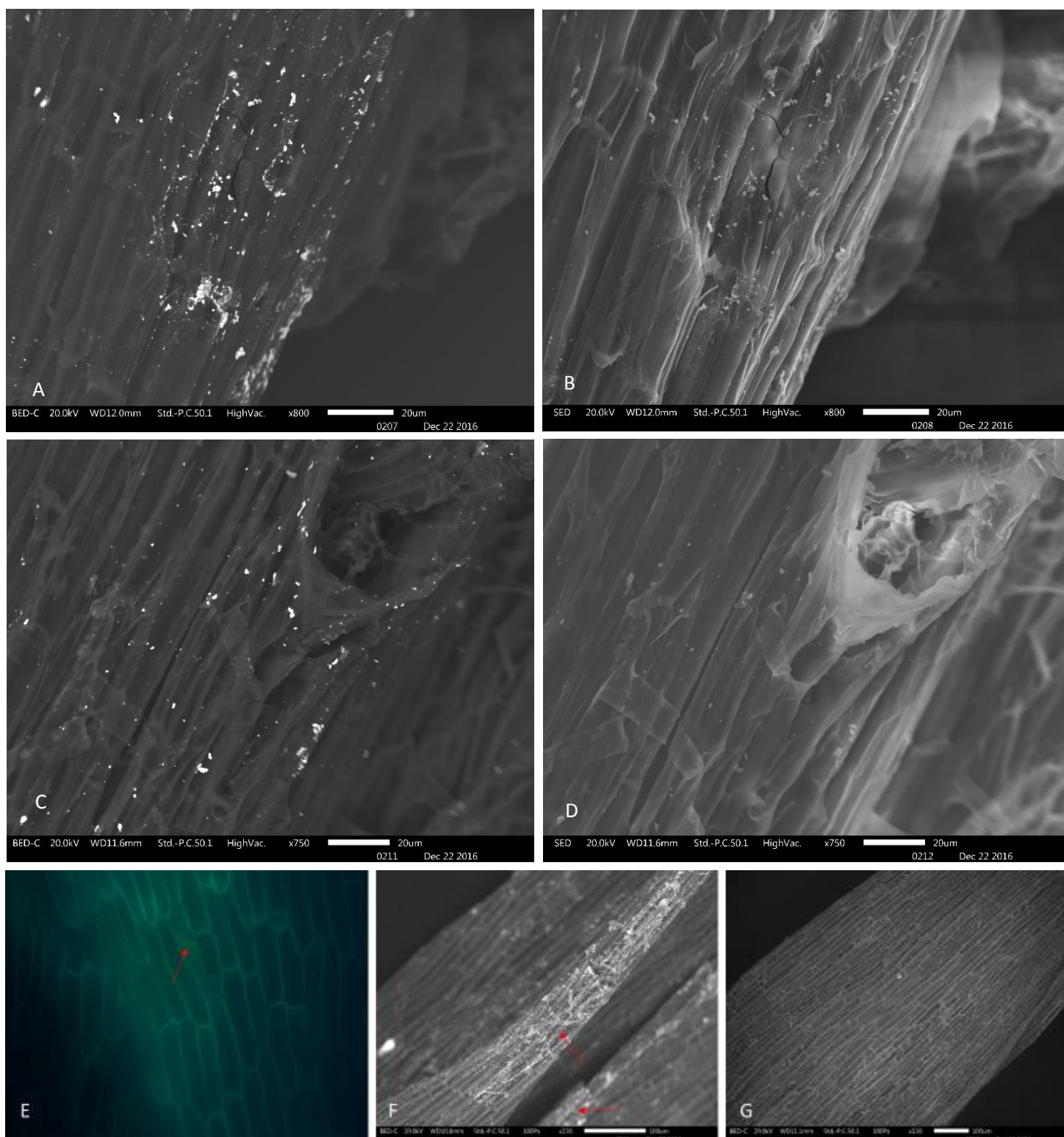
Figure 13: Detection of microorganisms on the surface soil-grown rice roots with Gold-FISH via SEM. EUB mix was used to target all bacteria in combination with biotinylated tyramides and streptavidin conjugates covalently bound to the tyramides, labelled with a 1.4 nm nanogold particle and 2-3 Alexa Fluor 488. Gold particles are visualized via imaging of backscattered electrons via SEM. A) Bacteria around a crack on the surface of rice roots visualized via imaging of backscattered electrons. C) Bacteria in close proximity to a lateral root opening, also visualized via detection of backscattered electrons. B) and D) Corresponding detection of secondary electrons. E), F), and G) are Gold-FISH analyses with NonEUB probes E) Red arrow indicates unspecific deposition of biotinylated tyramides visualized via fluorescence microscopy F) Red arrow indicates

unspecific deposition of enhanced gold particles on thin parts of the root as root hairs, visualized via SEM. G) Gold FISH with a nonEUB probe. No indication of unspecific deposition can be observed.

Gold-FISH analysis of rice roots via correlative microscopy. In order to correlate single microbial cells on the surface of rice roots via Gold-FISH, fluorescence microscopy was used to detect and visualize stained cells that were subsequently imaged via SEM. The approach for this correlative microscopy was to search for prominent regions on the surface of rice roots via CLSM (e.g. lateral root junctions, cracks) that can be found in the SEM afterwards. In Figure 14, a correlating region on a root surface is visualized via both imaging tools. It was challenging to detect microorganisms via fluorescence microscopy on the rhizoplane, indicating that the root was barely colonized (Figure 14A). The detection of weak signals via fluorescence microscopy is reflected in SEM, where gold signals are barely visible. Prior to SEM, the root has to be de-mounted from the microscopic slide, prepared for metallographic enhancement, and air-dried. The crucial point of drying the root implies a deformation of the root tissue, which makes the relocation of points of interest via SEM challenging. An overlay of both images (Figure 14C) illustrates that the pictures do not fit due to the deformation of the plant cells
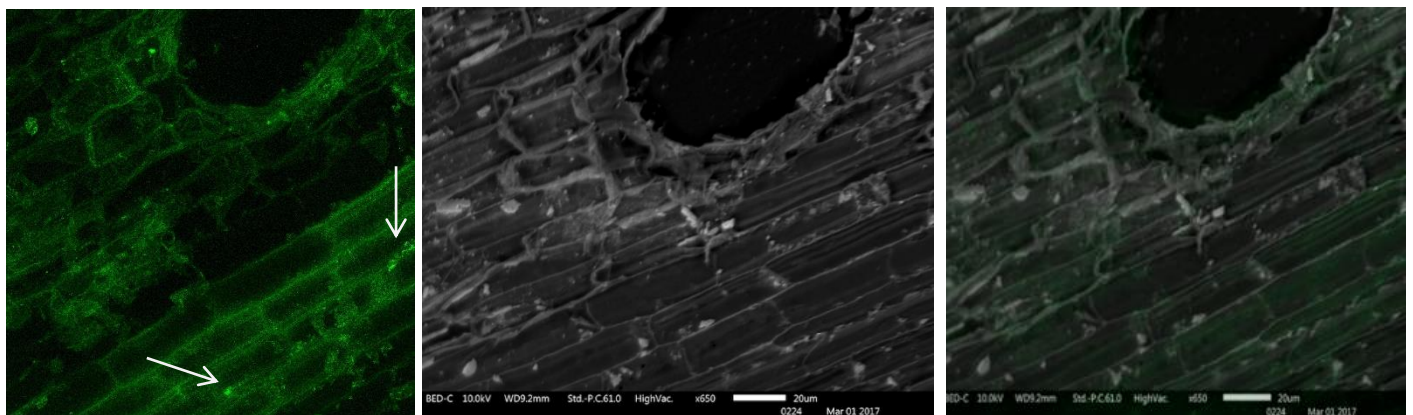

Figure 14: Correlative microscopy of single microbial cells via fluorescence microscopy and SEM. Microorganisms are labelled with an EUB I-III probe mix via Gold-FISH. In A) single bacterial cells are visualized, marked with an arrow. In B) the correlating image was obtained via imaging of backscattered electrons. Bacteria are not sufficiently labelled and therefore, gold-signals are barely visible. C) Represents an overlay of A) and B). Plant cells do not stack correctly, indicating a deformation of plant tissue between fluorescence microscopy and SEM

Discussion

Isolation of N-fixing microorganisms associated with rice

BNF is an important mechanism for the global N-cycle, and in agricultural soils the process could eventually alleviate the N-shortage of plants. Rice is the most important crop plant in terms of human nutrition (Ladha *et al.*, 2016) and therefore, scientists aim to isolate diazotrophic microorganisms from the paddy-soil environment, characterize their physiology, and re-associate them with rice plants (Ladha *et al.*, 1983; Jha *et al.*, 2009; Liu *et al.*, 2011). Recently it was shown for another cereal crop (wheat) that around 60% less urea fertilizer was necessary for plants in association with a genetically engineered Azotobacter species to sustain similar wheat yields (Bageshwar *et al.*, 2017). One goal of my experiments was to isolate and cultivate free-living and plant-associated diazotrophs from a paddy-soil and the rice-rhizosphere, respectively. These environmental samples were streaked onto N-free plates that were solidified with Gellan Gum, a highly transparent polysaccharide (Hashidoko *et al.*, 2002), for which it was reported to enhance the N-fixing activity of cultured diazotrophs (Hara *et al.*, 2009). For all further transfers onto fresh plates, Agar was employed to lower the financial costs of the experiment. I was successful in isolating 11 different microorganisms (listed in Table 4, marked with '+' or '*') by applying this approach. All the isolates have in common of being heterotrophic, because of the variety of sugars used for the preparation of NFB medium, discriminating against the growth of autotrophs. The isolates belonged to the phylum of *Firmicutes, Actinobacter* or *γ-Proteobacteria*, isolated from rhizosphere, seed or bulk-soil samples. Bacteria from these phyla have commonly been isolated from soil and plant-associated environments (Jha *et al.*, 2009; Mirza *et al.*, 2012; Ji *et al.*, 2014). It was reported for *Bacillus* species to be plant-associated and PGPB (Ahmad *et al.*, 2008) and several Bacillus spp. were isolated previously from different parts of rice plants (Mano and Morisaki, 2008). For instance, I isolated a bacterium closely related to *Bacillus thuringiensis*, which is widely known for its insecticidal (Schnepf *et al.*, 1998) and PGPB activity (Bai *et al.*, 2002). Three of my isolates belonged to the phylum of *Actinobacteria* from the order of *Actinomycetales*. Species from this genus are known for their PGPB activity and some produce the phytohormone indole acetic acid in the rice rhizosphere (Harikrishnan *et al.*, 2014). Although my isolates grew without any additions of N, it was not possible to amplify *nifH* genes for most of the strains.

This observation was also made by Mirza *et al.* (2012), who reported the isolation of diazotrophic organisms in a similar approach. In their study, it was likewise not possible to detect *nifH* sequences via PCR in 21% of their isolates that grew in NFB. Possible explanations for this observation are (i) that the PCR primers did not match the *nifH* genes of the isolates, (ii) that the PCR amplification was inhibited (e.g. due to low concentration of target sequence or due to the sample matrix), or (iii) that contaminations with nitrogenous substances led to growth of non-diazotrophic microorganisms. Some diazotrophs can store N-nutrients (Romans *et al.*, 1994). This may be an explanation, however, I transferred active colonies at least five times into fresh medium, which makes contaminations of N as well as storage of N unlikely as a continuous, reoccurring problem. Regarding the coverage of the primers, it seems unlikely that both primer pairs we used (IGK3-DVV (Ando *et al.*, 2005) and Ueda19F-R6 (Ueda *et al.*, 1995; Marusina *et al.* 2001) would not cover the majority of our isolates, as all isolates were closely related to previously known heterotrophic *Proteobacteria*, *Actinobacteria*, or *Firmicutes* that, according to the classification of Zehr *et al.* (2003), belong to the *nifH* cluster 1. According to Gaby *et al.* (2012), the primer pair IGK3-DVV should perform well for the amplification of *nifH* genes originating from cluster 1 and its sub-clusters. I have tested both primer pairs (IGK3-DVV and Ueda19F-R6), the performance of Ueda19F/R6 was slightly better for the amplification of *nifH* genes in my isolates (supplementary Figure 5). Furthermore, PCR-inhibition is often reported to represent a problem, especially in samples with an extremely low abundance of the target sequence (Zehr *et al.*, 2003b). I have enriched diazotrophic target organisms from soil via cultivation, therefore I can assure for high abundance of the target sequence. Furthermore, the sample matrix also seems unlikely to be the PCR inhibiting factor, because no inhibitions were observed in the amplification of 16s rRNA sequences. The isolates I obtained grew in NFB repeatedly, however, to find clear evidence for the diazotrophic activity of our isolates, it would have been necessary to conduct a functional analysis that helps to quantify BNF *in-situ* (e.g. ^{15}N stable isotope assay).

Kosakonia sacchari

K. sacchari was the first organism that was obtained in the attempt to isolate free-living diazotrophs from soil. The organism belongs to the genus *Kosakonia*, which was classified as a novel genus in the family *Enterobacteriaceae* (Brady *et al.* 2013). It consists of the species *K.*

cowanii (Inoue *et al*., 2000), *K. radicincitans* (Kämpfer *et al*., 2004), *K. oryzae* (Peng *et al*., 2009), *K. arachidis* (Madhaiyan *et al*., 2010), *K. sacchari* (Zhu *et al*, 2013), *K. oryziphilus* and *K. oryzendophyticus* (Hardoim *et al*., 2013). Except for *K. cowanii*, all the other species are N-fixing bacteria isolated from plant-associations. I obtained *K. sacchari* from the bulk soil of the Sri Lanka paddy-soil. The 16S rRNA gene sequence of my isolate is 99% similiar to *K. sacchari* strain SP1^T (isolated from a sugarcane stem (Zhu *et al*., 2013)) and the *nifH* nucleotide sequence is 94% similar to strain R4-724 (Madhaiyan *et al*., 2015). *K. sacchari* is well-known as a N fixer associated with sugar cane (Zhu *et al*., 2013), and the complete genome sequence of the type strain SP1^T is available (Chen *et al*., 2014). In 2002, an endophyte was isolated from sweet potato stems, the diazotrophic activity of the organism was verified via ARA and the organism was classified as *Klebsiella oxytocoa*, based on a partial sequence of the 16S rRNA (Adachi *et al*., 2002). Recently it was revealed via whole genome sequencing, that this isolate is a *K. sacchari* strain BO-1, sharing 98.67% average nucleotide identity to strain SP1^T (Zhu *et al*., 2013). Whole genome sequencing would be a potential option to clarify the identity of my isolated *K. sacchari* species as well the closest relative. A clear identification would be important as, to the best of my knowledge, there is no report in the literature of other N-fixing *Kosakonia* species that were isolated from a bulk soil environment and are also capable of forming associations with rice plants. This implies that either *K. sacchari* in general has the potential to inhabit both environments (bulk soil and plant-associated), or that my isolated strain differs from the other *K. sacchari* species. My successful efforts in re-associating the *K. sacchari* isolate with rice plants under gnotobiotic conditions, plus the origin of the species from a bulk soil sample suggests that this organism might be capable of inhabiting both niches. In conclusion, I observed the growth of our isolate in NFB multiple times without variations, making the isolate a reliable model organism for the study of BNF.

Rice grown under gnotobiotic conditions

One aim of my work was to develop a model system for the cultivation of rice plants under gnotobiotic conditions. Initially, I tried to cultivate rice in open test tubes (Table 5, 'OA), however, it was not possible to achieve gnotobiotic conditions via this approach. The seeds were surface-sterilized before planting, but this system was open to the surrounding atmosphere, due to which contaminations could not be avoided. Therefore, I established a

closed gnotobiotic system, based on the research conducted by Shrivastava in 2015. Surface-sterilized rice seeds were grown in a N-free nutrient solution solidified with Gellan Gum and sealed from the outer atmosphere of the greenhouse area (Figure 2). The rice roots were branching in all treatments (listed in Table 5; observable due to the high transparency of the Gellan Gum) and they developed morphologies similar to the soil-grown plants, indicating that the inoculation procedure did not hamper natural root development, and that the used concentration of 1.5% Gellan Gum was adequate to mimic physical soil properties. In previous studies, it was shown that surface-sterilized seeds may still contain endophytes that can spread upon the root surface and into the surrounding bulk soil area as the rice plant grows (Hardoim *et al.*, 2012). I did not detect microorganisms on the surface of surface-sterilized rice seeds via microscopy (Figure 5B), however, with this approach I would have not been able to detect endophytes within the seeds. To follow up on the sterility within the gnotobiotic experiments we investigated randomly chosen samples of sterile treatments (as described in the methodological section) via microscopy after one to three weeks of incubation. Contaminations were observed in one out of three replicates only after three weeks of rice growth (1CA-sterile; Figure 6H.1). To ascertain our results obtained via microscopy, three-week old root samples were incubated in four different media. The data shows, that if microorganism were detected on the root surface via microscopy, then another root from the same sample has led to turbidity within at least one out of eight different media controls (as described in the methodological section and visualized in Figure 2). Whereas a sample declared as sterile via microscopy did not lead to any turbidity in any of the media. This suggests that microscopy could serve as a valid tool for the detection of contaminants colonizing the rhizoplane of rice roots in our gnotobiotic system. However, it remains to be investigated if the contaminations that may arise are of extrinsic (during watering etc.) or intrinsic (seed endophyte) origin. Since the seedlings are pooled in the beginning of each experiment it is plausible that after an extrinsic input all seedlings would be contaminated. Since this was not the case in the experiments, I assume that only in some seeds endophytes remain unharmed from the surface-sterilization procedure and become visible after three weeks of growth under gnotobiotic conditions. It was reported that seed surface-sterilization procedures do not fully ensure sterility as microbiota in the endosperm and embryo of the seed could survive the disinfection (Robinson *et al.*, 2016). It was stated that the dispersal of the spermosphere (seed-born microbiome) onto the surface depends on extrinsic factors (e.g. rain event, age of the

seed) that have a strong influence on the potential of spermosphere-members to compete with resident soil microbiota (Shade *et al.*, 2017). Based on my observations I conclude, that I might not always be able to exclude intrinsic contaminations developing from the rice spermosphere with the procedure applied. However, plants can be tested for contamination as described above and an additional control can be established. I suggest to pre-germinate seeds on nutrient rich plates, instead of in the wells of a 12-well plate, before transplanting them into glass test tubes, which would allow for the separation of contaminated seeds from sterile seeds and therefore would be a further improvement of my experimental approach.

The newly isolated *K. sacchari* strain was used in re-association experiments with rice plants in the gnotobiotic model system. After one week of incubation the root surface was heavily colonized by microorganisms of the same morphotype (Figure 6). 16S rRNA gene amplicons were obtained from roots inoculated with *K. sacchari* (sampled after two and three weeks), digested via enzyme digestion, and compared to the restriction patterns in relation to a digested *K. sacchari* pure culture and a digested sterile sample (sampled after three weeks; Figure 8). The primers I used (8F and 1492R, see Table 1 for sequence) can bind to plant DNA (e.g. DNA from mitochondria and amyloplastids). I assume that plant derived DNA is responsible for the generation of amplicons from the sterile sample (Figure 8). Restriction patterns from 16S rRNA gene amplicons from roots inoculated with *K. sacchari* differ clearly from the sterile sample and resemble the digested positive control without any variations between samples. These results indicate that *K. sacchari* can colonize the root surface in our gnotobiotic approach in an early developmental stage without the influence of any other microorganism. Furthermore, it was verified that *K. sacchari* actively fixes $^{15}N_2$ *in-situ* within the gnotobiotic model system (Table 6). Furthermore, the enrichment of ^{15}N in the "wild" sample indicates the presence of diazotrophs in the spermosphere, introduced into the model system be the seed. Surprisingly, our analysis showed high $\delta^{15}N$ values not only in the rhizosphere, but also in the phyllosphere of both sample types ("wild" and in association with *K. sacchari*). Either the microorganisms were able to colonize the phyllosphere of the model system where they and assimilated $^{15}N_2$, or what we see is a translocation of fixed ^{15}N from the rhizosphere to the above-ground plant material. In a study by Knief *et al*. (2012) it was shown via metaproteogenomic analysis that dinitrogenase reductases were absent in the phyllosphere of rice and the authors concluded that the rice phyllosphere might not represent

an important niche for BNF. I therefore assume that in my experiments, the $^{15}N_2$ was fixed in the root compartment and translocated to the phyllosphere. This is in line with previous studies that reported the observation of ^{15}N fixed in the rhizosphere of rice and translocated to the aboveground biomass (Eskew *et al.*, 1981; Ito *et al.*, 1980). Nevertheless, this question still needs to be addressed microscopically and with further stable-isotope probing assays, to identify if microorganisms that eventually live in the phyllosphere are additionally involved in the process of N fixation or not. For such an analysis, RNA-SIP could be used to identify active utilizers of $^{15}N_2$ (Angel *et al.*, manuscript in preparation). Subsequently, FISH could be used to detect microorganisms of interest and verify their presence as endophytes in the phyllosphere of rice plants. According to the available genomic information of *K. sacchari* SP1[T] and other *Kosakonia* species, members of this genus have the potential to penetrate plant tissue and reside within the plant as an endophyte (Li *et al.*, 2017). In experiments with my newly isolated *K. sacchari* strain, I observed a re-occurring establishment of colonies at lateral root junctions and in trenches between plant cells. It is assumed that those are the locations where endophytes penetrate the plant tissue (Reinhold-Hurek and Hurek, 2011). I did not verify if my isolated strain resides within the plant endophytically, as no resin-embedding and sectioning of roots was done. In conclusion, my microscopic observations show that under gnotobiotic conditions my isolated *K. sacchari* strain efficiently associates with the rhizoplane of rice plants. Furthermore, we have verified the N-fixing ability of this *K. sacchari* strain in culture (via growth in N-free media) and in association with the rice plant (the latter via $^{15}N_2$ incubation measured by IRMS). To the best of my knowledge, this is the first report of active diazotrophy by a species from the genus of *Kosakonia* in association with a rice plant.

Gold-FISH

Gold-FISH is a recently developed method for the detection of specific microorganisms *in-situ* that allows for the combined use of fluorescence microscopy and scanning electron microscopy (Schmidt *et al.*, 2012). The method employs nanogold particles that were originally developed for the detection of small molecules at high resolution in histochemical research (Hainfeld *et al.*, 2004) but not for the detection of single microbial cells. It is because of the high atomic density of gold that the element can be used as a marker for detection via techniques employing electromagnetic radiation. The first successful application of such an

approach was conducted by Spring *et al.* in 1998, who employed nanogold and fluorescently labelled polynucleotide probes for the detection of magnetic bacteria via transmission electron microscopy (TEM). For the detection of microbes via SEM, several studies employed oligonucleotides linked to nanogold particles carrying molecules that are being introduced in a second hybridization step. For example, fluorescein labelled oligonucleotides for the subsequent binding of anti-FITC-immunogold conjugates (Gérard *et al.*, 2005), or biotin-labelled oligonucleotide probes for the subsequent binding of streptavidin-nanogold conjugates (Kenzaka *et al.*, 2005). Gold-FISH however, employs an enzymatic amplification of biotinylated tyramides to increase the number of deposited gold particles and thus to enhance the signal intensities (Schmidt *et al.*, 2012). Similar to CARD-FISH applications, Gold-FISH is based on 16S rRNA-targeting HRP-labelled oligonucleotide probes. In contrast to CARD-FISH, Gold-FISH employs biotinylated tyramides that bind to streptavidin conjugates carrying a fluorescent dye and nanogold particles. The fluorescent label allows for detection of microorganisms via fluorescence microscopy, with signal intensities comparable to those obtained with CARD-FISH (Schmidt *et al.*, 2012). The nanogold particles allow for detection of the same microorganisms by imaging of backscattered electrons via scanning electron microscopy (Eickhorst and Schmidt, 2016). I have tested the specificity in binding of probes, tyramides, and streptavidin conjugates and conclude that the hybridization, amplification and binding of streptavidin conjugates leads to specific signals (Figure 12). A mixed culture was employed (consisting of *B. subtilis* and K*. sacchari*) for a correlative microscopy approach. Fluorescence signals were specific for *Firmicutes* (detection of *B. subtilis* cells with a LGC354A probe (Meier *et al.* (1999)), and no signals of the *γ-Proteobacterium K. sacchari* was visible*.* Furthermore, the fluorescence signals correlated to the gold signals obtained via scanning electron microscopy. I analysed the chemical composition of this correlating image-spot via EDAX and the analysis verified that the potential gold signals truly consist of gold particles (Figure 12E). However, nanogold particles need to be enlarged, as they are usually too small to otherwise be detected via scanning electron microscopy (Hainfeld *et al.*, 2004). This enlargement from 1.4 nm to approximately 10 to 100 nm can be achieved via autometallographic enhancement (Hainfeld *et al.* 2004), and the size of gold particles correlates to treatment duration (Schöfer *et al.*, 2002). It was furthermore shown that a gold enhancement lasting longer than 30 minutes results in the formation of gold clusters randomly scattered across specimens (Schmidt *et al.*, 2012). I have observed such unspecific depositions

of gold particles, often residing on very thin parts of the roots (e.g. root hairs) or on the edges and endings of filter pieces and roots (Figure 13F). Via scanning electron microscopy however, those gold clusters were easily distinguishable from typical cell morphologies and therefore did not hamper the identification of gold-stained microorganisms. In rare cases, we also observed unspecific deposition of tyramides prior to the autometallographic enhancement via fluorescence microscopy (Figure 13E). This nonspecific binding of tyramides to the root tissue was also observed for CARD-FISH procedures, in which they remain distinguishable from positive signals due to their shape (Schmidt *et al*., 2014). The same principle of differentiation from positive Gold-FISH signals applies to our observation. In conclude that the probe-hybridization, the binding of tyramides, and of streptavidin-nanogold conjugates are specific (Figure 12). Furthermore, I successfully employed Gold-FISH for the visualization of microorganisms on the surface of rice roots (Figure 13). The pictures illustrate microorganisms in close proximity to a crack within the plant tissue (Figure 13 A, B) and near a lateral root junction (Figure 13 C, D).

Due to the high spatial, chemical and molecular resolution, NanoSIMS is very promising for measuring complex interactions of soil microbiota and plants in the rhizosphere (Pett-Ridge and Firestone, 2017). As an alternative to halogenated oligonucleotide probes (Behrens *et al*., 2008; Musat *et al*., 2008), gold markers can be used for detection of microorganisms via NanoSIMS directly. Such an approach was already proposed by Kubota *et al*. in 2014 (Gold-ISH), who used oligonucleotide probes conjugated with an Undecagold particle to analyse sulfate-reducing bacteria in anaerobic sludge samples. It was possible to detect the gold markers via NanoSIMS, however, not without prior autometallographic enhancement of the gold particles. Furthermore, the researchers state, that Gold-ISH might not suffice to detect microorganisms with a low rRNA content in oligotrophic environmental samples (Kubota *et al*., 2014). Gold-FISH in combination with NanoSIMS could be better suited for the detection of specific microorganisms with low rRNA contents in complex environments, as for instance in soil or on the rhizoplane. Furthermore, it would be interesting to analyse if the enzymatic amplification of biotinylated tyramides, that serves to enrich the number of deposited nanogold-particles in the target organism (as employed for Gold-FISH) would suffice to detect the gold label via NanoSIMS, without the necessity of autometallographic enhancement.

It was the aim within our group to establish Gold-FISH in combination with NanoSIMS as an alternative to FISH-NanoSIMS analysis. Such an approach would start with the addition of isotope-labelled substrates to an environmental sample. Afterwards specific microorganisms of interest would be detected via Gold-FISH and SEM instead of FISH and fluorescence microscopy. The problem that arises after fluorescence microscopy is, that specimen would need to be de-mounted, washed in ethanol and water to remove the mounting medium, and dried before analysis via NanoSIMS. For roots, this has a crucial implication, as between the analyses cells would get lost due to the washing, and the roots would change their structure due to the drying, which extremely hampers a correlative analysis. Gold-FISH allows for the possibility to identify microorganisms on dry samples via SEM *in-situ*. Therefore, no biasing preparations of the root would be necessary for the subsequent analysis of stable isotopes via NanoSIMS, which facilitates a correlative analysis. For the detection and identification of diazotrophs on the surface of rice-roots, Gold-FISH-NanoSIMS could prove to be very powerful. In such an analysis, rice plants would be incubated in a $^{15}N_2$-enriched atmosphere. Afterwards, microorganisms of interest could be detected via SEM and incorporations of $^{15}N_2$ into single microbial cells could be detected via NanoSIMS. Via correlation of the SEM images with the NanoSIMS images it would be possible to identify active diazotrophs on the surface of rice roots.

Final thoughts on the ecology of free-living diazotrophs associated with rice plants

Since the terrestrial net primary production is often limited through the availability of N, diazotrophs inherit a key position as they have the potential to alleviate the shortage of N by accessing N_2 from the atmosphere and transforming it into accessible NH_3. BNF thereby increases the productivity of whole ecosystems (Hunt *et al.*, 1988). However, it remains unclear how diazotrophs position themselves in micro-niches and how they cope with dynamic circumstances in paddy-soil used for rice cultivation. Rice paddy-fields can be characterized by a redox stratification of the anoxic zone beneath the oxic surface (Ratering *et al.*, 2000). Thereby different niches are created for bacteria and archaea exploiting different redox potentials in the oxygen depleted zone (Lüdemann *et al.*, 2000). However, the rice plant can introduce O_2 from the atmosphere into deeper layers of the soil via aerenchyma. This process adds to the complexity of niches and the dynamic of the system by re-oxidizing reduced

compounds in the rhizosphere (Liesack *et al.*, 2000). Our special interest was dedicated to free-living N-fixing microorganisms with the ability to associate with rice plants. Under gnotobiotic conditions and without amendments of N we showed that *K. sacchari* colonizes the rhizoplane of rice cultivar IR 64. Furthermore, we have shown that the organism fixes N_2 in the model system. Species of the genus *Kosakonia* are described as N-fixing PGPB (Chen *et al.*, 2014). However, they are cultivated representatives of a biologically diverse environment. The question is, if *K. sacchari* engages in a diazotrophic role in a natural environment, or if it is another organism that takes over a dominant diazotrophic position. Furthermore, what would its micro-niche be and what strategies and mechanisms determine its successful colonization?

Recently a metaproteomic study investigated microorganisms in association with rice plants under limited conditions of N (Bao *et al.*, 2014). The researchers showed that type II methanotrophs of the family *methylocystaceae* associate with the plants while simultaneously producing nitrogenases as well as methane monooxygenases. Thereby it was theorized that under conditions of low N availability methanotrophic bacteria mediate N_2 fixation and CH_4 oxidation (Minamisawa *et al.*, 2016). Thus, their strategy is the following: they receive CH_4 produced from methanogenic archaea for energy production and additionally assimilate N_2 to antagonize the shortage of N. However, methanotrophy is an aerobic process, while the nitrogenase is sensible against oxygen. Via CARD-FISH it was shown, that methanotrophic bacteria often reside on the basis of lateral roots (Schmidt *et al.*, 2014). These areas are thought to be oxygenated areas. Methanogenic archaea were also found in near range. It is assumed that the archaea can survive there by colonizing anaerobic microniches, where the oxygen has already diffused or was consumed by aerobic microorganisms on the rhizoplane (e.g. the methanotrophs; Schmidt *et al.*, 2014). It would also seem plausible if methanotrophic bacteria could colonize different micro-niches themselves – a hypoxic one in which they assimilate N_2, and an aerobic one, where they oxidize methane. Gold-FISH could be employed as a method that allows for detection of methanotrophic microorganisms with the simultaneous possibility to visualize the physicochemical environment, that is their habitat, on a very small scale. Additionally, it should be investigated if in the broader sense methanotrophic bacteria actually fix N_2 to enter a mutualistic association with rice plants. To answer this question, a survey employing a $^{15}N_2/^{13}CH_4$ stable isotope tracer assay is necessary. Such an experiment could be conducted on rhizosphere samples exposed to an atmosphere

containing $^{15}N_2$ and $^{13}CH_4$. A setup was already developed to analyse the fraction of oxidized $^{13}CH_4$ in the rhizosphere of rice via IRMS (Groot *et al.*, 2003). An analysis of incorporated ^{15}N and ^{13}C isotopes simultaneously via FISH-NanoSIMS would allow for the visualization and identification of active diazotrophs and methanotrophs in their respective micro-niches. Thereby it could be determined where methanotrophic cells fix N or oxidize methane, or if they are capable of doing both simultaneously. In preparation of a measurement via NanoSIMS it is very common to collect as much physicochemical and topographic information concerning the specimen that shall be measured. This information can be used for the optimization of the primary and secondary ion beam (Williams, 2006). To gain insight concerning the ultrastructure of a sample, scanning electron microscopy can be employed. Gold-FISH would fit into this pipeline, as the method additionally delivers the possibility to detect specific microorganisms of interest *in-situ* via scanning electron microscopy.

Furthermore, N fixation has often been reported as an active process in rice fields that are not N-poor but fertilized (Valiente *et al.*, 1997). What is the strategy behind diazotrophy in the presence of easily available N species? It was recently found that enzymes for the degradation of high molecular weight nitrogenous substances (HMW N) are phylogenetically widespread among free-living diazotrophs in soil. The researchers theorize a diazotrophic strategy that takes the acquisition of N originating from HMW N into account. According to their "LAH N-acquisition strategy", once inorganic N sources are unavailable, free living diazotrophs withdraw N from soil first from low molecular-weight nitrogenous substances (LMW N), then from the atmosphere, and then from high molecular-weight N (HMW N) (Norman and Friesen, 2016). It was shown that non-diazotrophic organisms start producing N-acquiring exoenzymes while low-molecular weight N is available at small concentrations (Geisseler *et al.*, 2010). The production of exoenzymes demands a high investment of cellular N (Burns *et al.*, 2013), therefor non-diazotrophs must use the last resources of LMW-N to produce exoenzymes. Diazotrophs however have an advantage, they can use up the entire LMW-N pool and start synthesizing exoenzymes afterwards, as they can match the cellular costs for those via BNF (Norman and Friesen, 2016). This theory could be in line with observations from Knief *et al*. in 2012, who showed that rice associated microorganisms have a repertoire of ABC transporter systems for the import of different amino acids. Additionally, diazotrophs might have a further ecological advantage, as they could degrade specific LMW-N sources and conduct BNF in

parallel (Norman and Friesen, 2016), because not all LMW-N compounds would repress diazotrophy (Hartmann *et al*., 1988). *Azoarcus sp*. Strain BH72 is a diazotrophic model organism for the investigation of endophytic colonization in plants (Reinhold *et al*., 1987; Hurek *et al*., 1994). Recently the transcriptome of the organism was analysed in pure culture under the influence of NH_3 and without. Under conditions of BNF one gene, essential for the synthesis of type 2 secretory pathways (azo0805), was upregulated ~21 fold compared to non-diazotrophic conditions. Proteins excreted by the type 2 secretion system include proteases, cellulases, lipases, pectinases, phospholipases and toxins (Sarkar et al., 2014). It should be questioned if *Azoarcus sp* strain BH72 prepares for the release of such proteins as it prepares for the degradation of HMW N substances in the environment, or if it would use such proteins for the degradation of plant tissues to enter an endophytic state. We have developed a gnotobiotic model that could help answering this question. Our system allows for the investigation of colonizing processes of diazotrophic microorganisms in association with rice plants via microscopy. It could be powerful to complement such an investigation with a metatranscriptomic and metaproteomic survey to elucidate the interplay of rice plants and specific diazotrophs under gnotobiotic conditions.

Conclusion and Outlook

My efforts in isolating N-fixing microorganisms from rice paddy-soils has led to the cultivation of *K. sacchari*, a key component for the subsequent development of my gnotobiotic model of BNF in association with rice. This reductionist system had the purpose to facilitate our view on BNF in the rhizosphere environment. The results suggest, that within the system *K. sacchari* efficiently re-associates with rice plants by colonizing the surface of their roots, where it assimilates N_2. The rapid formation of biofilms, the high $\delta^{15}N$-values measured via IRMS, as well as the successful detection of *K. sacchari* on the highly autofluorescent root surface via standard FISH provide indications that *K. sacchari* is highly active in my gnotobiotic model. I have verified its capability to assimilate N_2 *in-situ* via IRMS, the next step will be to measure the diazotrophic activity, and to analyse the translocation of biologically fixed N towards the plant via NanoSIMS.

Additionally, I further evaluated Gold-FISH, also regarding its advantages for subsequent NanoSIMS investigations compared to the combination of NanoSIMS with epifluorescent

detection of microorganisms. The outstanding characteristics of the method are (1) the potential to detect specific microorganisms via SEM at a resolution and magnification beyond light microscopy. This gives important additional insights concerning the composition and complexity of the habitat. And (2), Gold-FISH can be easily combined with chemical imaging tools, such as NanoSIMS. The advantage Gold-FISH has over FISH is, that the *in-situ* identification of microorganisms via SEM does not require embedding of the specimen, which facilitates a correlative analysis. NanoSIMS is one of the most promising tools for the study of nutrient cycles in microbial ecology, for it combines a high spatial and mass resolution allowing for the separation of several Isotopes simultaneously (Wagner, 2009). Gold-FISH could be used to identify active diazotrophs in association with rice plants as follows: In an environmental sample previously incubated with $^{15}N_2$-enriched atmosphere, specific microorganisms would be detected via gold-FISH and SEM. Subsequently, the incorporation of ^{15}N into single microbial cells would be investigated via NanoSIMS. By correlation of the images obtained from both methods, it would be possible to visualize and identify active diazotrophs associated to rice roots.

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Appendix
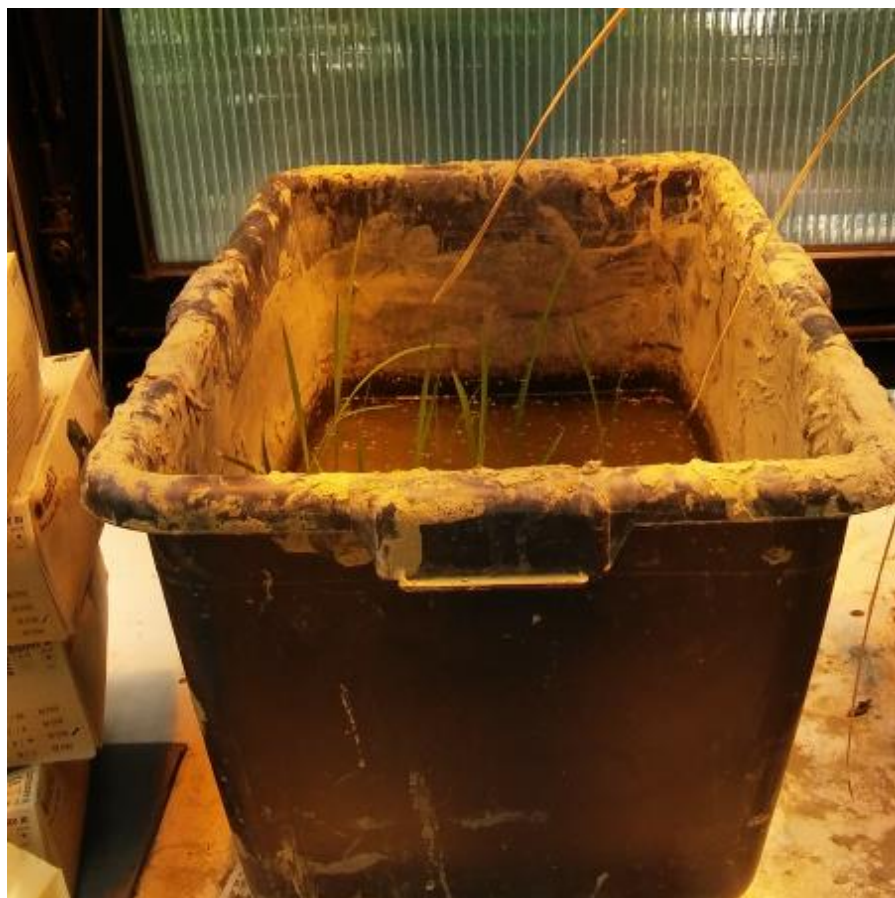
Supplementary Figure 1: Cultivation of wetland rice IR64 in paddy soil. This box was used for keeping the Italy- or SriLanka-soil in the greenhouse of the University of Vienna, under subtropical conditions.
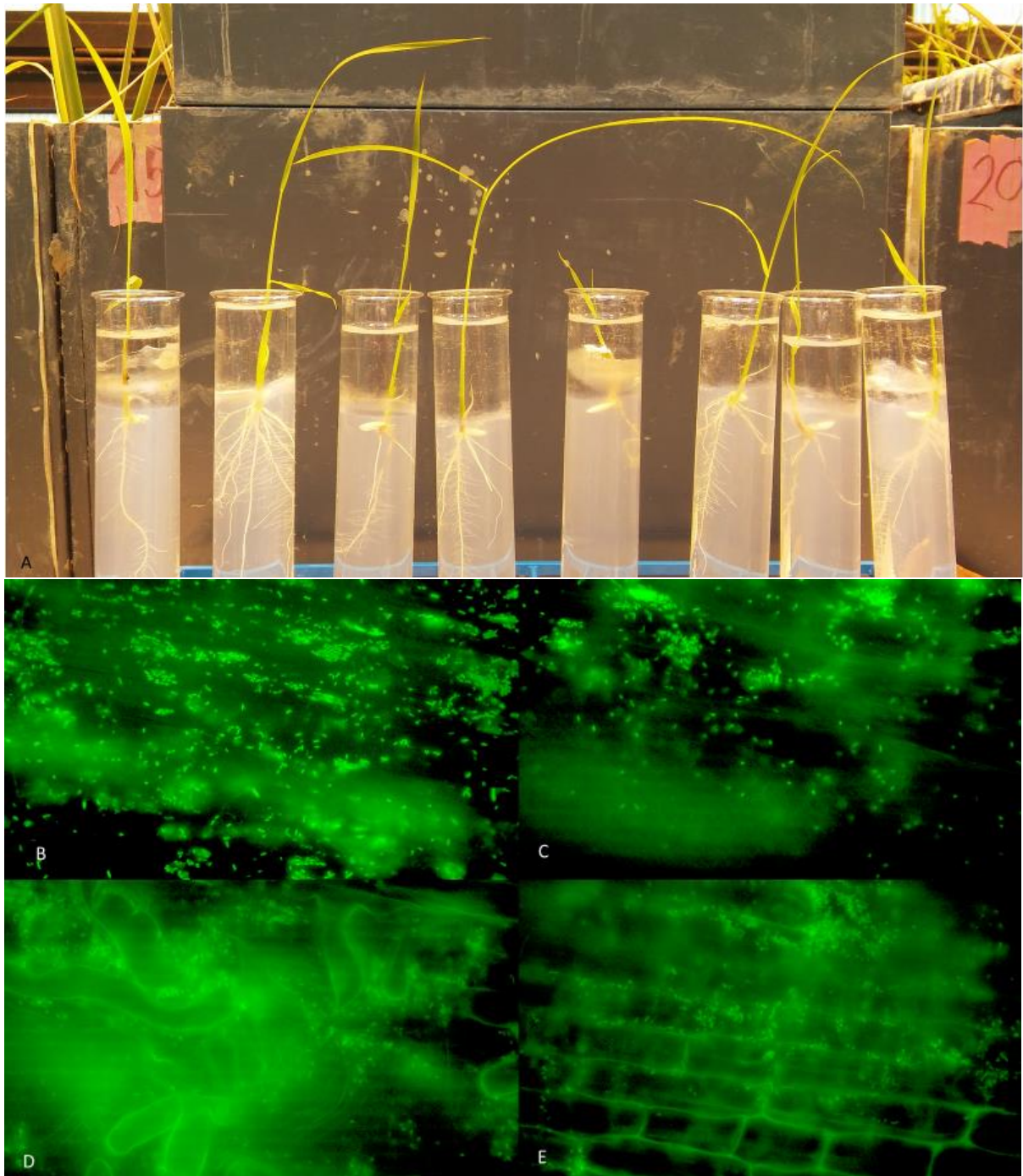
Supplementary Figure 2: Attempt to cultivate rice plants under gnotobiotic conditions in a "open test-tube" approach. In a several such plants are visualized in test tubes. In some a contamination in the surface water can already be seen by eye. The contaminations were analysed viaf SYBR green (absorption $\lambda_{max} = 494$ nm, emission $\lambda_{max} = 521$ nm, green) staining and epifluorescence microscopy. Microorganisms can be detected on all sample types: B) and D) surface sterilize seeds without association with K.sacchari; C) and D) surface sterilized seeds associated with K.sacchari

Supplementary Figure 3: The rhizosphere of 2 gnotobiotic rice plants in association with K. sacchari ("closed test tubes"). The organism forms a dense biofilm around the roots that can be seen by eye. The root branching is equal to the branching observed in natural environments.
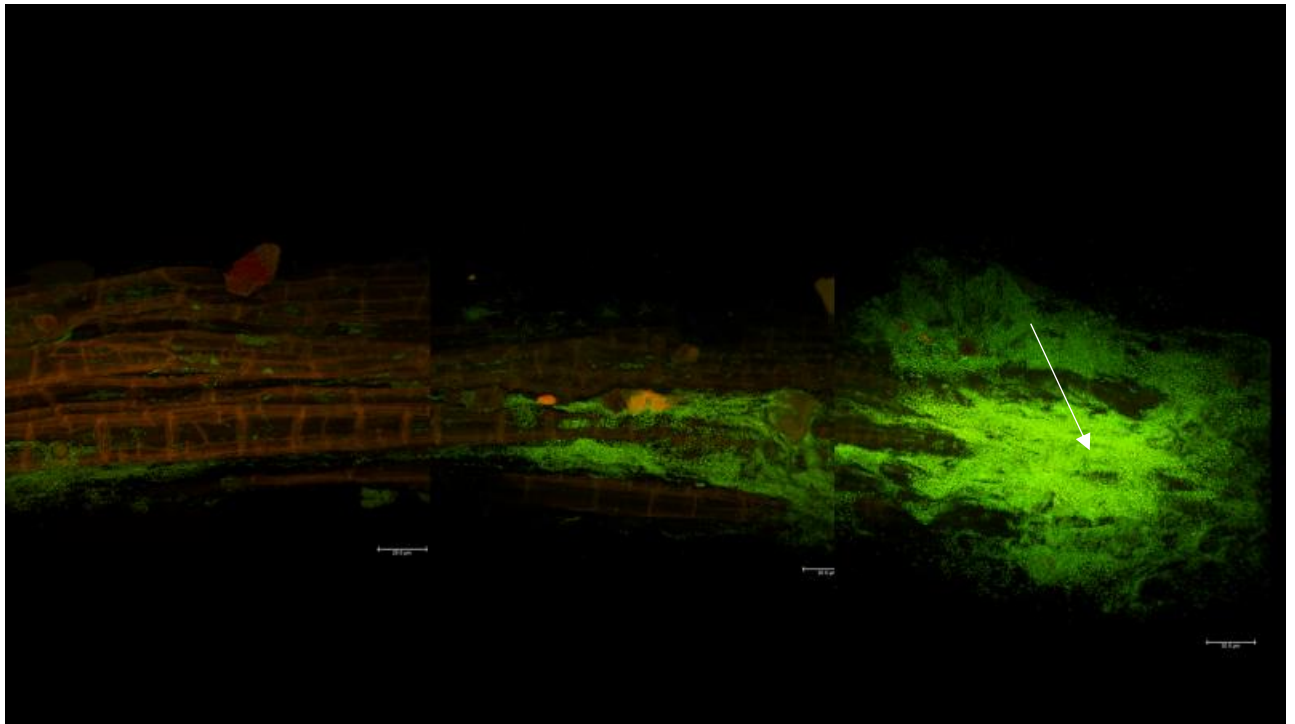
Supplementary Figure 4: *K. sacchari* cells on the rhizoplane of gnotobiotic rice roots, visualized with SYBR green. The Cy3-channel was used to image the root tissue, as it shows strong autofluorescence at 590 nm excitation (red). White arrow marks the position of the root tip. Images were obtained with CLSM and subsequently compiled into the present mosaic. The distribution of K. sacchari cells in this example is rather untypical (Comparison to Figure 7). This is the only observation I made where the organism has surrounded the root tip like visualized here.
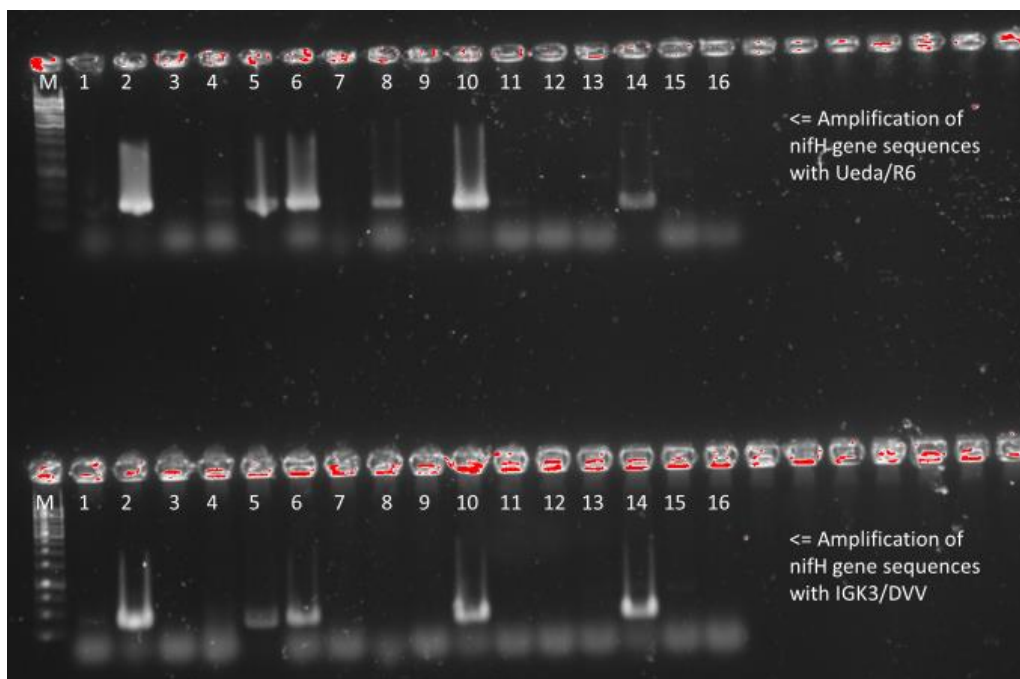
Supplementary Figure 5: Comparison of 2 different primer sets for the amplification of nifH gene sequences from cultivated diazotrophs. The results obtained with the Ueda/R6 primer set are shown in the upper lanes, the results obtained with the IGK3/DVV primer set are shown in the lower lane. The numbers refer to distinct isolated diazotrophic samples. M = Marker, 1kb + DNA ladder.

Supplementary Table 1: Samples weighed in at fine scale by Dr. Schmidt, before IRMS analysis

Operator:	Hannes Schmidt				
Datum:	22.12.2016				
Rackbezeichnung:	Hannes Dome Rice				
Seitenzahl im Buch:					
Probentyp:	Plant material, milled				
Natural abundance / markiert:	N2 and 15N2				
Rack-Position	Einwaage ug	Probenname	Sample Nr.	Comment	Label
A1	1.494	2CA-wild	1	plant material	Natural abundance
A2	1.357	2CA-kosa	2	plant material	Natural abundance
A3	0.797	2CA-wild	3	plant material	Natural abundance
A4	1.322	2CA-kosa	4	plant material	Natural abundance
C1	1.307	2CA-wild	5	plant material	15N2
C2	1.254	2CA-kosa	6	plant material	15N2
C3	0.641	2CA-wild	7	plant material	15N2
C4	1.206	2CA-kosa	8	plant material	15N2