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DISSERTATION

INTERACTIONS OF NITROGEN-RELATED, GROWTH PROMOTING BACTERIA WITH *MISCANTHUS* × *GIGANTEUS*: IMPACT AND MECHANISMS

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List of abbreviations

%	Percent
ACC	1-aminocyclopropane-1-carboxylic acid
AMO	Ammonia monooxygenase
BNF	Biological nitrogen fixation
C	Carbon
C4	Four-carbon molecule
Ca	Calcium
cm	Centimeter
CO2	Carbon dioxide
Corg	Orginic Carbon
Ct	Total carbon
Ctrl	Control
DM	Dry matter
EU	European Union
g	Gram
GFP	Green fluorescent protein
H.	Herbaspirillum
ha	Hectare
Hf	Herbaspirillum frisingense
K	Potassium
Kg	kilogram
m	Meter
M.	Miscanthus

N	Nitrogen
N0	Zero nitrogen fertilizier
N_2O	Nitrous oxide
N80	80 kg N $ha^{-1}a^{-1}$ application
NaOH	Sodium hydroxide
Nmin	Mineral nitrogen
NO	Nitric oxide
Nt	Total nitrogen
OTU	Operational taxonomic unit
P	Phosphorus
rDNA	Ribosomal DNA
Zome	Rhizome

Abstract

The highly nitrogen-use efficient biomass grass *Miscanthus* is a host of the bacterial endophyte *Herbaspirillum frisingense*. While *Herbaspirillum frisingense* has the genetic competence to fix nitrogen, the plant-associated microbiome may also contribute to this nitrogen efficiency. Furthermore, the costly field establishment of the sterile perennial *Miscanthus* × *giganteus* from rhizomes is a severe constraint for expanding the production area of this commercial biomass crop.

In this study, the effect of *Herbaspirillum frisingense* inoculation on stem-cutting sprouting, shoot biomass and other yield parameters was investigated. I studied how the inoculation impacts on the *M.* × *giganteus* associated microbiome and how the long term differences in nitrogen fertilizer amount modulated the *M.* × *giganteus* associated microbiome. This was studied in a 14 year-old field trial of *M.* × *giganteus* fertilized with various amounts of nitrogen.

Stem cutting inoculation improved the shoot sprouting and establishment success of *Miscanthus* × *giganteus* in the greenhouse. In a small field trial, plant height and biomass from inoculated sites were significantly larger in the second year after establishment, but already after one year after inoculation, the bulk soil, rhizosphere, root and rhizome microbiomes were almost devoid of *Herbaspirillum.* This *beta-proteobacterium* may colonize the shoot of *Miscanthus* × *giganteus* more efficiently. Major differences between bacterial communities were determined by plant-soil compartments and less by the plant organs, while both inoculation and nitrogen had little effects on these communities. Compared to the little effect on the soil, rhizosphere and root microbiomes, the rhizome microbiome was massively modulated by both inoculation and nitrogen level. In the

rhizome, several proteobacteria, which are associated with plant growth promoting functions, were enriched by inoculation, while N_2-fixing-related bacterial families were favored by long-term nitrogen-deficiency plots, but denitrifier-related families were depleted. The studies suggest that *H. frisingense* inoculation may improve establishment of *Miscanthus* stem cuttings and has long-lasting effects on the rhizome microbiome diversity, despite low rhizocompetence and low root abundance. Meanwhile, the rhizome could be a potential nitrogen fixation factory. The organ-specific, nitrogen-related bacterial communities are modulated by long-term different nitrogen supply and are mainly shaped by the plant, which provides guidance for optimizing *Miscanthus* sustainable cultivation.

Zusammenfassung

Das stickstoffeffiziente Biomassegras Miscanthus wird unter anderem vom bakteriellen Endophyten *Herbaspirillum frisingense* besiedelt, welches die genetischen Grundlagen für Stickstoff-fixierung besitzt. Das mit Miscanthus assoziierte Mikrobiom, welches ebenso zu seiner Stickstoffeffizienz beitragen könnte, war zu Beginn der Arbeit noch nicht bekannt. Darüber hinaus ist das Etablieren des sterilen, mehrjährigen *Miscanthus* × *giganteus* aus Rhizomen ein hoher Kostenfaktor und damit ein schwerwiegendes Hindernis für die Erweiterung der Produktionsfläche dieser kommerziellen Biomassepflanze.

In dieser Arbeit wurde die Wirkung der Inokulation mit *Herbaspirillum frisingense* auf das Keimen von Knospen an Stängeln, die Wirkung auf die Sprossbiomasse und auf andere Ertragsparameter untersucht. Darüber hinaus wurde untersucht, wie sich die Inokulation auf das mit *M.* × *giganteus* assoziierte Mikrobiom im Feld auswirkt. Das mit Miscanthus assoziierte Mikrobiom wurde ausserdem von einem 14-jährigen Dauerfeldversuch bestimmt, bei dem die Pflanzen ohne Stickstoff (N), oder jährlich mit 80 kg N gedüngt wurden.

Die Beimpfung von Stängelabschnitten mit *Herbaspirillum frisingense* regte das Keimen von Knospen an und erhöhte den Etablierungserfolg von *Miscanthus* × *giganteus* im Gewächshaus. In einem kleinen Feldversuch waren Pflanzenhöhe und Biomasse von beimpften Rhizomen im zweiten Jahr nach der Etablierung signifikant größer, aber bereits nach einem Jahr nach der Inokulation waren die Mikrobiome des Bodens, der Rhizosphäre, der Wurzel und des Rhizoms nahezu frei vom beimften *Herbaspirillum*. Dieses beta-Proteobakterium kann offenbar den Spross von *Miscanthus* × *giganteus* effizienter besiedeln. Die Hauptunterschiede zwischen den Bakteriengemeinschaften wurden durch

die unterschidlichen Umweltbedingungen der Kompartimente, also zwischen Pflanzen und Boden und weniger durch die Pflanzenorgane bestimmt. Sowohl die Inokulation, als auch die N-Düngung hatten nur geringe Auswirkungen auf diese Gemeinschaften. Im Vergleich zu den geringen Auswirkungen auf Boden, Rhizosphäre und Wurzelmikrobiome wurde das Rhizom-Mikrobiom sowohl durch Inokulation, als auch durch den N-Dünger, am meisten verändert. Im Rhizom wurden mehrere Proteobakterien, die bekanntermassen zur Förderung des Pflanzenwachstums beitragen, durch Inokulation angereichert, während N_2-fixierende Bakterienfamilien im Rhizom durch Langzeit-N-Mangel begünstigt wurden. Familien, in denen Stickstoff-Denitrifizierer gefunden werden, waren weniger im Rhizom repräsentiert. Die Arbeit legt nahe, dass durch die Inokulation mit *H. frisingense* die Etablierung von Miscanthus-Stängelabschnitten verbessert werden kann. Trotz geringer Rhizokompetenz und geringer Abundanz hat das Inokulat lang-anhaltende Auswirkungen auf die Rhizom-Mikrobiom-Diversität. Das Rhizom könnte als eine potenzielle Stickstofffixierungsfabrik dienen, und somit langfristig zur Stickstoffeffizient beitragen. Die organspezifischen und wenig durch das N-Düngeniveau regulierten Bakteriengemeinschaften werden hauptsächlich von der Pflanze geprägt und könnten eine Rolle bei der Optimierung der nachhaltigen Kultivierung von Miscanthus spielen.

1 Introduction

1.1 High nitrogen-use efficiency of biomass grass *Miscanthus*

Biomass grass is a major part of the renewable energy sources, which developed as an alternative energy of non-renewable energy-fossil fuels. The properties of sustainable energy sources, decrease of environmental pollution and carbon emission have promoted the development of bioenergy crops in the past decades. *Miscanthus*, in particular the hybrid *Miscanthus* × *giganteus* is one of the outstanding bioenergy crops, with the traits of low investment and long term of stable high yields (Heaton *et al.*, 2004). *Miscanthus*, originated in East Asia, is a perennial C4 grasses (Lewandowski *et al.*, 2000). *M.* × *giganteus* yields up to 25 ton dry matter (DM) ha^{-1} yr^{-1} in Europe and lasts a long term (Lewandowski *et al.*, 2000; Cadoux *et al.*, 2012; Iqbal *et al.*, 2015). It shows remarkable adaptability to a wide range of climate and soil conditions. So far, *M.* × *giganteus* has been established in broad locations throughout northern hemisphere, especially in Europe and America (Christian *et al.*, 2008; Lewandowski *et al.*, 2003).

Sustainable high yield production with low anthropogenic energy inputs, such as low N fertilizer and pesticide, is always a desirable requirement for biofuel feedstocks. As an outstanding biomass crop, *M.* × *giganteus* was found to harvest relative high biomass yield uninterruptedly in both short-term and long-term field experiments with low nitrogen fertilizer addition (Heaton *et al*., 2004; Cadoux *et al*., 2012; Maughan *et al*., 2012; Iqbal *et al*., 2015). In extreme cases with no N input fertilization while continuous biomass (included N) removal, *M.* ×*giganteus* fields produced considerable biomass (Dohleman *et al.*, 2012; Iqbal *et al*., 2015).

A major reason of the high nutrient efficiency is contributed to its perennial lifestyle and its active translocation of N from aboveground shoot to belowground storage organ rhizome at the end of the annual growth phase (Beale & Long, 1997, Liu *et al.*, 2014). Harvesting biomass products after senescence, could translocate more than half of leaf N to the plant storage organs (van Heerwaarden *et al.*, 2003). Delayed harvest of miscanthus in the winter after its translocation has demonstrated to markedly minimize N removal and reduce atmospheric pollutants (Lewandowski & Kicherer, 1997; Heaton *et al*., 2009).

Meanwhile, biological nitrogen fixation (BNF) is proposed to explain part of the high nitrogen efficiency. Multiple non-legume energy crops likely benefit from BNF under N deficient condition. Several nitrogen fixing bacteria have been successfully isolated from sugarcane, a close relative of *Miscanthus*. Further, both greenhouse and field studies have demonstrated that sugarcane benefits large amounts of nitrogen from BNF by using ^{15}N-labelled isotope dilution and $^{15}N_2$ incorporation (Sevilla *et al*., 2001; Thaweenut *et al*., 2011; Urquiaga *et al*., 2012; Baptista *et al*., 2014). In certain soil conditions for some sugarcane genotypes, nitrogen source from BNF may occupy more than three quarters of total nitrogen consumption (Baptista *et al*., 2014). Similarly, *Miscanthus* related nitrogen fixing bacteria have been successfully isolated (Kirchhof *et al*., 2001). Modeling studies on the nitrogen-balance of *Miscanthus* fields propose that biological nitrogen fixation type N contribute to the biomass product (Christian *et al.*, 2008; Davis *et al.*, 2010). Further, field experiments using ^{15}N-isotope trace indicate that BNF-source nitrogen supply more than sixteen percent of the *Miscanthus* N requirements (Christian *et al.*, 1997; Keymer & Kent, 2014). Moreover, the DAYCENT model suggests that the capacity of *M.* × *giganteus* BNF is weaker than sugarcane but significantly stronger than other non-legume biomass crops, such as switchgrass (Davis *et al.*, 2010).

1.2 *M.* × *giganteus* propagation and establishment

M. × *giganteus* is a naturally infertile hybrid of *M. sinensis* and *M. sacchariflorus*. The triploid sterile property has weakened invasiveness of *Miscanthus* × *giganteus* (Nishiwaki *et al.*, 2011). However, that *M.* × *giganteus* is incapable to directly reproduce via seeds further limits the biomass plantations establishing to a large-scale. To meet the ever-increasing requirement of *M.* × *giganteus* plantation size, three main propagation systems are used to produce numerous plants, consisting of rhizome-based propagation, micropropagation and stem-based propagation.

Currently, the main propagation method is via rhizomes. Rhizome is defined as a modified subterranean stem with emitting roots and shoots from its nodes. Firstly digging out rhizome of the soil, then breaking up the rhizome into several pieces, finally planting rhizome sections into the field, the process is relatively easy and favored by the farmers. Rhizome propagation system is moderately costly, however destroys the propagation fields and is relatively low efficient, with only 1:10-50 multiplication rate, cause that currently only about 20 000 ha in Europe are planted with *M.* × *giganteus* (Xue *et al.*, 2015; Lewandowski *et al.*, 2016; Clifton-Brown *et al.*, 2017).

Micropropagation is a rapid reproduction of whole plant through tissue culture, where a callus tissue is dedifferentiated from a single explant, and massive offspring can be produced by plant hormones under favourable conditions (Gubišová *et al.*, 2013; Figure 1). Three-step procedure is included: first tissue culture, then greenhouse culture, finally transplant in the field. The labor-intensive micropropagation has the advantages of high multiplication rates (about 1000 times) and prevents the transmission of diseases (Lewandowski, 1998; Xue *et al.*, 2015). However, micropropagation is not commercial application because the complicated procedure requires high establishment costs.

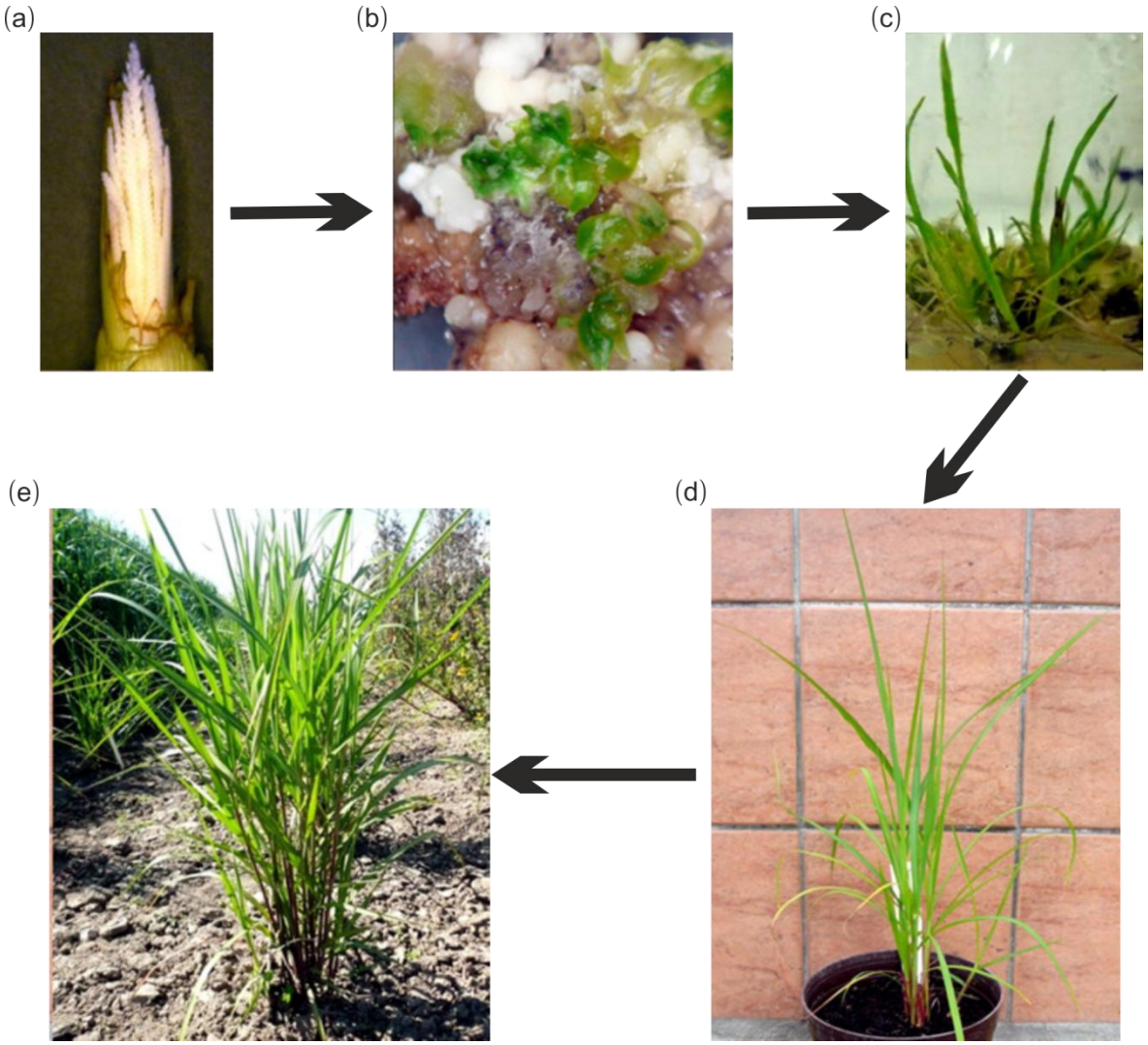


Figure 1: Schematic micropropogation of *Miscanthus* × *giganteus*.

(a) Immature inflorescence chosen for micropropogation; (b) callus culture; (c) plant induced; (d) mature plant in the control environment; (e) finally transplanted in the field. Figure has been modified after Gubišová *et al.*, 2013.

Propagation via stem cuttings with nodes has also been suggested with high multiplication rates and is readily available (Hong & Meyer, 2007; Atkinson, 2009), which is also environmentally friendly (Meyer & Hong, 2011; Boersma & Heaton, 2012). However, previous attempting to directly plant stem-cutting propagules in field conditions is failure

because of its sensitive to the environment stress and rooting hardly (Meyer & Hong, 2011). So stem sections are firstly pre-grown in greenhouse and then transplanted into the field, which increases cost and consumes time (Figure 2). Vertically planted stem-cuttings (Meyer & Hong, 2011) may be less promising than horizontally placed cuttings (Boersma & Heaton, 2012). And recently, directly planting of 1.8 m long stems was successfully established in the field with horizontal pattern (O'Loughlin *et al*., 2017). So the potential of horizontal pattern to shorten the two-step stem-cutting propagation needs further investigation.

Figure 2: Stem cutting propogation of *Miscanthus × giganteus*.

Stem cutting of *M. × giganteus* sprouted and rooted from the node below the soil after planted 4 weeks.

1.3 Plant associated soil microbiome

The root system of terrestrial plants can absorb nutrients and water from the soil, and interact with microbes. The plant–microbe interactions are primarily accomplished in this complex plant-soil-microbiome environment (Chaparro *et al.*, 2012; Figure 3). An understanding of how plant and soil factors manipulate and reshape the soil microbiome, and how the microbiome in turn affects to the plant health and productivity is essential. The progress of exploration of soil microbiomes was slow in the past century, however, because the majority of soil microbes are uncultivable and the mechanisms of their functions remain unclear. Recent advances in high-throughput sequencing can help us to classify the plant–microbe interactions and investigate how their benefit plant productivity (Morales & Holben, 2011).

The root system is a key energy supplying element to the plant associated microbiomes (Bais *et al.*, 2006). The plant root supports the soil mircrobiomes (especially rhizosphere microbiome) by the release of root exudates, such as carbohydrate, amino acids, peptides, and fatty acids. Meanwhile, the root cap drop and mucilage decomposition can also provide energy (Bertin *et al.*, 2003; Bais *et al.*, 2006). Plants also secrete chemical compounds, such as flavonoids and antimicrobial, to response the chemical signals of soil microorganisms and then recruit special microbe or eliminate. Release of these compounds varies depending on the plant species, plant development, plant nutrient condition, and abiotic factors (Flores *et al.*, 1999; Micallef *et al.*, 2009; De-la-Pena *et al*., 2010). Further, series of studies indicated a tightly relationship between rhizosphere microbiome communities and host plants (Broeckling *et al.*, 2008; Badri *et al.*, 2009; Micallef *et al.*, 2009).

The forces of soil physicochemical properties also markedly shape the soil microbial

diversity. Different types of Soil are provided series of habitation to diverse communities of microorganisms with the quantities of 10^8 ~ 10^{10} microbes per gram of soil (Schloss & Handelsman, 2006). Recent evidence suggests that out of all these factors, soil texture, nutrient content, soil water content and soil pH are the main factors influenced soil bacteria and fungi. The soil microbial community is shaped by a combination of all these factors. While both Fierer & Jackson (2006) and Rousk *et al*. (2010) indicated that soil pH outcome other factors in shaping the soil bacterial community. While N fertilizer changed the soil physicochemical properties and plant nutrient situation, plant related soil mircobiome should be reshaped by N fertilizer. If the N fertilizer induce a special trend in the related microbiome composition diversity, and this trend benefits the miscanthus or not, deserve further research.

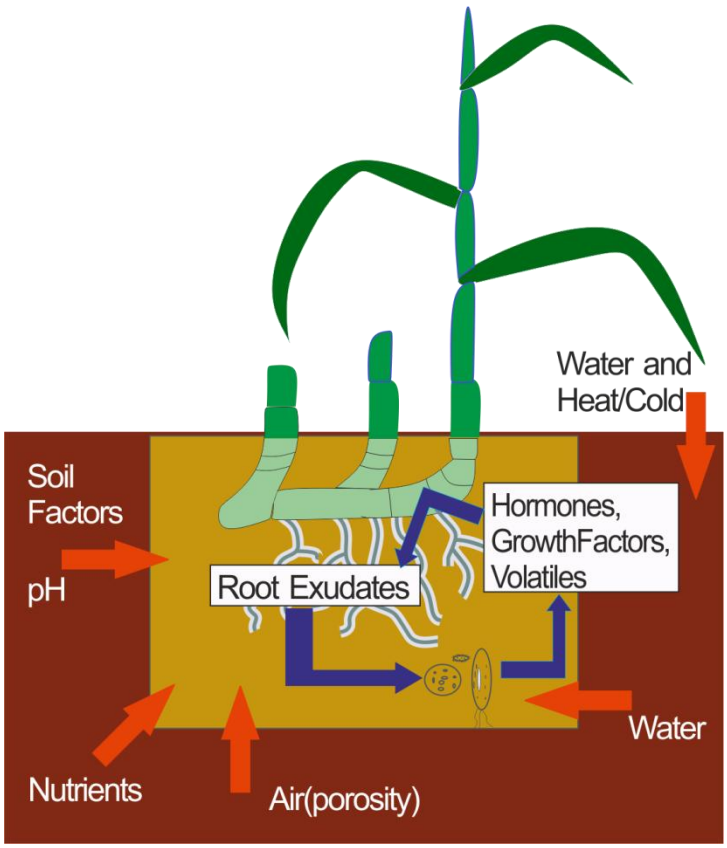


Figure 3: Schematic illustration of soil-plant root-soil bacteria system.

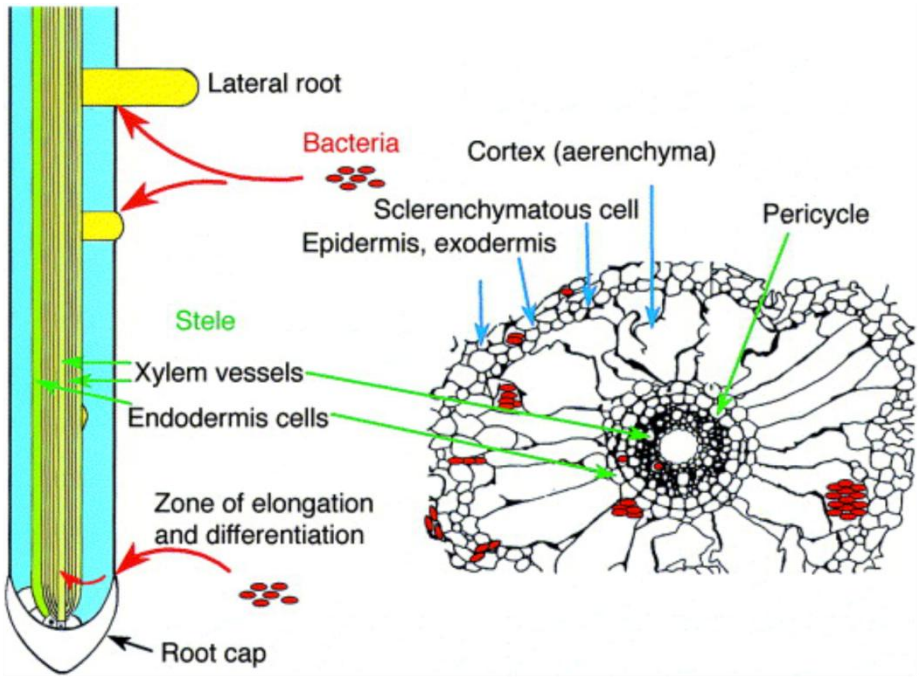
Both Soil factors and plant exudates influence soil microbes, which in turn reshape the soil environment and affect the plants through a dynamic exchange of chemical responses. Figure has been modified after Chaparro *et al.*, 2012.

1.4 Plant associated endophytic bacteria

An Endophytic bacterium is that resides within plant tissues and don't cause any visible symptoms for at least part of its lives (Compant *et al.*, 2010; Monteiro *et al.*, 2012). Typically they are non-pathogenic, but include latent pathogens cause disease occasionally (James & Olivares, 1998). Endophytes are considered to be originated from rhizosphere microbiome (Compant *et al.*, 2010), but they exert different characteristics, such as invasive, from rhizospheric bacteria, indicating that only a small part of special rhizospheric bacteria could enter in the plant tissue and evolve special functions to adapt to the plant internal environment (Ferrara *et al.*, 2012).

Previous studies indicated several candidate sites and putative pathways of endopytic bacteria entering into root tissue (Reinhold-Hurek & Hurek, 1998a; Reinhold-Hurek & Hurek, 1998b; Figure 4). One entry site is at the root tip among the elongation zone and differentiation zone. From this site, the bacteria can invade through the cells or cell-layers and finally into the central tissue, which latterly differentiates into stele (endodermis cell or xylem vessel). Another entry locates at the lateral roots emerging sites, from these points bacterial cells could invade into the cortex of the main root and lateral root. *Herbaspirillum* is described to infect the miscanthus with the second pattern of invasion (Straub *et al.*, 2013a). The infection process including several processes: such as Type IV pili mediating

attachment to the host, and pectic enzymes and cellulolytic enzymes digesting the cell wall (cell-wall-degrading enzymes (CWDEs)) (Reinhold-Hurek *et al.*, 1993; Reinhold-Hurek & Hurek, 1998b). *Herbaspirillum spp.* possesses T3SSs to excrete plant CWDEs (Monteiro *et al.*, 2012).



Reinhold-Hurek & Hurek.(1998);Trends in microbiology,6:139-144 Trends in microbiology
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Figure 4: Schematic illustration of endophytic bacteria infection and colonization in plant root.

Colonization sites of endophytes are described by red ovals, and colonization path are indicated by red arrows (Reinhold-Hurek & Hurek, 1998b).

Endophytic bacteria are capable of influencing the plant health and productivity in different mechanisms (Fitzsimons & Miller, 2010; Lau & Lennon, 2011; van der Heijden

et al., 2008). Endophytic bacteria could fix nitrogen and supply directly to the plant. Because nitrogen fixation consumes high energy, the plant could supply ample photosynthetic products to the endophytic bacteria for nitrogen fixation (Glick, 2012). And the endophytic bacteria fixing nitrogen have been confirmed in sugarcane by $^{15}N_2$ and isotopic dilution (Bhattacharjee *et al.*, 2008; Momose *et al.*, 2009). Plant hormones regulate plant growth and development and adjust the plant response to the environment stresses. However, much nonlethal stresses induced hormones regulation can limit plant growth. The endophytic bacteria may modulate phytohormones level and further affect the plant's response to stress (Glick *et al.*, 2007). Many studies have indicated that a lot of endophytic bacteria can produce cytokinins, gibberellins, auxins, and modulate ethylene production (Williams & De Mallorca, 1982; Garc á de Salamone *et al.*, 2001; Blaha *et al.*, 2006; Spaepen *et al.*, 2007). Moreover, studies have proved the endophytic bacteria inoculation promoting plant growth among a broad plant species, such as miscanthus, rice, maize (Bhattacharjee *et al.*, 2008; Straub *et al.*, 2013a; Nautiyal *et al.*, 2013). However, how the endophytic bacteria regulate the production of plant hormones in the plant is currently unclear. Furthermore, the endophytic bacteria suppress pathogens or trigger induced systemic resistance (ISR) to maintain plant health and promote plant growth indirectly (Eyles *et al.*, 2010; Pieterse *et al*., 2014).

1.5 Diazotroph and other N-cycle bacteria in agriculture ecosystem

Nitrogen is an essential macronutrient for plant growth and participates in biosynthesis of amino acids and nucleic acids. The nitrogen cycle mainly consists of three processes — N_2 fixed to ammonia (N_2 fixation), ammonia nitrified to nitrate (nitrification), and nitrate denitrified to NO, N_2O and N_2 (denitrification). And accordingly, microbes participated in

N_2 fixation are labeled as ‘nitrogen fixers’, participated in nitrification labeled as ‘nitrifiers’ and in denitrification labeled as ‘denitrifiers’’.

Atmospheric N_2 is fixed by microorganisms that carry nitrogenase into NH_3. Nitrogenase is only in prokaryote, and consumes 16 molecules of ATP to fix one molecule of dinitrogen. Molybdenum-iron (MoFe) nitrogenase is the primary one, and consumes less ATP than the other two — iron-iron (FeFe), vanadium-iron (VFe) nitrogenases (Zehr *et al*., 2003). *NifH* encoded the iron-containing nitrogenase reductase is used as a gene marker to detect and quantify the environmental nitrogen-fixing microorganisms (Bothe *et al*., 2010).

Denitrification describes the process of reduction of nitrate to nitrite (NO_2^-), further NO, and N_2O to N_2 in the anaerobic condition. Nevertheless, many denitrifiers express only partial denitrifying inventories, and result in the release of greenhouse gas, especially N_2O to the environment, causing air pollution.

In the agriculture system, for example the *Miscanthus* biomass crop field, the N balance depends on the input of N fertilizier and non-legume biological nitrogen fixation (BNF), and the output of biomass harvest, nitrate leaching and nitrogen denitrification (Figure 5). Due to the reduced N fertilizer input, we believe the nitrate leaching is small. So the bacteria related nitrogen fixation and denitrification, and how N fertilizer and plant affect on them need deep investigation.

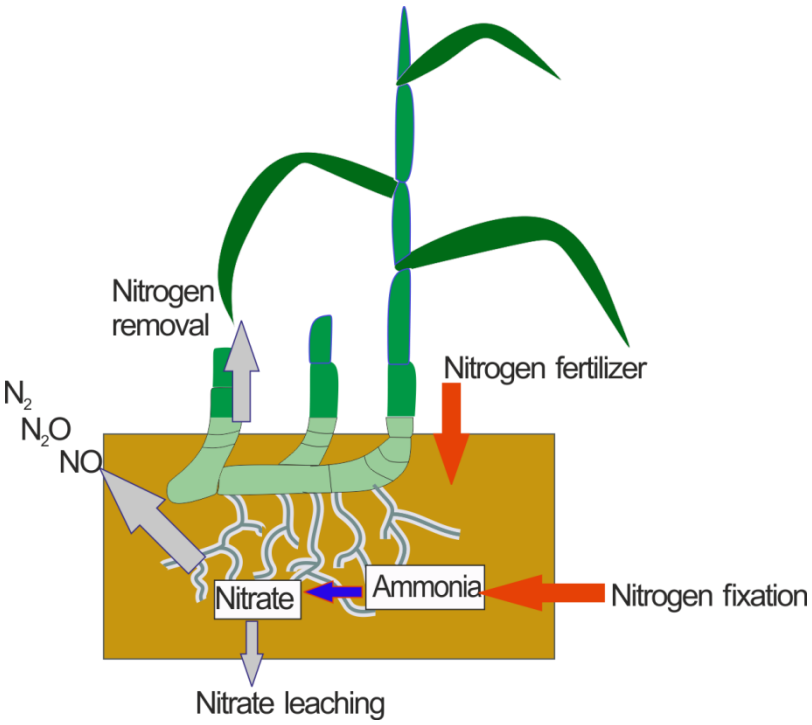

Figure 5: Schematic illustration of N-cycle in the agriculture ecosystem.

Nitrogen removal, denitrificaiton (NO, N_2O, N_2) reduce soil nitrogen; while bacterial nitrogen fixation and fertilizer addition increases soil nitrogen in miscanthus field.

1.6 High-throughput sequencing applied in plant associated microbiome

Distinct from the symbiotic nitrogen fixation in the Legume-Rhizobium system and Frankia -Actinorhizal plants system, plants associate with other group of diazotrophs and other functional bacteria. However, most investigations still concentrate on the single isolate interacting and promoting *M.* × *giganteus*, causing relative weak positive effects, especially in the field experiment most (Kirchhof *et al.*, 2001; Davis *et al.*, 2010; Straub *et al.*, 2013b). The traditional culture-independent 16S rDNA-denaturing gradinent electrophoresis (DGGE) or a hybridization-based method (PhyloChipTM) identified the profile of *Miscanthus* related bacteria (Li *et al.*, 2016; Cope-Selby *et al.*, 2017), but still only get a limited coverage of the whole *Miscanthus* associated microbiome. So, while *M.*

× *giganteus* associated bacteria, including both the soil microbiome and endosphytic microbiome, have indicated to play a key role in the biomass productivity and crop health, the knowledge of these *M.* × *giganteus* associated bacterial communities is still limited.

Since the roche 454 pyrosequencing was first introduced to the DNA research, a series of high-throughput sequencing platforms, such as Illumina, SOLiD platform and Ion Torrent, were developed to sequence the whole genome DNA, transcriptome and environment metagenome, with the advantages of cost-effective, faster and deeper sequencing than traditional Sanger sequencing (Goodwin *et al.*, 2016). Simultaneously, the relevant bioinformatics software tools and analysis procedure were developed to analyze the generated big data and further open some new frontiers, such as the environmental microbial community analysis. Currently, the Illumina sequencing platforms dominate the short-read next generation sequencing markets, such as 16S rRNA gene sequencing, which providing the identified microbial composition and distribution in test samples. The Illumina platforms utilize sequencing by synthesis approach with the cyclic reversible termination (CRT) PCR strategy (Figure 6), with the advantages of low homopolymer errors and high resolution.

High-throughput sequencing of 16S rRNA gene studies in *Arabidopsis*, sugarcane, sorghum and many other plants have demonstrated that the bacterial communities associated with plant are strongly influenced by plant organ type and soil type (Lundberg *et al*., 2012; Lavecchia *et al*., 2015; De Souza *et al*., 2016; Li *et al*., 2016). A much weaker effect on the bacterial communities is imposed by different plant growth stages and genotype (Lundberg *et al*., 2012; Lavecchia *et al*., 2015) or agricultural practice such as ploughing, while different nitrogen fertilization often had only minor effects on the plant associated bacterial communities (Babin *et al*., 2019; Yeoh *et al*., 2016).Therefore, to

achieve the high resolution profile of *M.* × *giganteus* associated soil bacterial community and endophytic bacterial community, and to classify whether N fertilizer application or diazotroph inoculation massively reshape these communities, the high-throughput sequencing is needed.

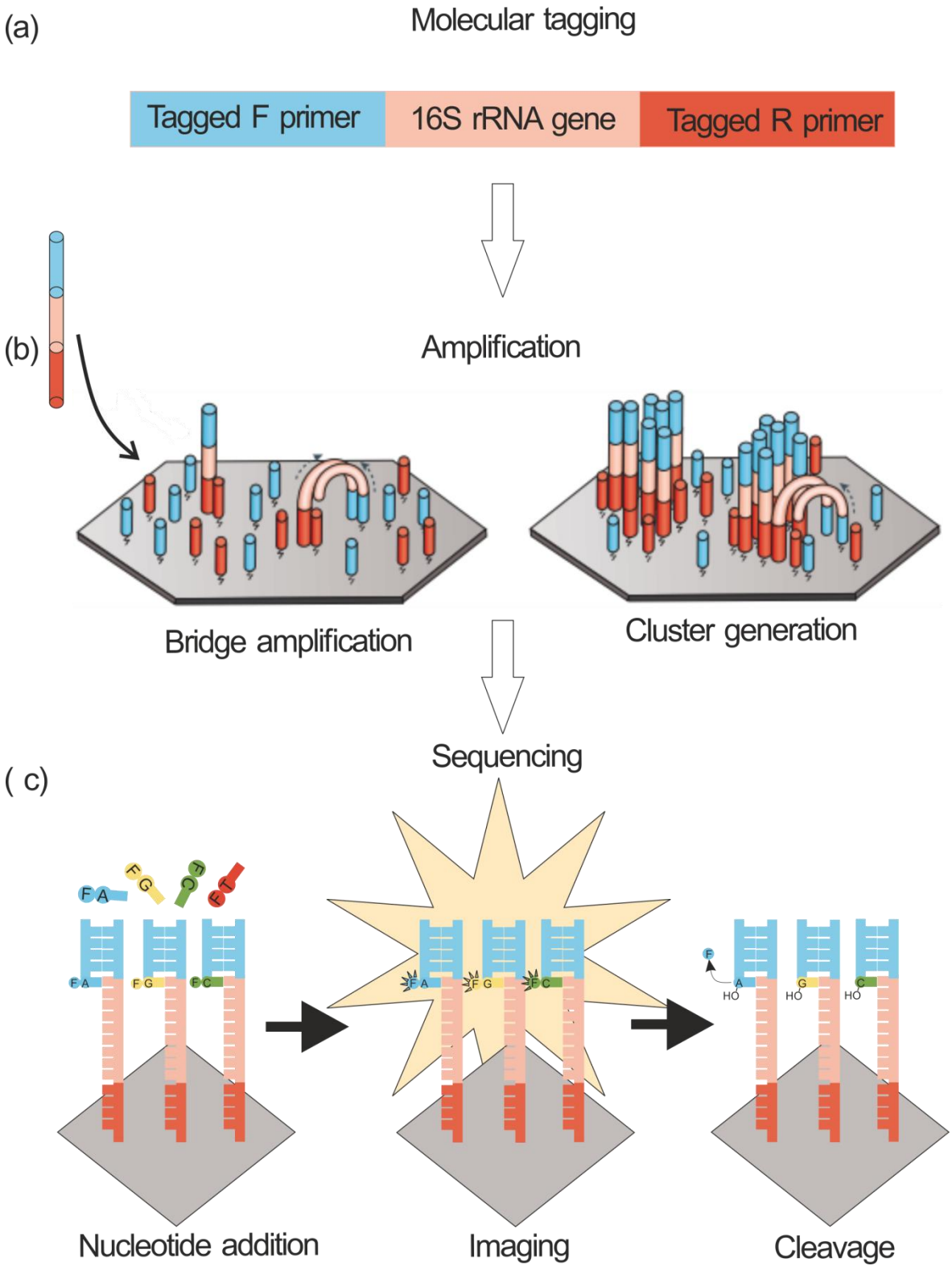
(a)
Molecular tagging
Tagged F primer
16S rRNA gene
Tagged R primer
Amplification
(b)
Bridge amplification
Cluster generation
Sequencing
(c)
Nucleotide addition
Imaging
Cleavage

Figure 6: Schematic illustration of high throughput full length 16S sequencing workflow.

A general process of Illumina 16S rRNA amplicon sequencing is shown. (a) Barcode, adaptor and primer were tagged to the target gene with PCR amplicaiton. (b) Bridge amplification was used to enrich templates. (c) Enriched templates were sequenced by cyclic reversible termination approach. Figure has been modified after Goodwin *et al.*, 2016.

1.7 Objective of this research project

For the development of biomass crops, such as *Miscanthus*, sustainable production and high biomass yield are contradictory and need to be coordinated. High biomass removal will deplete soil fertility, harm the soil chemical elements recycle, cause poor physical structure, and in turn decrease the biomass yield. Even though the ash returned to the soil could release the phosphorus and potassium, the nitrogen gap keeps as the main problem (Lanzerstorfer, 2019). Besides late harvest and other field managements reducing the nitrogen removal, the N-related bacterial communities will also provide a potential N pool to minimize the nitrogen gap. Simultaneously, other *Miscanthus*-associated microbes could also result in an integrated effect on the plant growth and health. However, the knowledge of the composition and distribution of *Miscanthus* related bacterial communities is still limited, which inhibits the application of potential beneficial bacterial communities.

Moreover, *H. frisingense*, a plant growth beneficial nitrogen fixing bacterium isolated from *Miscanthus* (Kirchhof *et al.*, 2001), was chosen as a example of potential beneficial bacterial communities to investigate the promoting function on the *Miscanthus* stem cutting propagation and the inoculation effects on related bacterial communities.

In this research project, three main objectives were purposed: The first objective was to test whether long term field nitrogen fertilizer will reshape the *Miscanthus* × *giganteus* associated bacterial communities, especially for the nitrogen-related bacterial group. The second objective was to test whether *H. frisingense* inoculation can help to establish new miscanthus plants from nodes in stem cuttings and whether this has long-lasting effects. The last objective was whether *H. frisingense* inoculation can help to dominate the special niche and reshape the *Miscanthus* × *giganteus* associated bacterial communities.

Therefore, the high throughput sequencing Illumina Miseq was used to investigate *Miscanthus* × *giganteus* associated bacterial communities from a long term field experiment and a second year established *H. frisingense* inoculated field experiment. Four different underground fractions, the bulk and rhizosphere soil, roots and rhizomes, were collected from both in the long-term N fertilizer *M.* × *giganteus* field trials and in the *H. frisingense* inoculated field trials in southern Germany, respectively. Following samples collected, DNA extraction, sequencing and analysis were carried out. For the last objective, two planting dates, three node positions, different planting patterns with *H. frisingense* inoculation were used to test the stem propagation and biomass yield of *M.* × *giganteus* both in the greenhouse and in a small field experiment.

It was hypothesized that:

- Different soil-endosphere fractions provided different niches for bacterial species and recruit different bacterial communities.

- A distinct long-term nitrogen fertilizer application shifts the *Miscanthus* × *giganteus* associated bacterial composition.

- The genetic properties of *H. frisingense* are ideal to improve biomass yield of *M.* ×

giganteus.

- *H. frisingense* inoculation will lead to substantial occupation of an endosphere niche in miscanthus, has long lasting effects and will benefit stem cutting propagation.

To our knowledge, the present survey of underground bacterial communities of field-grown *Miscanthus* is the first high resolution community profiling investigating the effects of compartment type, N fertilizer application and *H. frisingense* inoculation on *Miscanthus*. The obtained community profiling could help to select the beneficial bacterial groups and further may help to utilize the associated beneficial microbes to improve bioenergy crops yield and sustainability. Meanwhile, the *H. frisingense* inoculation improved stem cutting of *M.* × *giganteus* establishment could help to expand the *M.* × *giganteus* cultivation area.

2. Materials

2.1. Culture Substrates

Nutrient-poor artificial mixture substrates were used to culture cutting stems of *Miscanthus × giganteus* in greenhouse culture.

Table 1: Mixture substrates compositon and its corresponding properties.

mixture substrates	soil chemical properties	
Calcareous Loess subsoil (80%)	pH	7.6
	C_{org}	< 0.3%
	N_{total}	0.02 %
	extractable P	5 mg/kg
	Favorable soil moisture	20 %
quartz sand(20%)	Particle diameter	0.6–1.2 mm

The soil moisture was adjusted daily to 24% (w/w) = 80% substrate water holding capacity (WHC).

Herbaspirillum frisingense GSF30^T cultures were performed on Liquid Luria–Bertani medium.

Table 2: Liquid Luria–Bertani medium compositon.

Reagent	Amount to add
H2O	950 mL
Tryptone	10 g
NaCl	10 g
Yeast extract	5 g

The liquid medium was adjusted to pH = 7.0 with NaOH and then adjusted to 1 L with H_2O. The liquid medium was sterilized by autoclaving for 20 min. When it was used to culture *Herbaspirillum frisingense*, 50mg l^{-1} of kanamycin was added.

2.2. Plant and bacterial materials

The stems collected from *M.* × *giganteus* Greef et Deuter plots, established in 1997 at the experimental station 'Ihinger Hof' (48.75 ℃N, 8.92 ℃E) (Clifton-Brown *et al.*, 2001), were used. Leaves were removed from the stem to reveal axillary buds and the three most basal nodes were excised as single node segments with around 4 cm of stem at each side. The basal node segment closest to the soil surface was considered as '1st node', the more apical nodes were counted as 2nd and 3rd node. *Herbaspirillum frisingense* GSF30^T (hereafter *H. frisingense*) was a growth promoting bacterium isolated from miscanthus with the functions of nitrogen-fixation, ACC deaminase (Rothballer et al., 2008; Straub et al., 2013c).

2.3. Kits

Table 3: Kits used for microbial DNA extraction and purification.

Kit	Purpose	Merchant
MO BIO's PowerSoil DNA Isolation Kit	DNA extraction from miscanthus underground samples	Qiagen
QIAquick Gel Extraction Kit	extract and purify DNA from gel	Qiagen

2.4. Instrumental equipment

Table 4: Instrumental equipment used.

Instrument	Purpose	Merchant
Nanodrop 2000c Spectrophotometer	Quantification and quality check of DNA from miscanthus underground samples	Thermo Fisher Scientific
Qubit Fluorometric Quantitation	Quantification and quality check of DNA from miscanthus underground samples	Thermo Fisher Scientific
PCR Thermal Cyclers	Amplification of 16S rDNA V4 gene	Thermo Fisher Scientific
Illumina Miseq 2 x 250	Sequencing of 16S rDNA samples	Illumina (provided by Beijing Genomics Institute, HongKong, China)
CFA Evolution II	Measurement of Ammonium and Nitrate content	Alliance Instruments GmbH

2.5. Bioinformatic applications

Table 5: Bioinformatic tools used for microbial 16S rDNA analysis.

Bioinformatic tools	Purpose	Reference
FastQC	Quality check of clean data	www.bioinformatics.babraham.ac.uk
FLASH	Overlap paired-end reads to generate the consensus sequence	Magoč & Salzberg, 2011
USEARCH	Cluster consensus sequence to OTU	Edgar, 2013.
RDP Classifier	Taxonomically classify OTU representative sequences	Cole *et al*., 2009
QIIME	Analyze 16S rRNA gene sequences	Caporaso *et al*., 2010
NCBI	Providing access to 16S rRNA annotation	www.ncbi.nlm.nih.gov/

2.6. 16S rDNA reference databases

Table 6: 16S rDNA reference gene and annotation data used in the analyses.

Database	Purpose	Reference
Greengene (V201305)	Taxonomically classify OTU representative sequences	DeSantis *et al*., 2006

2.7. Services

Library preparation and sequencing of 16S rDNA samples was done by Beijing Genomics Institute (BGI), HongKong, China. The raw data filtered to eliminate the adapter pollution and low quality to obtain clean reads were also done by BGI.

3. Methods

3.1. Stem propagation directly in the field

The field experiment was conducted at the experimental station 'Heidfeldhof' (48.71 °N, 9.19 °E), with planting date and *H. frisingense* inoculation as treatment factors. In 2014 and 2015, mean annual air temperatures were 11.0 °C and 10.9 °C, and precipitation was 654.1 mm and 492.1 mm respectively (weather station 'Hohenheim', LTZ Augustenberg, 2016). The arable soil at the site is a loess-derived stagnic Luvisol with silty loam-texture, total C content of 11.4 g C/kg soil dry weight and pH 6.8. The experiment was conducted in randomized incomplete block design with each treatment replicated three times, which is a total of 12 plots. The field was harrowed before planting. In each 1.5 m × 0.6 m plot, five node segments were planted with 0.33 m spacing between plants within the rows. Stems used in this experiment came from stems with 7–8 nodes on June 3rd, 2014, and stems with 11-12 nodes on July 14th, 2014, respectively.

The first planting was conducted on June 3rd, 2014. For the inoculation treatment, each side of the half of fresh cut node segments was inoculated with *H. frisingense* for 60 minutes (Straub et al., 2013a). The *H. frisingense* inoculated solution was prepared by grown in liquid Luria–Bertani medium, harvested by centrifugation, and resuspended in distilled water to a final OD 600 of ~0.1. Meanwhile, each side of the rest node fragments was inoculated in distilled water for 60 minutes as control. After that, propagules were planted horizontally at a soil depth of 3 cm in the same day. The plots were irrigated twice per week for the first two months after planting. Then, no additional water was given during the whole experimental period. Weeding was conducted several times by hand. The second planting was conducted on July 14th following the same progress.

The establishment success was assessed at the end of the 2014 growing season (10th December 2014), and stems were harvest by cutting 10 cm above the soil surface in mid-March, 2015. Finally the overwintering survival rate was assessed in June, 2015.

Finally, morphological measurements were carried out on 4th December 2015, including plant height, stem number and stem diameter. For the measurements, two strongest plants per plot were selected. Plant height was measured from the soil surface to the node of the uppermost fully expanded leaf on the highest stem of each selected plant. Stem diameter was measured on the same stem between the collar and the first internode. For stem number per plant, all stems with a height of at least 10 cm were counted and the number divided by the planting density. Then plants were harvested and weighed per plots by cutting 10 cm above the soil surface.

3.2. Stem propagation in the greenhouse

Meanwhile, the greenhouse experiment was conducted with node position, planting pattern and *H. frisingense* inoculation as treatment factors. The experiment was conducted in completely randomized design with each treatment replicated three times, including 3 node position (1st, 2nd and 3rd node), 2 planting pattern (vertically and horizontally), and 2 inoculation situations (*H. frisingense* inoculated or not). The greenhouse experiment started on 13th August, when the stems had 13-15 nodes (Figure 7). To simulate marginal lands, nutrient-poor artificial mixture substrates of 20% washed quartz sand (0.6–1.2 mm Ø) and 80% calcareous Loess subsoil (pH: 7.6; C_{org} < 0.3%; N_{total} 0.02%; $CaCO_3$: 23%) were used. For the vertical pattern, 10 node segments were vertically planted with the axillary bud at the soil surface in 18 pots (25 cm diameter), which were filled with 8 kg artificial substrate. In contrast, for the horizontal pattern, 10 node segments were horizontally planted just below the soil surface in trays (50 cm (length) × 30 cm (width) ×

6 cm (height)), which was also filled with 8 kg substrate. The *H. frisingense* inoculation followed the same procedure as in field Experiment. The soil moisture was adjusted daily to 24% (w/w) = 80% substrate water holding capacity (WHC). The greenhouse condition was 16 h/8 h light/dark period and a 25 ℃/20 ℃ day/night temperature during the experiment period. Before sprouting, each pot and tray was covered with a thin transparent foil to avoid water loss that was removed after sprouting, then pots and trays were watered twice per week.

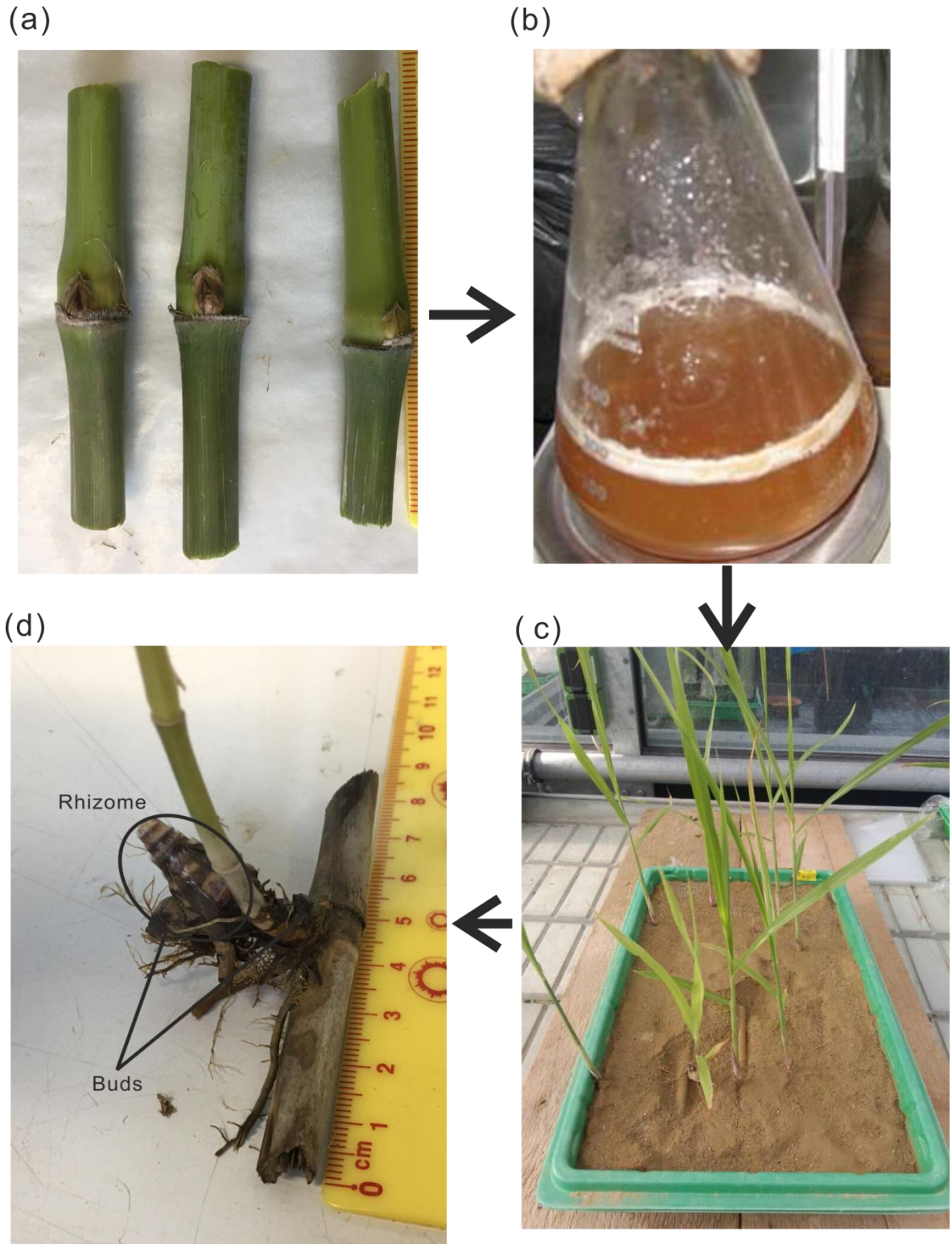

Figure 7: Planting of *M.* ×*giganteus* stem cuttings in greenhouse.

(a) Stem cuttings; (b) *H. frisingense* inoculation; (c) cutting nodes sprouting; (d) *M.* × *giganteus* underground situation after 4 months.

Node sprouting was defined as the elongation of the bud to 1 cm out of the soil surface. The sprouted nodes were counted every day for 35 days. The establishment success, plant height and plant yield were carried out after planting for 4 months. The five strongest plants per tray were selected for morphological measurements. Plant height was measured from the soil surface to the node of the uppermost fully expanded leaf on the highest stem of each selected plant. Then stems were harvest by cutting 2 cm above the soil surface and oven-dried (60 ℃ for 7 days) and weighed for dry matter biomass yield assessment.

The sprouting index was modified from the germination index (Siddiqui & Al-Whaibi, 2014), and estimated according to the following formula:

$$Si = \sum_{i=1}^{t} \frac{St}{Dt} \ (i = 1,2 \dots t) \qquad (1)$$

where *St* is the number of sprouted nodes within the day, *Dt* is the number of sprouting days and t is the total sprouting period (d).

3.3. N fertilizer treatment sites description

Miscanthus × *giganteus* plots located at the University of Hohenheim (Germany) experimental station 'Ihinger Hof' (48.75 ℕ, 8.92 ℍ), which were established in the year 2001 by Boehmel *et al*. (2008). Depending on the FAO classification, the soils were classified as *Haplic Luvisols* with a silty clay texture (approximately 40% clay) and overlying loess loam. When measured in 2002, Soil Ct was about 0.99% of soil and soil Nt was about 0.10%. The field trial was established as a split plot with 3 different N levels (0, 40, and 80 kg $ha^{-1}a^{-1}$) and 4 replicates (180 m^2 each). Fertilizer (ENTEC®, 80 kg N ha^{-1}) was applied in late spring to the N80 plots, while this was omitted in the N0 plots. This long-term experiment and the block design are described in detail in Iqbal *et al*. (2015).

3.4. Sample collection

Sample collection was performed in the middle of July, 2015, when *Miscanthus* grew maximal.

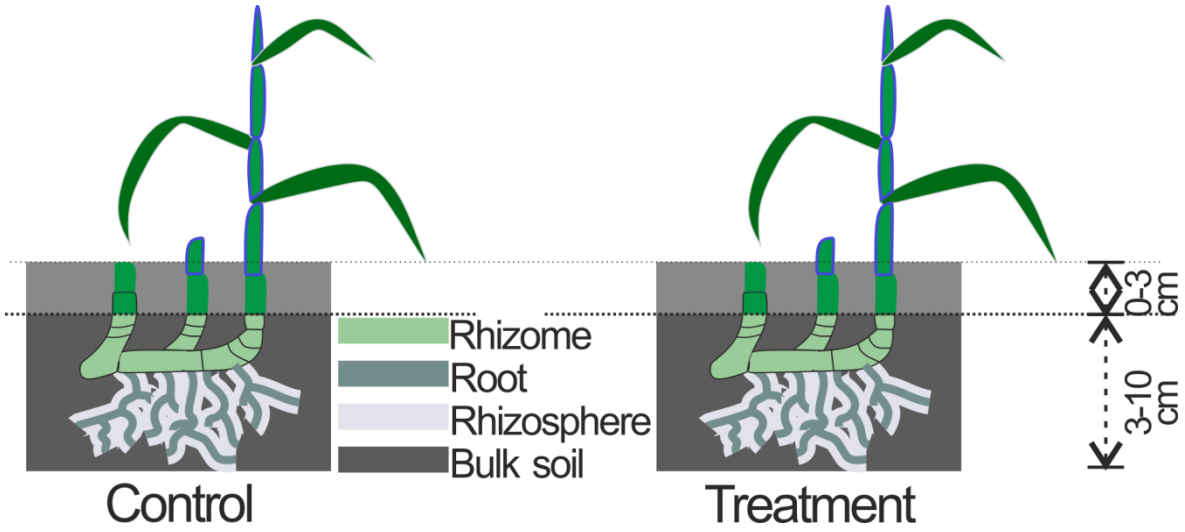

Figure 8: Samples collection of Miscanthus.

Illustration of *Miscanthus* plants depicting the bulk soil, rhizosphere, roots and rhizomes sampled from the top 3-10 cm soil layer. Control, 0 nitrogen fertilizer or without inoculation; Treatment, 80 kg N $ha^{-1}a^{-1}$ application or *H. frisingense* inoculation.

For the *H. frisingense* inoculated trials, bulk soil, rhizosphere, root and rhizome (Figure 8) were sampled from the top 3-10 cm in the soil layer of the *H. frisingense* inoculated plots and the control plots in the field experiment respectively. For per treatment, all the three replicates were sampled twice, and combined into two composite samples. Adding with two different planting date treatment as replicates, that is a total of 16 samples.

For the N fertilizer treatment trials, four replicates of bulk soil, rhizosphere, root and rhizome (Figure 8) were sampled from the low nitrogen fertilizer level (N0) and high

nitrogen fertilizer level (N80) respectively. These four replicates were mixed and pooled, yielding two composite samples. Adding with two different N fertilizer treatments as replicates, that is also a total of 16 samples.

The top 3-10 cm soil with at least 5 cm distance from surrounding *Miscanthus* root was crushed and sieved through a 2-mm mesh in the field for collection of corresponding bulk soil samples. 100g bulk soil per treatment was used to measure the Nmin (nitrate and ammonium) by continuous-flow analysis technique (only for the N fertilizer trials). Loose soil was manually removed from the roots by gently shaking with sterile gloves. Soil tightly adhering to the roots was defined as rhizosphere soil sample (Lavecchia *et al.*, 2015). The bulk soil samples and rhizosphere soil samples were stored at -20℃ for subsequent DNA extraction. Root samples were standardised by taking the fresh lateral roots. Roots and rhizome from the top 3-10 cm in the soil for each independent block were vigorously washed with sterilized deionized water and sonicated in order to remove all soil from the root surface. The washing steps were repeated twice to avoid soil contamination in the root type samples. Subsequently, rhizome and roots were separated with sterilized scissors. The rhizome and root samples were gently dried with clean soft tissue, immediately frozen in liquid nitrogen and stored at -80℃ for downstream DNA extraction.

3.5. Sample DNA extraction and sequencing

PowerSoil DNA isolation kit (MoBio, Carlsbad, CA) was used to extract DNA from 300 mg soil sample or 100 mg plant samples following the manufacturer's instructions, with an extending vortex mixing period for 25 min. After quantified with the Thermo Scientific Nanodrop 2000c Spectrophotometer, the DNA samples with about 800 ng DNA per sample were sequenced by Beijing Genomics Institute (BGI, China). Sequencing libraries were constructed by the BGI, including barcodes and adaptors. The qualified library was

amplified with 250 bp paired-end amplicon sequencing on the Illumina Miseq 2 X 250 paired-end platform. Briefly, 16SrRNA V4 region was amplified with universal prokaryotic primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (TAATCTWTGGGVHCATCAGG) as in Caporaso *et al*. (2012).

3.6. Sequence classification and bioinformatics analysis

The raw sequence reads were filtered to obtain clean data by the procedure showed in Fadrosh *et al.* (2014). Two paired-end reads overlapping to the consensus sequence were generated by FLASH (v1.2.11) and chimeras were filtered out with UCHIME (v4.2.40). Finally, 625.403 consensus sequences with average length of 252 bp were obtained in the total 32 samples. Then sequences were clustered into organismal tauxonomic units (OTUs) with a 97% threshold by using UPARSE and the taxonomy was assigned with Ribosomal Database Project (RDP) Classifier v.2.2, with reference to the Greengenes database V201305. By removing OTUs assigned to the plant chloroplast and mitochondria, a matrix containing OTU read counts and taxonomic assignments for all samples was generated for further taxonomic analysis.

Before analyses, rare OTUs represented by ≤5 reads in ≤ 3 samples, were filtered out from the OTU table, according to the modified procedure of Gottel *et al*. (2011). The OTU relative abundance was calculated by dividing the absolute abundances by the total sequence counts per sample. Fold change tests and false discovery rate (FDR)-corrected Student's *t*-Test were conducted to compare the treatment effects (*H. frisingense* inoculation or N fertilizer) on relative abundance in a given plant fraction, respectively. The enriched and depleted relative abundances of bacterial taxa among soil-plant fractions

were determined based on fold changes (≥ 2).

Principal coordinates analysis (PCoA) was performed to visualize the sample relations using package 'ape' of software R (v3.1.1), based on the Bray–Curtis similarity matrix calculated by the QIIME. The effects of different factors on the bacterial communities were tested using permutational multivariate analysis of variance (PerMANOVA) with R package 'vegan'.

Venn diagrams visually display the number of common/unique OTUs in multi-samples. The core microbiomes of different environments were based on the OTU abundance and Venn diagrams were drawn by VennDiagram of software R (v3.1.1).

Species heat map analysis was done based on the relative abundance of each species in each sample. To minimize the differences degree of the relative abundance value, the values were all log10 transformed. If the relative abundance of certain species was less than 1%, it was pooled in the low abundance fraction. If the relative abundance of a certain species was 0, the minimum abundance value was substituted by -2.3, which means 0.005% relative abundance. Heat maps were generated using the package 'gplots' of software R(v3.1.1) and the distance algorithm 'euclidean', with the clustering method 'complete'.

The Shannon diversity index was estimated as the diversity at family level with R package 'vegan'. The Tukey's post-hoc test was conducted to compare the Shannon diversity among all treatments. Taxonomy histograms were created with the software R (v3.1.1). All taxonomic groups less than 0.5 % were combined in the 'low-abundance' category. Differences at the family level were tested by the Student's *t*-Test. Raw bacterial 16s rDNA sequencing data were deposited at the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA575500 (*H. frisingense* inoculation) and PRJNA527123 (N fertilizer), respectively.

3.7. Microscopy

A GFP-tagged *H. frisingense* GSF30^T strain (Rothballer et al., 2008) was used in the greenhouse experiment and plant material was observed by confocal microscopy (Leica DMRE microscope equipped with a confocal head TCS SP; Leica, Wetzlar, Germany).

3.8. Statistical analysis

Data analysis was performed using the software R (v3.1.1). The effects of different factors on the yield biomass and plant traits were tested using two-way ANOVA with R and Tukey's post-hoc tests were used to identify significantly different treatments. As establishment rate and survival rate in each trial are binomially distributed, a generalized linear model (glm) was performed in R using the link function "logit". The sprouting percentages were simulated using the following logistic model (Chatterjee *et al.*, 2000; Lattin *et al.*, 2003):

$$Y = \frac{c}{1+e^{a-b*X}} \qquad (2)$$

where *c*, *a* and *b* are variables to be estimated.

4 Results

4.1. Inoculation with *Herbaspirillum frisingense* promoted the establishment and yield of *M.* × *giganteus* stem-cuttings in the field

We first tested whether *H. frisingense* inoculation under field conditions and the planting date (June or July) affect the establishment of *M.* × *giganteus* from nodes. The field establishment success was quantified twice, in December 2014 and after the winter in June 2015 (Figure 9). In detail, single nodes of *M.* × *giganteus* stem cuts were established in the field by direct horizontal planting and *H. frisingense* significantly promoted the establishment (Table 7), no matter whether planted in June or July (Figure 9). After the first year, the second year's establishment and survival rate were similar as in the first year (Figure 9b). When harvested at the end of the second year, the biomass yield per plant was mostly affected by the planting date, but *H. frisingense* inoculation also had an effect (Table 1). Biomass was significantly higher when planted in July and with this planting date substantially affected by inoculation (Figure 9c). When considering major plant traits that affect biomass yield: plant stem number, plant height and stem diameter, all of them were significantly affected by the planting date. By contrast, only plant height was also significantly affected by *H. frisingense* inoculation (Table 7). Furthermore, the stem number per plant was significantly bigger when nodes planted in July 2014, compared to nodes planted in June, while no significance between control and *H. frisingense* inoculation was observed (Figure 9d). The similar trend was also found in stem diameter (Figure 9f). The plant height was increased by *H. frisingense* inoculation only at the second plantation date (Figure 9e). In short, *H. frisingense* inoculation significantly promoted establishment of *M.* × *giganteus* nodes from cut stems in the field, with the planting date

was the main factor determining the second year's yield of *M.* × *giganteus*, while the second important factor was *H. frisingense* inoculation.

Table 7: Field establishment statistics analysis.

Logistic regression and overall ANOVA for the significance of the main effects and their interactions (*Hf* inoculation, planting date, *Hf* *date) on yield and plant traits for *Miscanthus* × *giganteus* stem cutting culture in field trials (a = 0.05).

	Establishment rate		Biomass yield		Stem number		Height		Diameter	
	z-value	Pr>|z|	F-value	Pr>F	F-value	Pr>F	F-value	Pr>F	F-value	Pr>F
Hf	3.045	0.002	6.53	0.021	0.53	0.479	28.53	<0.001	3.24	0.091
Date	0.576	0.564	123.8	<0.001	108.1	<0.001	94.57	<0.001	13.95	0.002
Hf × Date	0.576	0.564	3.80	0.069	0.16	0.692	5.78	0.029	0.11	0.743

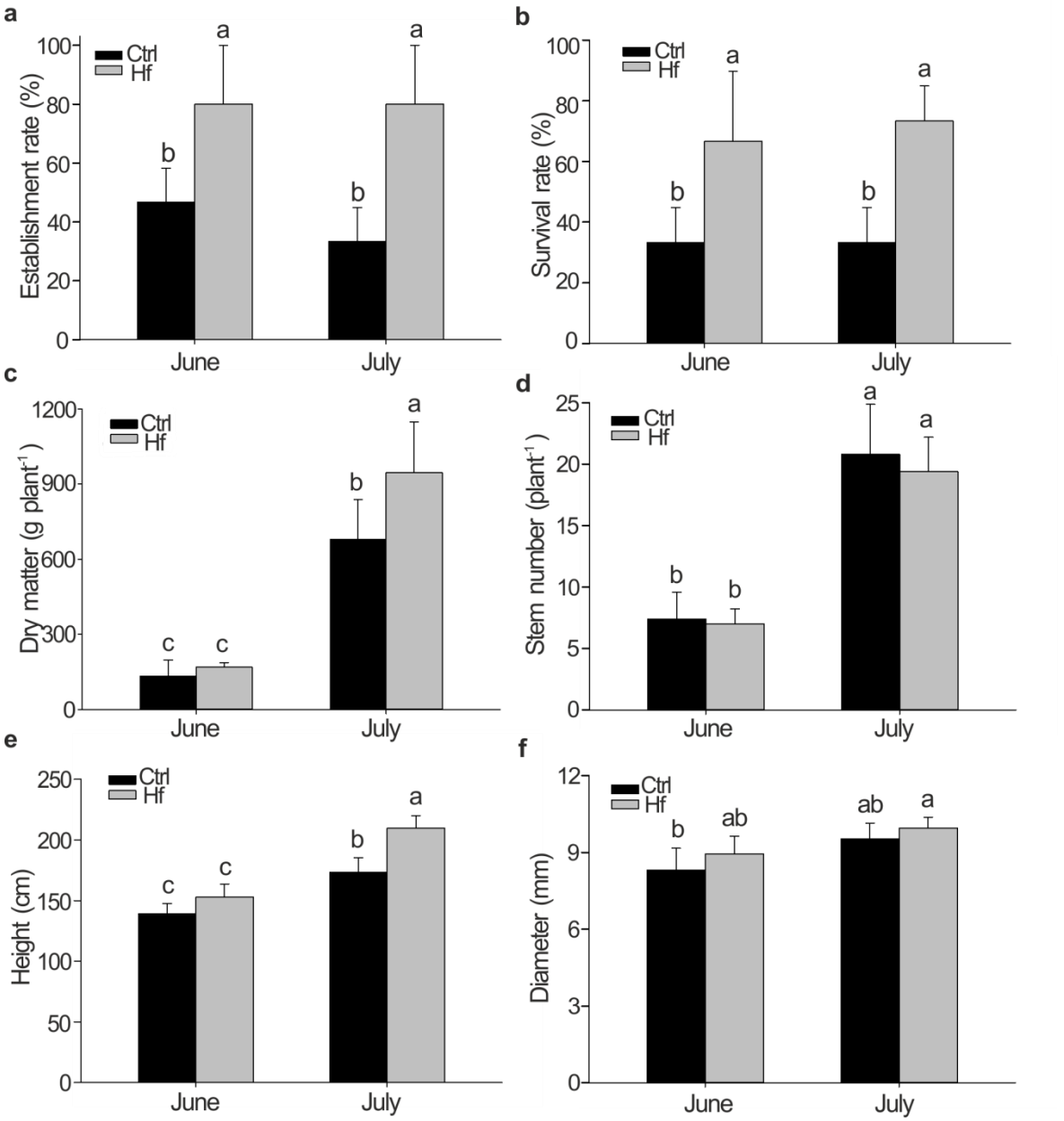

Figure 9: Field establishment results.

Establishment rate in the first year (a), survival rate in the second year (b), dry matter yield [g plant^{-1}] (c), stem number (d), height (cm) (e) and stem diameter (mm) (f) from stem cuttings with one node planted directly into the field at two dates (June and July 2014). Significant differences are indicated by different lower-case letters. Level of significance was a = 0.05. Error bars represent standard deviation in different plant traits. Ctrl, control treatment; Hf, *Herbaspirillum frisingense* inoculation; June, stem planted in June, 2014;

July, stem planted in July, 2014.

4.2. Sprouting and plant growth of *M.* × *giganteus* stem-cuttings in the greenhouse from inoculation with *Herbaspirillum frisingense*

The effect of *H. frisingense* inoculation on the emergence and establishment of *M.* × *giganteus* shoots from different node positions was investigated in greenhouse conditions. Only the first three nodes counted from the base of the plant were considered. Stems were harvested in August. Emergence of *M.* × *giganteus* from vertical and horizontal plantings was also compared, but as emergence from vertically placed stem-cuttings was extremely poor, only horizontally planted stem cuttings were considered in the following analysis.

Overall, nodes started to sprout after 8 days up to 30 days (Figure 10). Both *H. frisingense* and node position had significant effect on the sprouting progress, which showed that base node 1 sprouted fastest, followed by node2 and node 3, while *H. frisingense* promoted the sprouting especially of node 3 (Figure 10, Figure 11b).

The greenhouse establishment success determined was only affected by *H. frisingense* inoculation (Table 8). *H. frisingense* significantly promoted the establishment rate for both node1 and node2, but not of node3 (Figure 11a). When harvest after 4 months of growth, the aboveground biomass yield per plant of the cultured nodes was notably affected by both node position and *H. frisingense* inoculation (Table 8). It was significantly higher with inoculation and notably decreased from node1 to node3 (Figure 11c). A similar trend was found for plant height (Figure 11d), which was also affected by both node position and *H. frisingense* inoculation (Table 8).

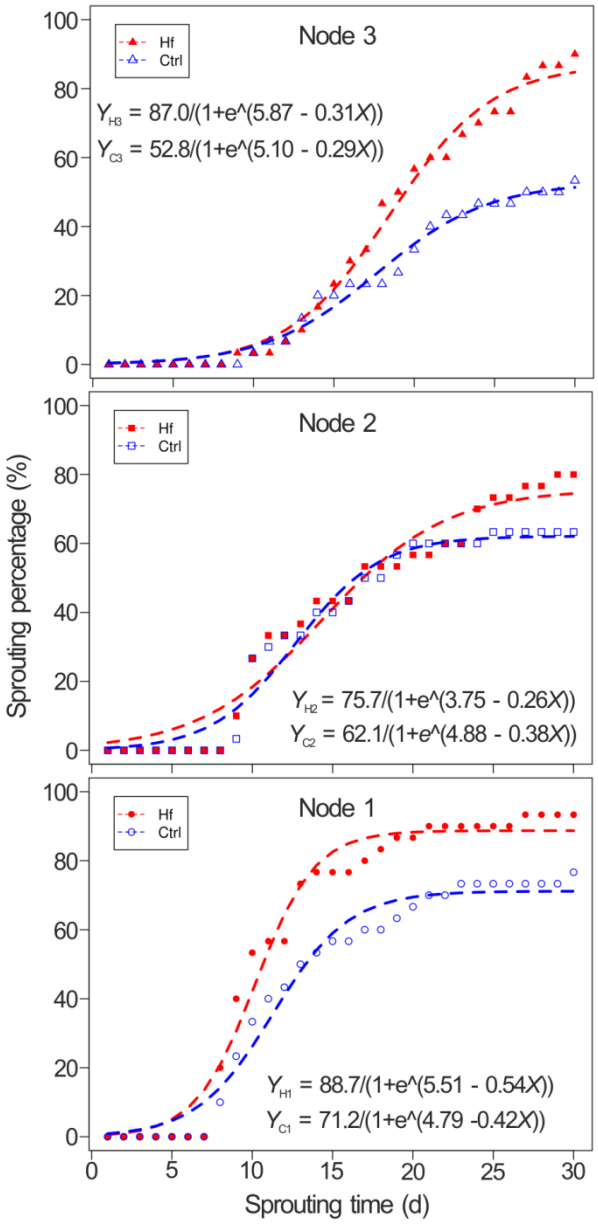

Figure 10: Sprouting percentage from the three most basal nodes planted in the greenhouse.

Sprouting percentage from the three most basal nodes planted in the greenhouse and dependence on *Herbaspirillum frisingense* (red) and controls (blue). Fits to the logistic model (see methods) were significant at Pr<0.001. Ctrl, control treatment; Hf, *Herbaspirillum frisingense* inoculation; Node1, basal node segment closest to the soil surface; Node2, node segment closest to Node1 upward; Node3, most apical node; H1,

inoculated Node1; C1, uninoculated Node1; H2, inoculated Node2; C2, uninoculated Node2; H3, inoculated Node3; C3, uninoculated Node3.

Table 8: Greenhouse establishment statistics analysis.

Logistic regression and overall ANOVA for the significance of the main effects and their interactions (*Hf* inoculation, node, *Hf* * node) on yield and plant traits for *Miscanthus* × *giganteus* stem cutting culture in greenhouse trials (a = 0.05).

	Establishment rate		Sprouting index		Dry matter yield		Height	
	z-value	Pr>|z|	F-value	Pr>F	F-value	Pr>F	F-value	Pr>F
Hf	2.243	0.025	10.32	0.007	37.98	<0.001	75.45	<0.001
Node	-0.217	0.828	20.32	<0.001	31.05	<0.001	23.16	<0.001
Hf × Node	0.981	0.107	1.44	0.275	12.94	0.001	10.75	0.002

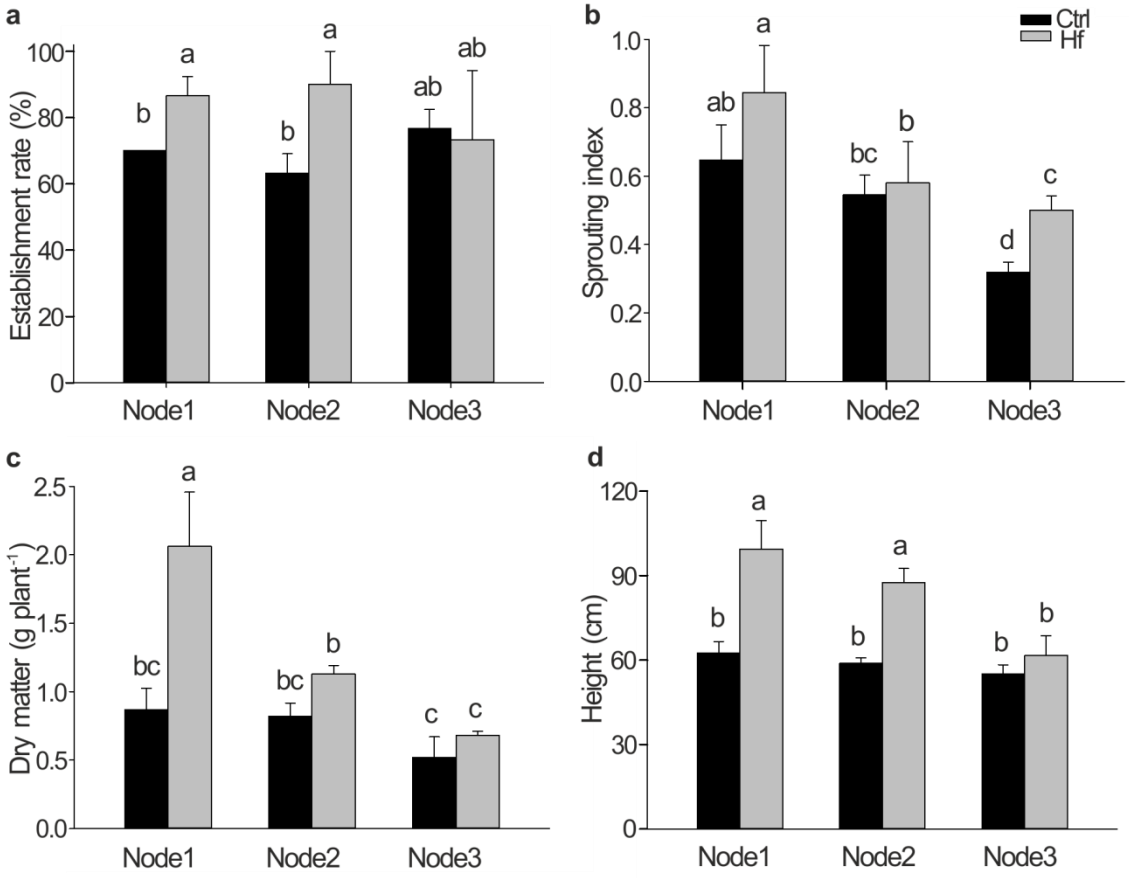

Figure 11: Greenhouse establishment results.

Establishment rate (a), sprouting index (b), dry matter [g plant^{-1}] (c), and height (cm) of 3 different cutting nodes planted in the greenhouse with *Herbaspirillum frisingense* inoculation (gray bars) or not (black bars). Significant differences are indicated by different lower-case letters. Level of significance was a = 0.05. Error bars represent standard deviation in different plant traits. Ctrl, control treatment; Hf, *Herbaspirillum frisingense* inoculation; Node1, node segment closest to the soil surface; Node2, node segment closest to Node1 upward; Node3, node segment closest to Node2 upward.

The localization of *H. frisingense* after sprouting from nodes was investigated using a GFP-labelled *H. frisingense* strain with confocal microscopy. The fluorescence of bacteria was primarilly detected as aggregates of fluorescent spots close to the veins of the first and

second leaves closest to the node. Very poor fluorescence was found in the roots, in agreement with the low abundance of *H. frisingense* in the root microbiome (Figure 12). Further leaves were again little colonized, suggesting that the competence to colonize and distribute within *M.* × *giganteus* was restricted.

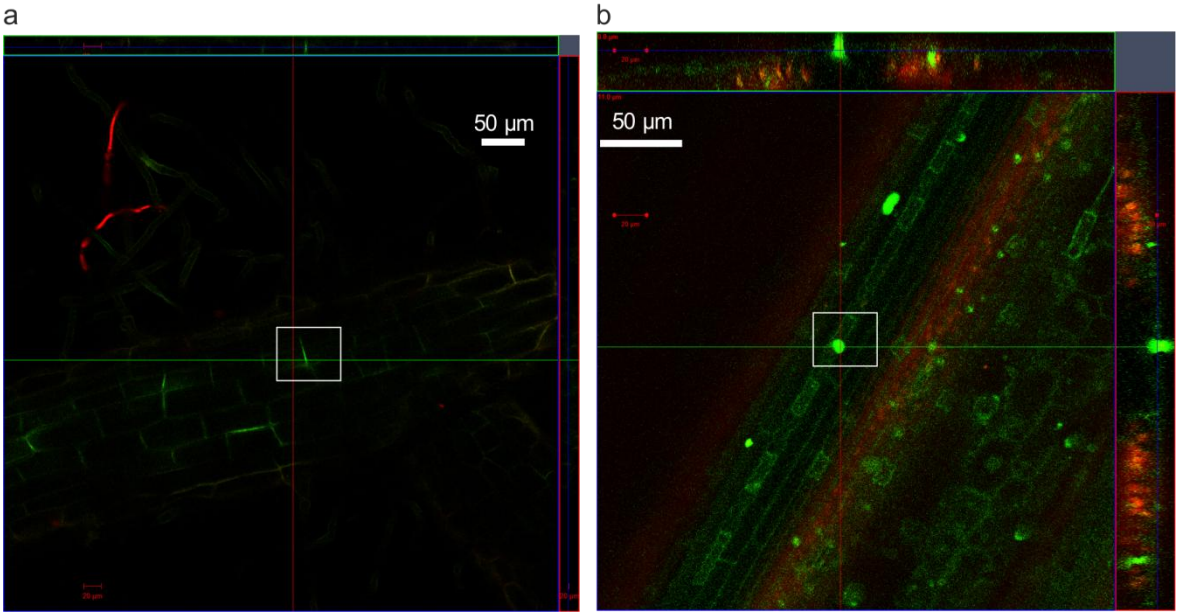

Figure 12: Presence of GFP-labelled *H. frisingense* in *M.* ×*giganteus* roots and leaves.

Green fluorescent spots in *M.* × *giganteus* roots (a) and veins of *M.* × *giganteus* leaves (b). The white square highlights potential aggregates.

4.3. Long-term effect of *Herbaspirillum frisingense* inoculation on soil and plant microbiota in the field

Bacterial 16S rDNA was extracted from bulk soil, rhizosphere, root and rhizome (Figure 8) from the above stem propagated *Miscanthus* field experiment in early summer two years after establishment. Stem cuttings used for propagation had either been inoculated with *H. frisingense* at the time of establishment time or not. Principal coordinates analysis (PCoA) showed that exophyte (soil and rhizosphere) and endophyte (root and rhizome) compartments were distinct in the first coordinate (PerMANOVA: R^2 = 0.44, p value =

0.001), while different plant organs (lateral roots and rhizome) were also separated (PerMANOVA: R^2 = 0.78, p value = 0.036) (Figure 13a). Overall, the *H. frisingense* effect was very small, as may have been expected from the long time between inoculation and sampling. However, there was potentially a minor influence of *H. frisingense* on endophytic bacterial communities (PerMANOVA: R^2 = 0.14, p value = 0.33) in contrast to the exophyte compartments (PerMANOVA: R^2 = 0.03, p value = 0.8). The Shannon diversity indices significantly decreased from soil samples (bulk soil and rhizosphere) to the rhizome, while similar Shannon indexes were found between inoculated sample and control (Figure 13b).

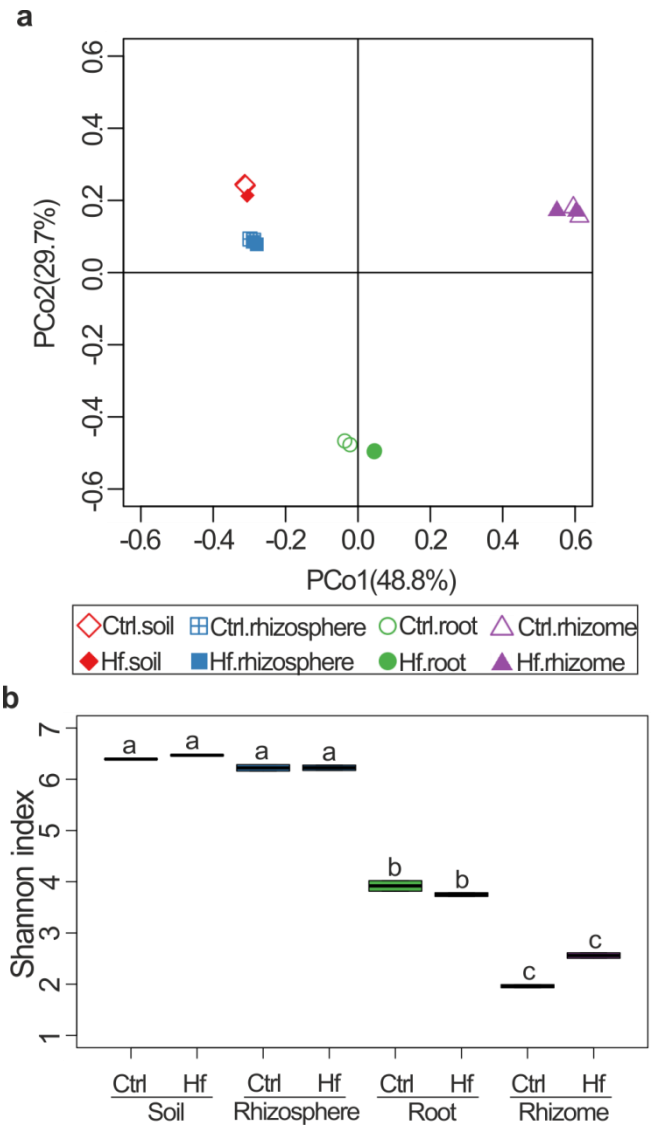

Figure 13: The effect of *Herbaspirillum frisingense* inoculation on the diversities of *Miscanthus* × *giganteus*-associated bacterial communities.

(a) Principal coordinates analysis (PCoA) of pairwise, Bray-Curtis distances between samples based on relative abundance of OTUs. (b) Shannon diversity is estimated as the OTU abundance. Significant differences are indicated by different lower-case letters. Level of significance was a = 0.05. Ctrl, control treatment; Hf, *Herbaspirillum frisingense* inoculation

The relative abundance of different bacterial taxa in the soil-endosphere fractions is displayed in histograms at the phylum level (Figure 14). The major phyla were *Proteobacteria, Acidobacteria, Verrucomicrobia, Actinobacteria* and *Bacteriodetes*. While similar taxonomic compositions were found between *H. frisingense* inoculated and controls in bulk soil, rhizosphere and root, *H. frisingense* inoculation significantly changed the bacterial composition in the rhizome, although *H. frisingense* was always detected only at residual levels, irrespective of the compartment. In rhizomes, the *Proteobacteria* were enriched and *Actinobacteria* depleted (Figure 15). Furthermore, *Pseudomonadaceae* (13.8%) and *Xanthomonadaceae* (10.8%), which both comprise many plant growth promoting *Proteorhizobacteria*, as well as *Oxalobacteraceae* (0.8%, identified as the beneficial *H. frisingense*) were enriched by *H. frisingense* inoculation (Figure 15c), while the family *Promicromonosporaceae* (6.3%), which is known to contain opportunistic pathogens, in *Actinobacteria* was depleted (Figure 15b).

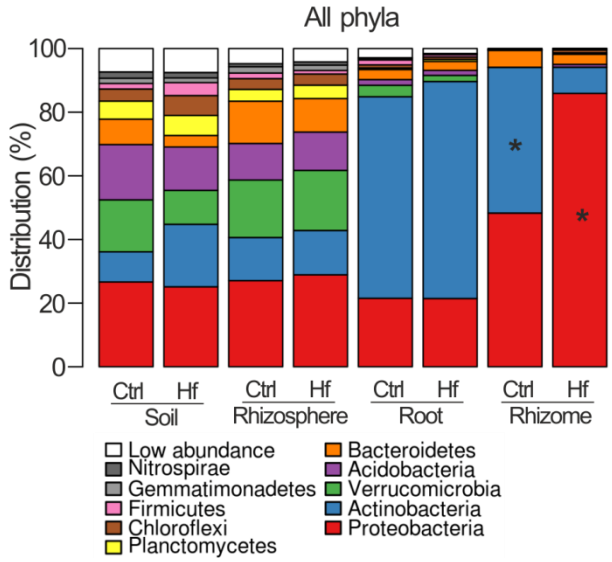

Figure 14: The effect of *Herbaspirillum frisingense* inoculation on the composition of *Miscanthus* × *giganteus*-associated bacterial communities.

Histograms showing the distribution of phyla present in bulk soil, rhizosphere, roots and rhizomes between *Herbaspirillum frisingense* inoculation and control. Asterisks indicate a significant difference using Student's t-test. Ctrl, control treatment; Hf, *Herbaspirillum frisingense* inoculation

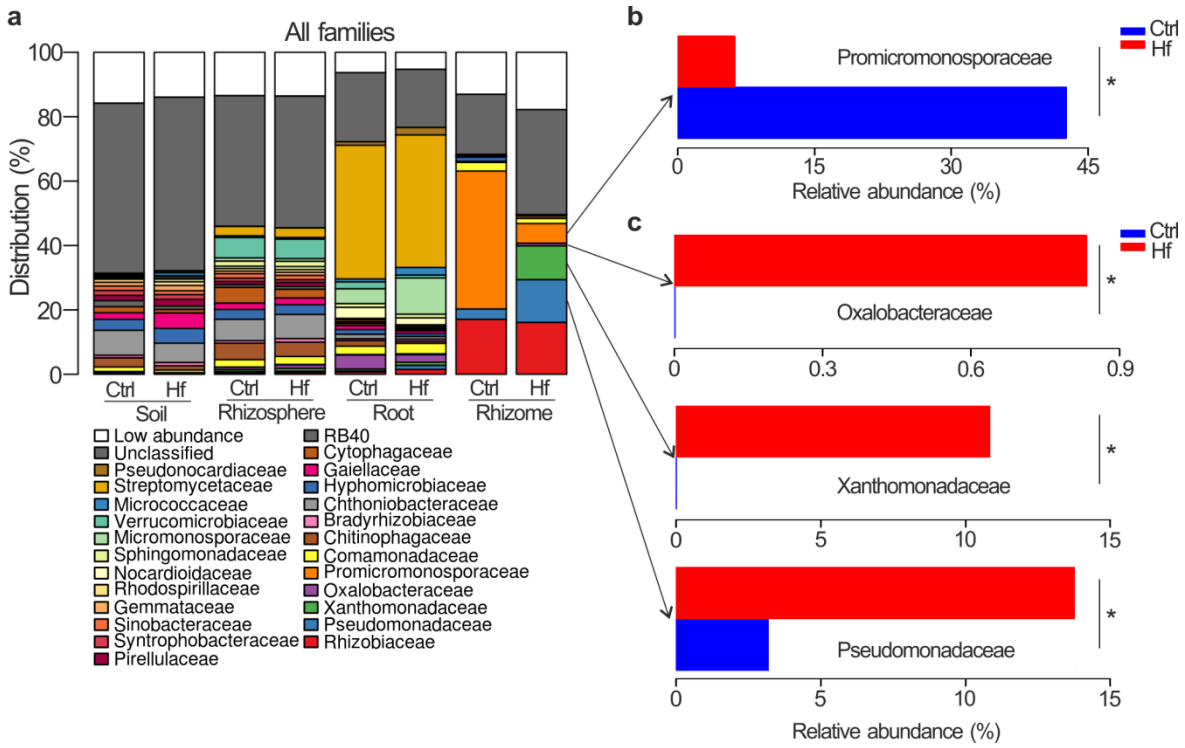

Figure 15: *Herbaspirillum frisingense* inoculation enriched *Proteobacteria* and depleted *Actinobacteria* in the rhizome of *M.* ×*giganteus.*

(a) Histograms showing the distribution of families present in bulk soil, rhizosphere, roots and rhizomes between *H. frisingense* inoculation and control. Distinct families depleted in inoculated relative to control rhizomes in *Actinobacteria* (b) and enriched in *Proteobacteria* (c). Asterisks denote significantly enriched families between inoculated and control rhizomes according to paired Student's t-test (*, $P < 0.05$). Ctrl, control treatment; Hf, *Herbaspirillum frisingense* inoculation.

4.4. Long-term effect of N fertilizer regimes on *M.* ×*giganteus* underground bacterial communities

Bacterial 16S rDNA was extracted from bulk soil, rhizosphere, root and rhizome from a 14-year old long term *Miscanthus* field experiment. Plants did either receive no N or were

fertilized with 80 kg $ha^{-1}a^{-1}$ nitrogen fertilizer. This resulted in almost 6-fold lower N_{min} levels in the N0 plots (2.88 mg ha^{-1}), compared to 16.4 mg ha^{-1} in N80 plots at the time of harvest, respectively, with nitrate levels substantially higher than those of ammonium (Table 9).

Table 9: Soil mineral nitrogen (N_{min}) for the 3–10 cm soil in the sampling sites.

Nmin	N rate (Kg N $ha^{-1}a^{-1}$)	
	0	80
NO_3-N (mg kg^{-1})	1.84	11.2
NH_4-N (mg kg^{-1})	1.04	5.20
Nmin (mg kg^{-1})	2.88	16.4

After sequencing, the sequence abundances from the four different compartments and two N-levels (resulting in a total of 8 sample types) were compared by conducting principle coordinates analysis (PCoA), using the resulting operational taxonomic unit (OTU) table from the QIIME program based on the v4 conserved region of the bacterial 16S rDNA. The PCoA showed that the *Miscanthus* exosphere (both bulk soil and rhizosphere soil) was separated from the root and rhizome, reflecting the distinct environmental conditions for bacterial populations (Figure 18). The first principle coordinate was dominated by the compartment type, containing exophyte and endophyte (PerMANOVA: R^2 = 0.39, p value = 0.002 **), while the second principle coordinate reflected, the different plant organ types, lateral root and rhizome (PerMANOVA: R^2 = 0.41, p value = 0.021 *). Overall, the nitrogen fertilizer effect was small, N0 fertilizer and N80 fertilizer treatment data clustered closely together. Meanwhile, the relationship between fertilizer and exophytic bacterial communities (PerMANOVA: R^2 = 0.20, p value = 0.19) was less pronounced than that for

endophytic bacterial communities (PerMANOVA: R^2 = 0.25, p value = 0.065). Taken together, PCoA showed that in the long-term nitrogen fertilizer application, bacterial taxonomic patterns were mainly imposed by compartment type and plant organ type, but not by the distinct nitrogen fertilizer amount.

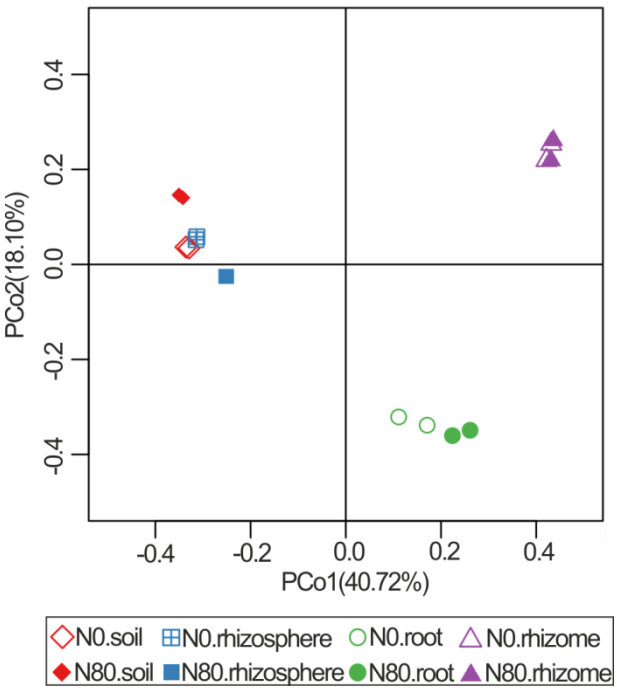

Figure 16: Soil compartment and plant organ type drive the microbial community composition of Miscanthus.

Principal coordinate analysis (PCoA) of pairwise, Bray-Curtis distances between samples based on relative abundance of OTUs. N0, no nitrogen fertilizer application; N80, 80 kg N $ha^{-1}a^{-1}$. Different symbols are color coded by fraction: soil, diamond; rhizosphere, square; root, circle, and rhizome, triangle. N80, solid; N0, hollow.

Using the 5 × 3 threshold, we identified 763 OTUs defined as general units of microbial taxonomic classifications under the N0 condition and 862 OTUs under the N80 condition (Figure 17). In both nitrogen fertilizer conditions, the OTU number reduced significantly from bulk soil to the rhizome, while about 62% of the endosphere OTUs were also found

in soil. However, both roots and rhizome showed specific OTUs (24 % in roots and 20 % in rhizome respectively), which were not detected in bulk soil and rhizosphere soil.

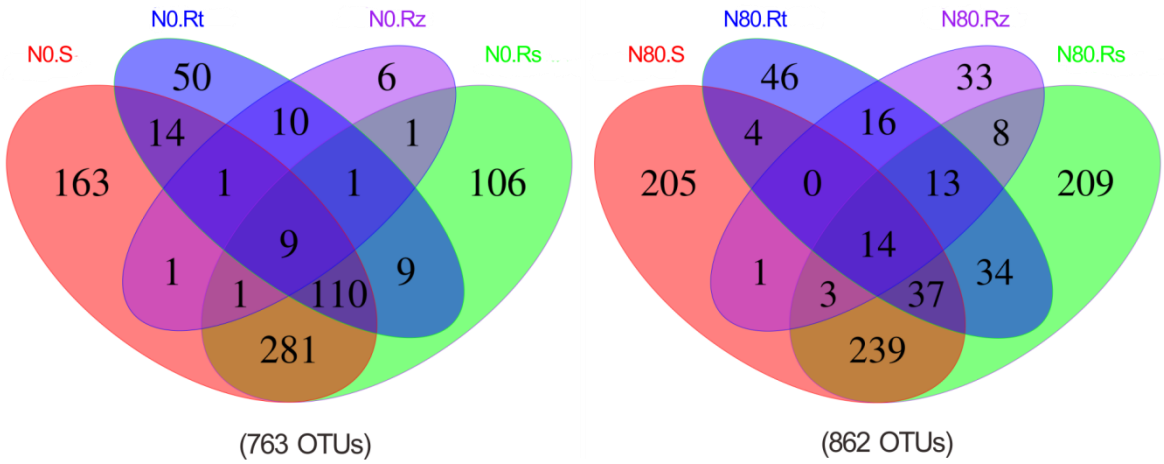

Figure 17: OTU differences among different fractions.

Numbers of shared and differentially distributed bacterial operational taxonomic units (OTUs) in bulk soil, rhizosphere, *M.* × *giganteus* roots and rhizomes, in N0 and N80. N0, no nitrogen fertilizer application; N80, 80 kg N $ha^{-1}a^{-1}$ application; S, bulk soil; Rs, rhizosphere; Rt, *Miscanthus* root; Rz, *Miscanthus* rhizome.

To access how the different soil-plant fractions and the nitrogen fertilizer level influence the taxonomic distributions of bacterial communities, the relative abundance of the 16s rDNA OTU tables in each taxanomic level was quantified after log10 transformation. Euclidean distance clustering of the data in a heatmap at the phylum level (Figure 18) and family level (Figure 19) indicated three distinct bacterial composition patterns. In both, bulk soil and rhizosphere, the major phyla were *Proteobacteria*, *Acidobacteria*, *Verrucomicrobia*, *Actinobacteria* and *Bacteriodetes*. The *Proteobacteria*, *Acidobacteria*, *Actinobacteria* and *Bacteriodetes* were mainly identified in the root samples. Finally, the rhizome was dominated by *Proteobacteria*, *Actinobacteria* and *Bacteriodetes* (Figure 18b).

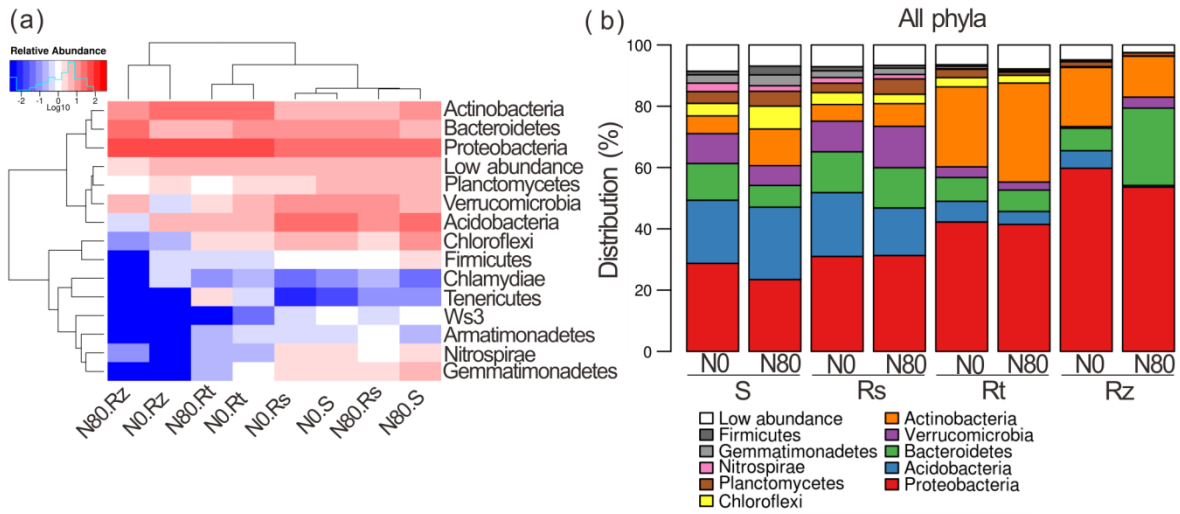

Figure 18: Bacterial phyla composition and distribution among different fractions.

(a) Heat map showing the distribution of bacterial phyla in the different fractions from log_{10}-transformed relative abundances. Samples were clustered on their Euclidean distance. The color key relates colors to the transformed relative abundance. (b) Histograms showing the distribution of phyla present in bulk soil, rhizosphere, roots and rhizomes. N0, no nitrogen fertilizer application; N80, 80 kg N $ha^{-1}a^{-1}$ application; S, bulk soil; Rs, rhizosphere; Rt, *Miscanthus* root; Rz, *Miscanthus* rhizome.

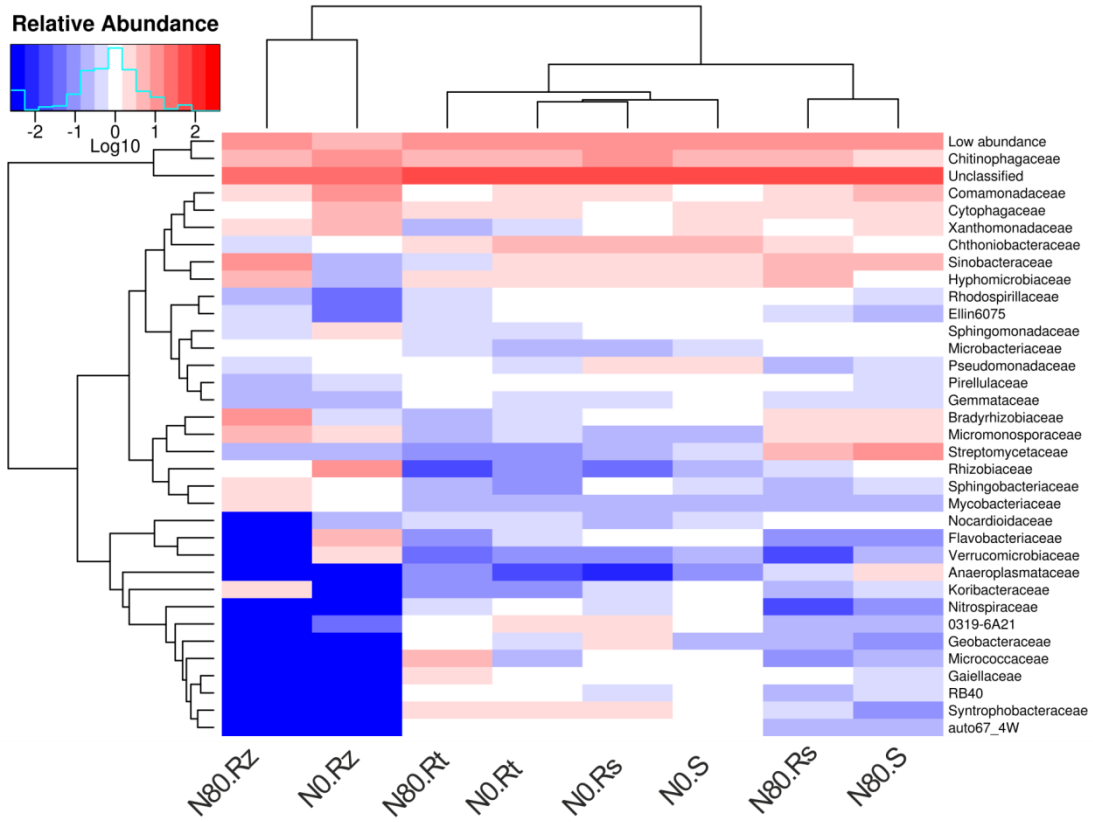

Figure 19: Heat map showing the bacterial samples relationship in the family level.

Samples and taxa are clustered on their Euclidean distances. The key relates colors to the transformed family relative abundance. N0, no nitrogen fertilizer application; N80, 80 kg N $ha^{-1}a^{-1}$ application; S, bulk soil; Rs, rhizosphere; Rt, *Miscanthus* root; Rz, *Miscanthus* rhizome.

To better understand the bacterial diversity and composition in the 16S rDNA data, first of all, we applied fold-change tests to assess whether OTUs enrichment or depletion occurred among other fractions, which were then compared to the bacterial OTUs in the bulk soil. The amount of depleted OTUs in the rhizome covered all depleted OTUs in the root, which also covered most of depleted OTUs in the rhizosphere, in both the compartment-depleted OTUs of N0 and N80 (Figure 20a, b), indicating a gradual filtering towards the plant compartments. However, the compartment-enriched OTUs of both N0 and N80 were

different among the rhizosphere, root and rhizome samples, respectively, with only a very small subset shared between them (Figure 20c, d). This suggests different preferences for the different OTU groups, which are driven by distinct environmental habitat properties.

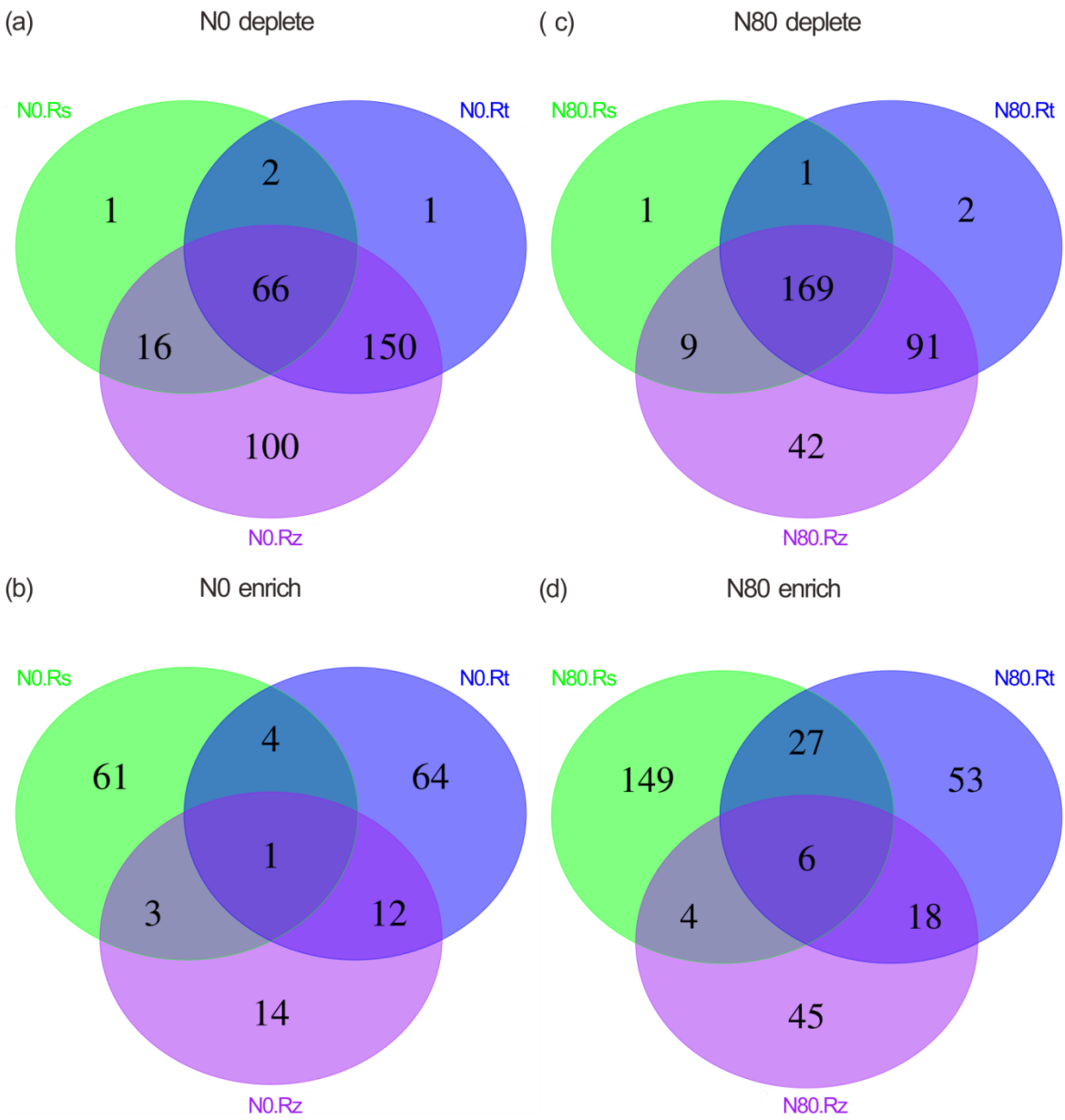

Figure 20: Compartment factor depleted and enriched bacterial OTU.

Numbers of depleted and enriched bacterial operational taxonomic units (OTUs) in the *M.* × *giganteus* rhizosphere, roots and rhizomes compared to the bulk soil. (a) Two-fold depleted and (b) enriched OTUs in N0; (c) Two-fold depleted and (d) enriched OTUs in

N80. N0, no nitrogen fertilizer application; N80, 80 kg N $ha^{-1}a^{-1}$ application; S, bulk soil; Rs, rhizosphere; Rt, *Miscanthus* root; Rz, *Miscanthus* rhizome.

Furthermore, the relative abundance of different bacterial taxa in all the soil-endosphere fractions is displayed in histograms at the phylum (Figure 21) and family level (Figure 22). For this analysis, the N0 and N80 samples were pooled together. Root-enriched OTUs mainly belonged to the phyla of *Proteobacteria* and *Actinobacteria,* while rhizome-enriched OTUs belonged to *Proteobacteria*, *Bacteriodetes* and *Actinobacteria*. Conversely, the phyla distribution of both the root-depleted OTUs and rhizome-depleted OTUs included *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteriodetes, Chloroflexi* and *Verrucomicrobia*, which resemble taxa typical of the soil fraction. The Shannon diversity of the root and rhizome fractions were 4.4 and 3.5, respectively, significantly lower than that of the soil fractions (5.7), which is consistent with the enrichment of a subset of dominant phyla.

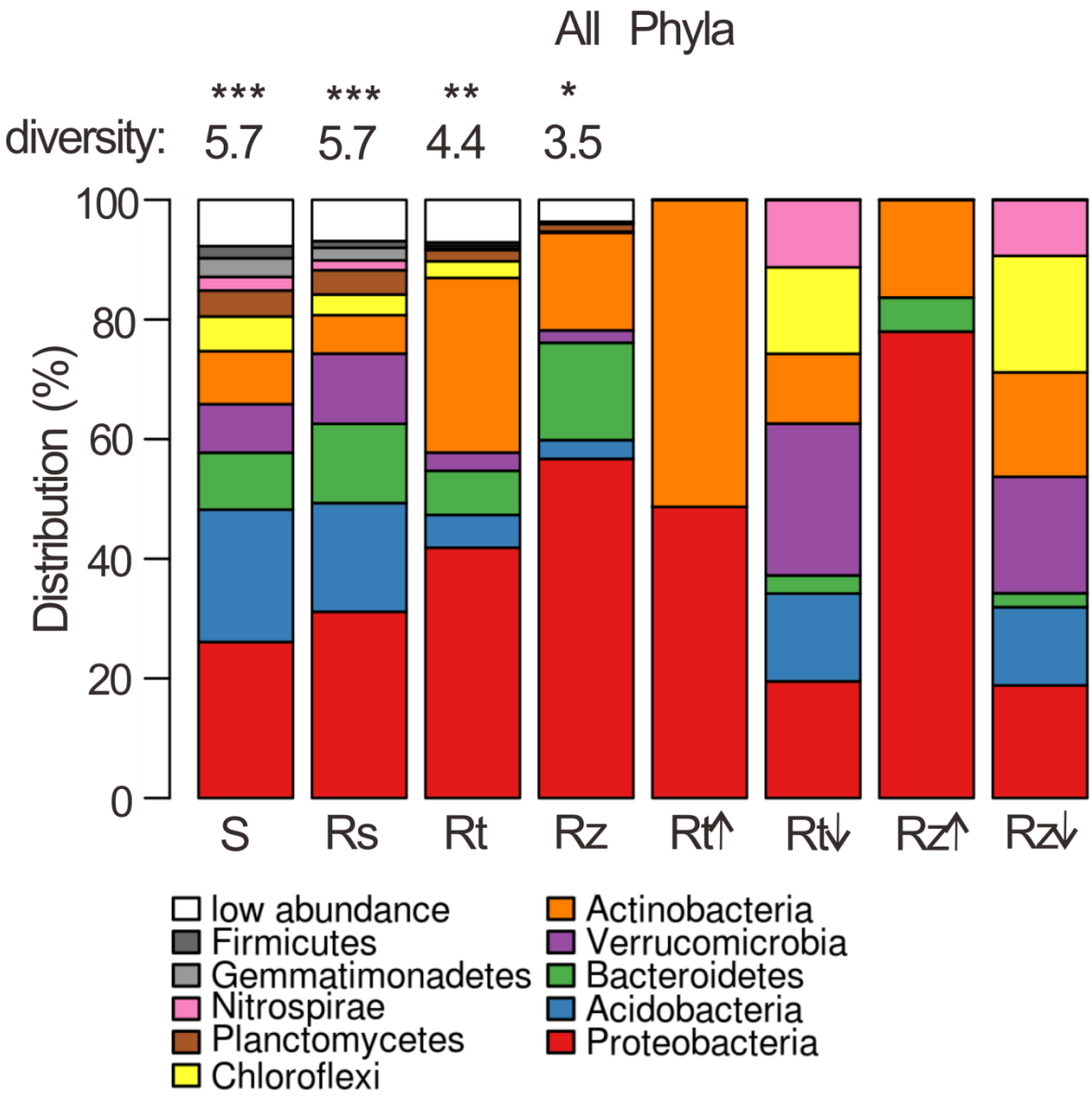

Figure 21: Differentiation of the *Miscanthus* ×*giganteus*-associated bacterial communities in phylum level.

Histograms showing the distributions of phyla present in the families in bulk soil, rhizosphere, root and rhizome compared with phyla present in the subset of root and rhizome families enriched (Rt↑ and Rz↑) or depleted (Rt↓ and Rz↓) relative to soil. Shannon diversity (considering phyla as individuals) is given above each bar. Letters above the diversity values represent significant differences ($p < 0.05$).

We were most interested in the endosphere-enriched phyla, predominantly *Proteobacteria* and *Actinobacteria*, as these are candidate phyla for the *Miscanthus* growth promotion with respect to N fixation. Family taxonomic analysis demonstrated that the enrichment of *Actinobacteria* in the root and rhizome was mostly due to *Streptomycetaceae* and *Micromonosporaceae* in the root, and *Mycobacteriaceae* and *Microbacteriaceae* in the rhizome (Figure 22a).

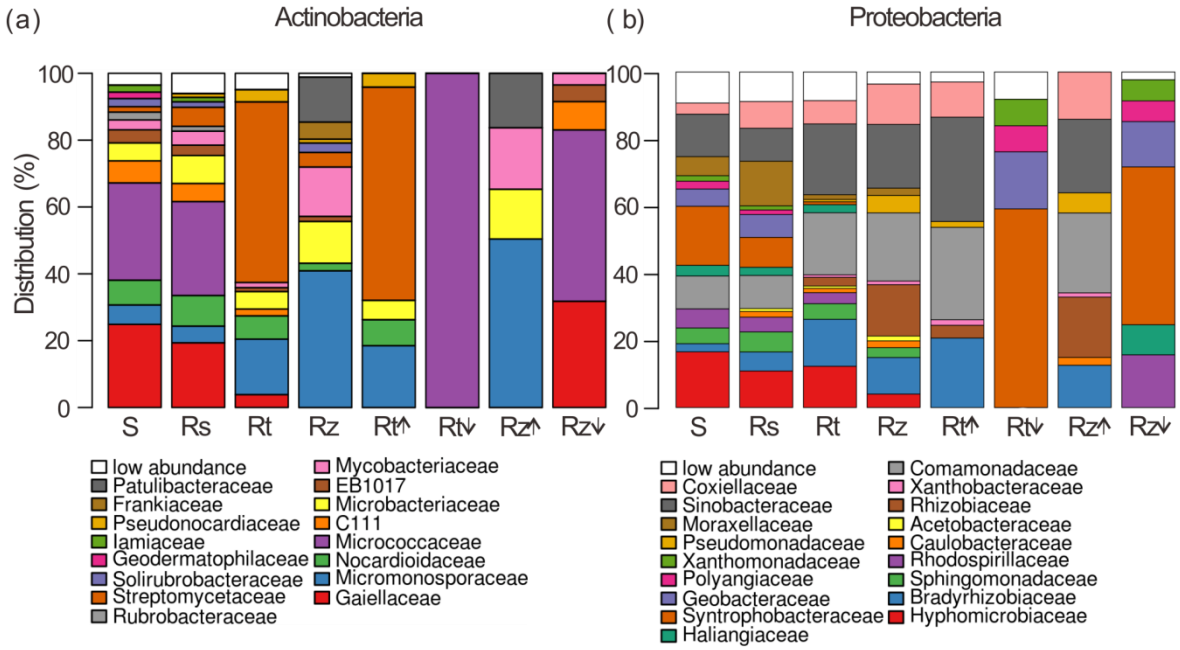

Figure 22: Distribution of families in the phylum *Actinobacteria* and *Proteobacteria*.

Distribution of families in the phylum Actinobacteria (a) and Proteobacteria (b). S, bulk soil; Rs, rhizosphere; Rt, *Miscanthus* root; Rz, *Miscanthus* rhizome.

The relative complex family changes in the *Proteobacteria* are shown in four domain classes in histograms (Figure 23). In the root fraction, *Bradyrhizobiaceae* dominated the enriched families in the *Alphaproteobacteria* class, while *Comamonadaceae* dominated enriched families in the *Betaproteobacteria*. Furthermore, *Sinobacteraceae* and

Xanthomonadaceae dominated enriched families in the *Gammaproteobacteria* class. In the rhizome fraction, a similar enrichment and depletion profile as in the root was found, except for one enriched *Alphaproteobacterial* family, the *Rhizobiaceae*. Compared to the bulk soil and rhizosphere, where the main phyla remained relatively stable, the plant fractions (root and rhizome) significantly filtered and reestablished a special subset of the bacterial communities from the soil pool.

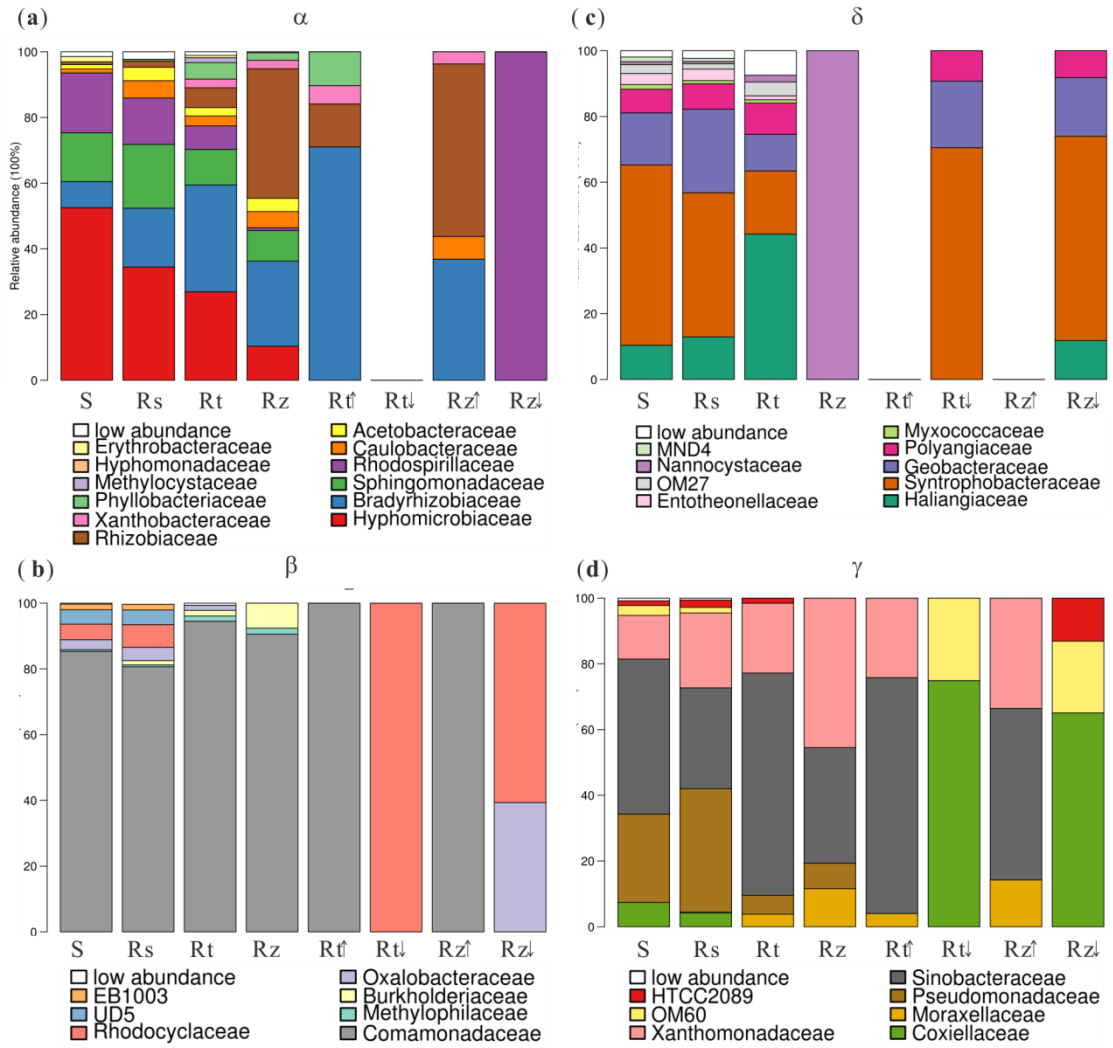

Figure 23: Histograms showing the distribution of families of four classes of the phylum *Proteobacteria*.

Alphaproteobacteria (α), Betaproteobacteria (β), Detaproteobacteria (δ) and Gammaproteobacteria (γ), respectively showing in figure a, b, c and d. Root and rhizome

compared with phylum *Proteobacteria* present in families enriched (Rt ↑ and Rz ↑) or depleted (Rt ↓ and Rz ↓) relative to bulk soil. N0, no nitrogen fertilizer application; N80, 80 kg N ha-1a-1 application; S, bulk soil; Rs, rhizosphere; Rt, Miscanthus root; Rz, Miscanthus rhizome

4.5. Long term nitrogen fertilizer application enriched and depleted bacterial taxa

We then applied a fold-change test to assess the enrichment and depletion of some taxa with respect to the two nitrogen fertilizer levels, from the bulk soil to the rhizome. A subset of OTUs was distinct between the two nitrogen fertilizer levels in each fraction, which is shown in Venn diagrams (Figure 24). Intriguingly, among the different fractions, there were only few shared OTUs, indicating that the nitrogen fertilizer had distinct effects in each compartment. Even though nitrogen fertilizer had only a minor effect on the phyla distributions between the N0 and N80 samples, we noted that several families differed in soil-plant fractions. Interestingly, these mainly belong to the phyla *Proteobacteria* and *Actinobacteria* (Figure 25a).

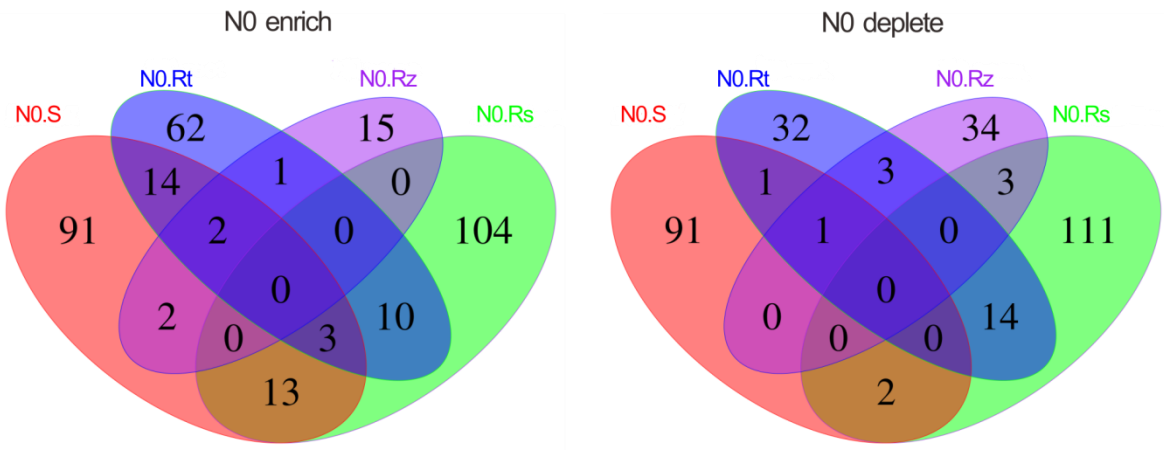

Figure 24: OTUs that differentiate the N0 microbiome from the N80 microbiomes of different compartments.

Numbers of differentially enriched and depleted bacterial operational taxonomic units (OTUs) in *M.* × *giganteus* bulk soil, rhizosphere, roots and rhizomes in N0 relative to N80, based on 2-fold change. N0, no nitrogen fertilizer application; N80, 80 kg N $ha^{-1}a^{-1}$ application; S, bulk soil; Rs, rhizosphere; Rt, *Miscanthus* root; Rz, *Miscanthus* rhizome.

We then classified the enriched and depleted *Proteobacteria* families, which are thought to comprise the most important bacteria to the nitrogen fixation. The relative complex family changes in the *Proteobacteria* are shown in histograms (Figure 25b) and significantly enriched and depleted families in the phylum *Proteobacteria* by N fertilizer are shown in Table 10.

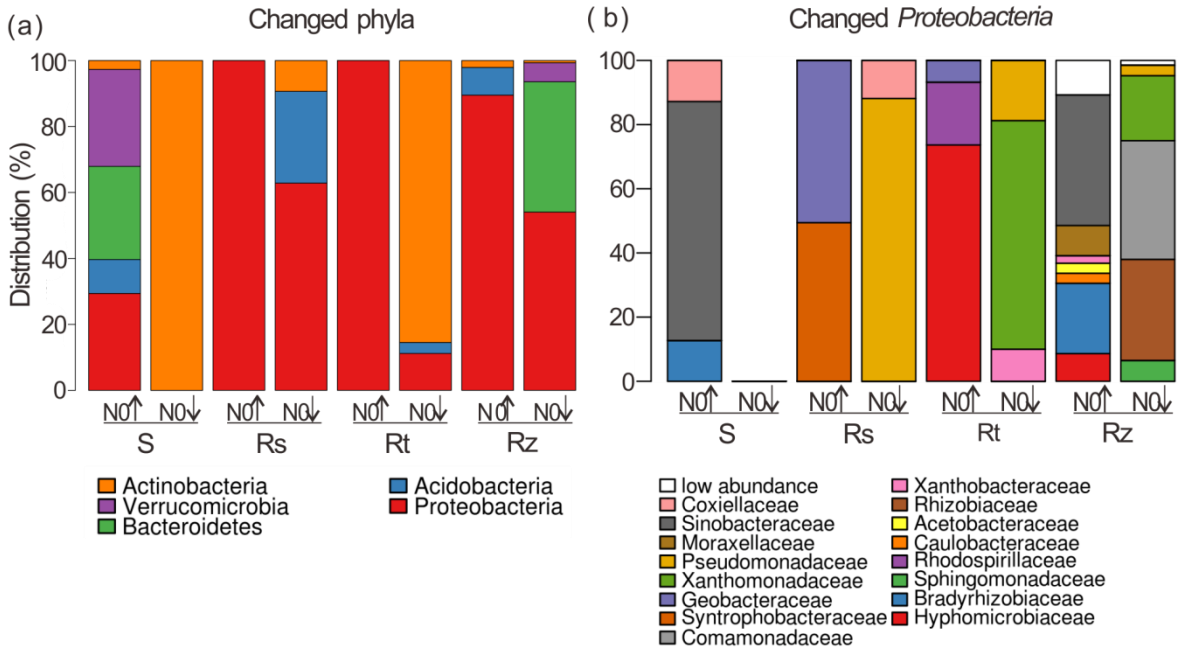

Figure 25: Distribution of N enriched and depleted phyla and families.

(a) Histograms showing the distributions of enriched (N0↑) or depleted (N0↓) phyla present in the families in soil, rhizosphere, root and rhizome in zero nitrogen fertilizer (N0)

relative to N80. (b) Distribution of enriched and depleted families in the phylum *Proteobacteria*. N0, no nitrogen fertilizer application; N80, 80 kg N $ha^{-1}a^{-1}$ application; S, bulk soil; Rs, rhizosphere; Rt, *Miscanthus* root; Rz, *Miscanthus* rhizome.

In order *Alphaproteobacteria*, the putative nitrogen fixing-associated *Hyphomicrobiaceae* (up to 3.9% in roots and 2.8% in the rhizome, respectively) were enriched in the root and rhizome in N0, while *Bradyrhizobiaceae* (0.5% in soil and 7.0% in the rhizome) were enriched in the bulk soil and rhizome, respectively. However, another putative nitrogen fixing family, the *Rhizobiaceae* (9.9% in N80), mainly consisted of the tumor-pathogen genus *Agrobacterium*, was depleted in the rhizome under N0. When considering the order *Betaproteobacteria*, only the putative denitrifier family *Comamonadaceae* (11.6% in N80) was depleted in the rhizome in N0. Of the *Deltaproteobcacteria*, intriguingly, both the putative nitrogen fixing family *Syntrophobacteraceae* (2.2%) and the *Geobacteraceae* (2.2%) were enriched in the N0 rhizosphere, respectively. Finally, from the *Gammaproteobacteria* the putative plant pathogen-associated family *Xanthomonadaceae* was depleted in the root (2.4% in N80) and rhizome (6.4% in N80) in the N0 condition. However, the *Pseudomonadaceae*, that include plant nitrogen fixing and denitrifying species, as well as some that are involved in disease and pathogen protection, was widely depleted in all fractions in the N0 condition, except for the bulk soil. At last, the *Sinobacteraceae*, putatively involved in ammonia oxidation, were enriched in the bulk soil (2.9% in N0) and rhizome (13.0% in N0) in the N0 condition.

In total, the N0 fertilizer condition significantly enriched putatively nitrogen-fixing bacteria among all fractions compared to the N80 fertilizer condition, 0.5% versus 0.2% in soil, 4.4% versus 1.2% in the rhizosphere, 5.3% versus 2.0% in the roots, and 9.8% versus 0.7% in the rhizome, while depleted putative denitrifiers in the rhizome, 3.0% versus

12.6%.

Table 10: Functional microbial family abundances in the *Proteobacteria* in soil-plant fractions.

Class family	Putative function	Average relative abundance (%)							
		soil		rhizosphere		root		rhizome	
		N0	N80	N0	N80	N0	N80	N0	N80
Alphaproteobacteria									
Hyphomicrobiaceae	N-fixing					3.9*↑	1.4	2.8*↑	0.2
Bradyrhizobiaceae	N-fixing	0.5*↑	0.2					7.0*↑	0.5
Rhodospirillaceae	N-fixing					1.0*↑	0.4		
Rhizobiaceae	Plant pathogen							0.8	9.9*↑
Betaproteobacteria									
Comamonadaceae	Denitrifier							2.5	11.6*↑
Detaproteobacteria									
Syntrophobacteraceae	N-fixing			2.2 *↑	1.0	0.4 *↑	0.2		
Geobacteraceae	N-fixing			2.2 *↑	0.2				
Gammaproteobacteria									
Xanthomonadaceae	Plant pathogen					0.7	2.4*↑	2.0	6.4*↑
Pseudomonadaceae	Denitrifier			1.6	3.2*↑	0.2	0.6*↑	0.5	1.0*↑
Sinobacteraceae	AMO, N-fixing?	2.9*↑	0.7					13.0*↑	0.0
Summary									
	N-fixing	0.5	0.2	4.4	1.2	5.3	2.0	9.8	0.7
	Denitrifier			1.6	3.2	0.2	0.6	3.0	12.6

Only families that occupied more than 0.5 % and that were significantly shifted in relative abundance of the entire bacterial composition are shown. Asterisks and arrows represent significant enrichment (*Student t-Test*: $p < 0.05$). AMO, ammonia monooxygenase; N-fixing, nitrogen fixing. N0, no nitrogen fertilizer application; N80, 80 kg N $ha^{-1}a^{-1}$.

5 Discussion

In this research, *M.* × *giganteus* propagated from stem nodes was identified to profit from inoculation with the diazotrophic *H. frisingense* both in the field and greenhouse. Meanwhile, the *Miscanthus* × *giganteus* associated microbiomes were charactered in different soil-endosphere compartments both from a long-term growth experiment with two nitrogen fertilizer levels and from a 2 year growth trial established by stem propagation with *H. frisingense* inoculationwith amplicon sequencing.

5.1. Node position and planting date influence establishment of *M.* × *giganteus* stem-cuttings

Our greenhouse studies identified almost no establishment success with vertically placed stems, while about 80% establishment was achieved with the horizontal pattern from nodes 1 to 3. Node position only very little affected the establishment (Figure 11a). The independence of node position is consistent with Boersma *et al*. (2012), but not with Hong & Meyer (2011). Hong & Meyer (2011) reported using vertical planting pattern that the most basal node exhibited the greatest establishment success; establishment decreased for nodes 2–4. Boersma *et al*. (2012) using horizontal planting found no significant difference in shoot emergence from nodes 1 to 5 with high sprouting success. Problems with the establishment from vertical cuttings was mainly attributed to quickly deterioration, especially under warm, dry field conditions (Xue *et al*., 2015). We also found this placement highly sensitive to environmental stress.

Even though ultimately the horizontal positioning did not affect final establishment success, the node position significantly affected the sprouting speed and sprouting index (Figure 10, Figure 11a). Besides more reserves in the lower nodes (Jones & Walsh,

2001), sprouting is likely affected by internal phytohormones, such as abscisic acid and shoot tip-derived auxin, as indicated by the linear decrease in sprouting index from node1 to node3 (Thimann & Skoog, 1933; Boersma *et al*., 2012). The notably higher sprouting index led to a significantly higher plants and increased dry biomass per plant, which is important for overwintering (Figure 11, Table 8).

In the field, *M.* × *giganteus* stem-cuttings are rarely well established, so that costly pre-growth under controlled conditions and then transplantation into the field was recommended (Boersma *et al*., 2014a; Boersma *et al*., 2014b; Xue *et al*., 2015). However, direct planting of stem cuttings had been successfully established in Ireland, with stem segments of 1.8 m length at a depth of 10 cm (O'Loughlin *et al*., 2017; O'Loughlin *et al*., 2018). Though our field experiment indicated that plants can directly be established from horizontal single node segments of only 7-8 cm length, we suggest to testing this promising method in more adverse environmental conditions.

The field establishment from stem cuttings did not differ between the two planting dates (June 4rd and July 14th), but the final harvested biomass, plant height, stem number per plant and stem diameter were strongly affected. The better success at the later date may simply be related to the more mature stem and node material. Hong & Meyer (2011) demonstrated that stem cuttings planted in September exerted significantly worse establishment compared to July, while O'Loughlin *et al*. (2018) found that stem cuttings directly planted in October and even juvenile stem cuttings harvested in April were successfully established in the field. However, high establishment success in Germany was from late July to late August (Jones & Walsh, 2001). Here, stem cuttings directly planted in June and July successfully overwintered, and the greenhouse experiment confirmed that four months of growth is enough for secondary shoot emergence.

5.2. *Herbaspirillum frisingense* inoculation influenced establishment of *M.* ×*giganteus* stem-cuttings

In the greenhouse, as well as in the field, *H. frisingense* inoculation significantly promoted establishment, sprouting index, plant biomass and plant height. However, no significant effects on stem numbers per plant and stem diameter were observed (Figure 9, Figure 10 and Figure 11). The sprouting index was mainly determined by the node emergence, which is promoted by cytokinins (Hirimburegama and Gamage, 1995). The *H. frisingense* genome encodes the whole pathway to synthesize the plant hormone cytokinin (Straub *et al*., 2013b). The establishment success is crucially determined by successful rooting and comparative work with sugarcane and bamboo suggests that root development from nodal stem cuttings is induced by auxin (Ramanayake et al., 2006). Even though *H. frisingense* produces indoleacetic acid (IAA) (Rothballer *et al*., 2008; Straub *et al*., 2013b), Straub *et al*., 2013c found that *H. frisingense* inoculation slightly repressed *IAA* genes in *Miscanthus sinensis*. Clearly, the mechanism of establishment promotion by *H. frisingense* (Table 7, Table 8) remains unclear and requires further research.

The growth promotion by *H. frisingense* (Figure 9 and Figure 11) resembles previous results from inoculated *Miscanthus sinensis* seeds (Straub *et al*., 2013c). ACC degradation by bacterial ACC deaminase may be a common strategy that affects plant growth. *H. frisingense* produces ACC deaminase, may reduce plant-available ethylene and hence promote plant growth (Contesto *et al.*, 2008; Rothballer *et al.*, 2008; Straub *et al*., 2013c). Intriguingly, if the large, two-fold higher establishment improvement in the *H. frisingense* inoculated stems in the field is due to ACC deaminase, this may indirectly protect plants from various stresses and indirectly improve establishment success (Glick, 2004).

5.3. Compartment type and plant organ type influence *M.* ×*giganteus* associated microbiota

The PCoA analysis showed the similar compartment pattern for microbiota in both the N fertilizer treatment and *Herbaspirillum frisingense* inoculation treatment trials, which indicated environment fractions (the endophytic compartments and exophytic compartments), as well as plant organs (root and rhizome) were the main factors to separate the different microbiota. Meanwhile, the Shannon diversity showed a similar pattern. These are consistent with the well-accepted view that the compartment type plays a key role in determining the structure and composition of bacterial communities; different plant organs recruit different microbial communities (Berg and Smalla, 2009; Lundberg *et al*., 2012; Johnston-Monje *et al*., 2016; De Souza *et al*., 2016; Yu *et al*., 2018). Meanwhile, bulk soil and rhizosphere samples clustered together in the PCoA plot and shared a similar shannon-diversity and relative abundance in both phylum and family profile. These results support that the rhizosphere associated microbiome is mainly dependent on the soil type, such as pH, soil moisture, organic matter and C/N ratio (Lundberg *et al*., 2012; De Souza *et al*., 2016; Reinhold-Hurek *et al*., 2015). The nitrogen level had very mild effects on the bacterial communities in the soil, consistent with previous analyses (Babin *et al*., 2019).

However, the endophytic compartment recruited a different microbial pattern and serves as a distinct niche for only a fraction of the bacteria found in the soil and rhizosphere. A similar core microbiome was found in the endophytic compartment for different treatments. Compared to the exophytic compartment, the two phyla *Proteobacteria* and *Actinobacteria* were significantly enriched in the endophytic compartments. Previous work on the endophytic bacterial communities of different immunity related *Arabidopsis thaliana* mutants and the endophytic fungal community of maize under different P fertilization

indicated that the endophytic microbial communities were mainly dependent on plant factors, mainly plant defense system, which selects against detrimental microbes (Lebeis *et al.*, 2015; Yu *et al.*, 2018). We had recently observed that inoculation of the endophytic bacterium *Herbaspirillum frisingense* GSF30^T induced plant defense signaling in *Miscanthus*, including the jasmonate signaling pathway and the ethylene pathway (Straub *et al*., 2013c). An OTU representing *Herbaspirillum frisingense* was identified, but it comprised only a very minor fraction of the exo- and endophytic bacteria, which is inconsistent with a major role of this bacterium in growth stimulation of *Miscanthus* (Straub *et al*., 2013c).

The plant organ type, root or rhizome, played a major further determinant role in the structure and composition of the internal bacterial communities, which was apparent in the second principal coordinate of PcoA. Both lateral roots and rhizome samples mainly recruited *Proteobacteria*, *Actinobacteria* and *Bacteriodetes*, while the roots had significantly higher Shannon-diverstiy and harbored more *Acidobacteria*. It is now well-established that different plant organs, including roots, stalks, leaves, seeds and even flowers recruit different microbial communities in sugarcane, maize, rice, *Arabidopsis* and grape (Lundberg *et al*., 2012; Paszkowski *et al*., 2013; Johnston-Monje *et al*., 2014; Zarraonaindia *et al*., 2015; De Souza *et al*., 2016). Fungal community distribution even diverged among different parts of the same maize organ, lateral and axial roots (Yu *et al*., 2018). Different filtering of bacterial communities by extra plant compartment (the rhizosphere) and further by the plant organs, supplying different environmental niches, is probably responsible for these properties of the communities (Hardoim *et al*., 2008; Reinhold-Hurek *et al*., 2015). The different bacterial communities between root and rhizome of *Miscanthus* are indicative of different physiological niches of these belowground compartments.

Intriguingly, even though the endophytes in the *M.* × *giganteus* rhizome, which has an outstanding rigid outer surface, are expected to derive mainly from the root, there were still some OTUs predominantly identified in the rhizome. It is possible that these dicectly are propagated and derived from the rhizome itself, as the sterile *M.* × *giganteus* is usually propagated via the rhizome. In non-sterile *Miscanthus varieties*, bacterial vertical transmission is accomplished via seeds, and the endophytes of the next generation are significantly affected by these seed-borne endophytes (Cankar *et al.*, 2012; Hardoim *et al.*, 2012). On the other hand, rhizosphere bacteria may be derived from the plant. Using GFP-tagged bacteria, it was found that rhizosphere bacteria may derive from plant endophytes when injected into the maize stem, indicating that vegetative organs are a source of additional bacteria (Johnston-Monje *et al.*, 2011).

5.4. Microbiota of *M.* ×*giganteus* with respect to previous inoculation

In the inoculated experiment, the microbiomes that were associated with *M.* × *giganteus* in soil, rhizosphere, root and rhizome did not show much indication of the previous *H. frisingense* inoculation, but this may be expected from the late sampling after more than one year after inoculation.

The PCoA analysis showed that *H. frisingense* inoculation had very small effects on the bacterial communities in the soil and rhizosphere, which is consistent with the endophytic lifestyle of *H. frisingense* (Kirchhof *et al*., 2001).Only in the rigid rhizome, which is well-protected from environmental influences, *H. frisingense* enriched the *Proteobacteria* and depleted the *Actinobacteria*, while *H. frisingense* itself was little abundant. The shifts in the rhizome community towards *Pseudomonadaceae* and *Xanthomonadaceae,* which are often associated with beneficial plant growth promoting functions, and the suppression of *Promicromonosporaceae*, in which opportunistic

pathogens are found, strongly suggests that inoculation positively affected the community structure towards a "healthier" community. Low root colonization is consistent with fluorescent screening with a GFP-tagged strain, but suggests that certain leaves are a preferred niche for this bacterium. Poor colonization with the GFP-tagged *H. frisingense* strain was also found in *M. sinensis*, despite growth promoting effects (Straub *et al*., 2013c).

Compared to the soil samples, the enrichment for *Actinobacteria* in roots and rhizomes mainly depends on their ability to form spores and grow extensive mycelia (Naylor *et al*., 2017). Meanwhile, the *H. frisingense* enriched families in rhizomes, namely *Pseudomonadaceae* promote plant growth by secreting auxin (IAA), via phosphate solubilisation or pathogen inhibition (Fang *et al*., 2012), while *Xanthomonadaceae* are often associated with pathogenic characteristics and auxin (IAA) secretion (Moreira *et al*., 2010; Lasudee *et al*., 2017). It is possible that the bacterial shifts in the rhizome are caused by initial jasmonate-induced defense signaling, which is found in Sugarcane and *M. sinensis* (Rocha *et al*., 2007; Straub *et al*., 2013c) upon inoculation.

5.5. Long-term nitrogen fertilizer shift N-related bacterial communities

Compared to the compartment type and organ type factors, the effect of the nitrogen fertilizer on the composition and diversity of the bacterial community is relatively small (Figure 16-23). However, we observed that the nitrogen fertilizer treatment had a significant effect on the rhizosphere and the endosphere bacterial abundance. This was most interesting when compared on the family and OTU level, especially in the rhizome. In sugarcane with two different nitrogen fertilizer levels for two years, nitrogen did not change the endophytic bacterial composition, although it significantly shifted the nitrification and denitrification associated bacteria in the soil (Yeoh *et al*., 2016). A major

difference to our results is not only the plant species difference, but also the different period of the nitrogen fertilizer application (a difference for 14 years). The succession of the nitrogen related bacterial community may thus be a gradual process that may mainly affect perennials, such as *Miscanthus*. Accordingly, two years of nitrogen fertilizer application may thus be not long enough to substantially shift the endophytic communities.

The main shifted phyla between different N applications were *Proteobacteria*, which contain the major diazotrophic and N-cycle associated microbe groups. Within the *Proteobacteria*, both enriched and depleted families were identified with respect to the N fertilizer application. Furthermore, within each compartment differences in the diazotroph community composition (Figure 25) and abundance were apparent (Table 10). Soil diazotrophs are significantly affected by nitrogen fertilizer in rice and sorghum (Coelho *et al*., 2008; Prakamhang *et al*., 2009). In agreement with that, such an N fertility effect is observed in the root and rhizome in the *Misacnthus* field.

In the bulk soil there was almost no difference in the composition of the bacterial communities, although the fold-change analysis showed that the nitrogen fixing family *Bradyrhizobiaceae* was slightly enriched in N0. Even though a number of non-legume associated nitrogen fixing bacterial families also express denitrifier function, such as *Bradyrhizobiaceae*, our analysis is most consistent with the idea that the nitrogen fixing function dominated in N0, while the denitrifier function dominated under the high N condition (Itakura *et al*., 2009) (Figure 25, Table 10). In the rhizosphere, two putative nitrogen fixing bacterial families, *Geobacteraceae* and *Syntrophbacteraceae* were enriched in the N0 condition and these comprise candidate rhizosphere diazotrophs of *Miscanthus* (Holmes *et al*., 2004; Bolhuis *et al*., 2010). *Pseudomonadaceae* were enriched in the N80 rhizosphere, in accordance with their function in denitrification (Xun *et al*., 2009). By

contrast, stronger nitrogen fertilizer effects were apparent in the endophytic compartments. Two putative diazotroph families, *Hyphomicrobiaceae* and *Rhodosprilliaceae*, were enriched in the root to about 5% in N0, while these only accounted for 1.8% in N80. Other putative diatrophes, *Hyphomicrobiaceae* and *Bradyrhizobiaceae*, were enriched to about 10% in the rhizome, while these accounted for only 0.7% in N0. On the contrary, two putative denitrifier families, *Pseudomonadaceae* and *Comamonadaceae* were enriched to about 12.6% in N80 (only 3.0% in N0). The putative diazotroph families *Hyphomicrobiaceae*, *Rhodosprilliaceae* and *Bradyrhizobiaceae* might thus contribute nitrogen to *Miscanthus*, although this needs experimental confirmation. *Herbaspirillum frisingense*, a diazotroph previously identified from several biomass grasses with *Miscanthus*-growth promoting function (Straub *et al*., 2013c), was apparently always low abundant and likely has little contribution to the nitrogen efficiency of *Miscanthus* (Kirchhof *et al.*, 2001). Taken together, the yearly application of moderate N fertilizer enriched the relative abundance of denitrifiers and depleted diazotrophs compared to the non-fertilized *Miscanthus* field. This nitrogen fertilizer effect strengthened gradually from soil to rhizome, opening the possibility that the rhizome is a niche that supports nitrogen fixation.

Miscanthus × *giganteus* is intrinsically quite resistant against many pathogens, a property that allows growing this grass without chemical protection. Interestingly, the plant pathogen related family *Xanthomanodaceae* and *Rhizobiaceae* were more than two-fold depleted in N0, compared to N80, which is in agreement with previous findings that low nitrogen fertilizer promoted the bacterial nitrogen bacteria and suppressed the plant pathogens (Compant *et al*., 2005; Hayat *et al*., 2005). Surprisingly, several field experiments have found an antagonistic relationship between *Sinobacteraceae* and *Xanthomanodaceae* (Ho *et al*., 2017). *Sinobacteraceae*, which often express ammonia

monooxygenase (AMO), tended to enrich in the nitrogen deficient conditions, while the *Xanthomanodaceae*, mainly containing plant pathogens, were depleted in the nitrogen deficient conditions. As a consequence, *Sinobacteraceae* might contribute other plant growth promoting functions to *Miscanthus* under nitrogen deficient conditions.

6 Conclusion

Up to date, with the wide application of next generation sequencing platform, the research foci in plant-microbe system have transferred from single or few bacteria to the whole bacterial communities, to classify the global pattern of functional and beneficial plant associated microbial communities. To our knowledge, the high throughput sequencing applied in metagenomics and 16S rDNA of *mscanthus*, especially for the sterile *Miscanthus* × *giganteus*, is still uninvestigated. Therefore, in this research project, *Miscanthus* × *giganteus* associated microbiota affected by a long-term different nitrogen fertilizer regime and a one establishment year of *Herbaspirillum frisingense* inoculation were analyzed to investigate nitrogen fertilizer shift and *Herbaspirillum frisingense* inoculation reshaped bacterial group in different underground compartments. Simultaneously, stem node propagation with *H. frisingense* inoculation was established in the greenhouse and directly in the field to investigate plant-growth-promoting bacteria promotes the stem node propagation, following these major hypotheses (see 1.7):

- Different soil-endosphere fractions provided different niches for bacterial species and recruit different bacterial communities.

- A distinct long-term nitrogen fertilizer application shifts the *Miscanthus* × *giganteus* associated bacterial composition.

- The genetic properties of *H. frisingense* are ideal to improve biomass yield of *M.* × *giganteus*.
- *H. frisingense* inoculation will lead to substantial occupation of an endosphere niche in miscanthus, long lasting effects and will benefits stem cutting propagation.

Almost all the established hypotheses were confirmed by the research, except that *H.*

frisingense fails to dominate the miscanthus inside niche after one establishment year. And the more precise conclusions were shown as following summary.

6.1. Summary

The efficient establishment of the most popular *Miscanthus* genotype, *Miscanthus* × *giganteus* Greef et Deu., in the field is a severe bottleneck in widening the growth area of this highly productive, nutrient-efficient biomass crop. While the sterility of this genotype is considered as a bonus to reduce invasiveness of this grass, propagation in practice mostly relies on rhizome propagation. Propagation from stem nodes is an attractive alternative strategy (Hong and Meyer, 2007; Atkinson, 2009) and inoculation with the diazotrophic *H. frisingense* improves establishment were performed both in the field and in the greenhouse (Figure 9-11). Despite very promising, it must be noted that establishment from stem nodes strongly depends on the availability of mature stem nodes and is highly sensitive to environmental stress, suggesting that pre-culture under greenhouse conditions or transfer of stem cuttings from other environments may be necessary to integrate this propagation method in agricultural practice.

The endophytic *Herbaspirillum frisingense* appears to occupy only a minor niche in *M.* × *giganteus*, but inoculation appears to improve the establishment of stem cuttings in the field, leads to long-lasting growth promotion and altered microbial abundances in the rhizome. In details, combined with the field experiment and greenhouse experiment, *H. frisingense* inoculation could develop the sprouting speed, establishment rate, plant height and further plant biomass (Figure 9-11). Meanwhile, *H. frisingense* inoculation could enrich the *Proteobacteria* and deplete the *Actinobacteria* in the rhizome to promote the growth of *M.* × *giganteus* for a long time.

In this study, we also confirm that the stem cutting of *M.* × *giganteus* planted directly in

the field could be established successfully, especially with the *H. frisingense* inoculation, and the major reason confused before are the planting patterns. Planting horizontally is significantly better than planting vertically. The node positions and plating date also significantly affect the establishment of stem propagation. Overall, stem cuttings propagation is a promising and practicable propagation method for *M.* × *giganteus*. We suggest inoculated stem cuttings are directly horizontally planted in the wetting field with cutting nodes from node 1 to node 3 of the *M.* × *giganteus* established more than 4 years, from the middle July to the early August in South Germany.

The amplicon sequencing of 16S rDNA indicates that two similar main factors shaped the bacterial community both in the 14-years nitrogen fertilizer supplying and *H. frisingense* inoculating *M.* × *giganteus* fields. The compartment type was the primary factor, which separated the bacterial community into an exophytic and an endophytic community. On the other hand, the plant organ type was also important, separating the root and rhizome.

Previous work on two-year nitrogen fertilizer application on *Miscanthus* and sugarcane demonstrated how the nitrogen fertilizer factor shifted the soil nitrogen-related bacterial community, while similar microbial communities were retained in the endophytic compartment (Li *et al.*, 2015; Yeoh *et al.*, 2016). However, the long-term nitrogen fertilizer difference in the field of *Miscanthus* allowed us to observe that the nitrogen fertilizer could significantly enrich the nitrogen fixing bacterial abundance under the low nitrogen condition, especially in the rhizome, an organ that may allow physiological conditions for efficient N fixation, i.e. low oxygen pressure. By contrast, denitrification-related bacteria may be recruited by moderate or high N (Figure 26). We also observed that the rhizome of *M.* × *giganteus* hold more potentially beneficial bacteria and less potential plant pathogens under low nitrogen. Because the sterile *M.* × *giganteus* is mainly

propagated via the rhizome, it may even be beneficial to propagate this plant by choosing the rhizome from low nitrogen plots.

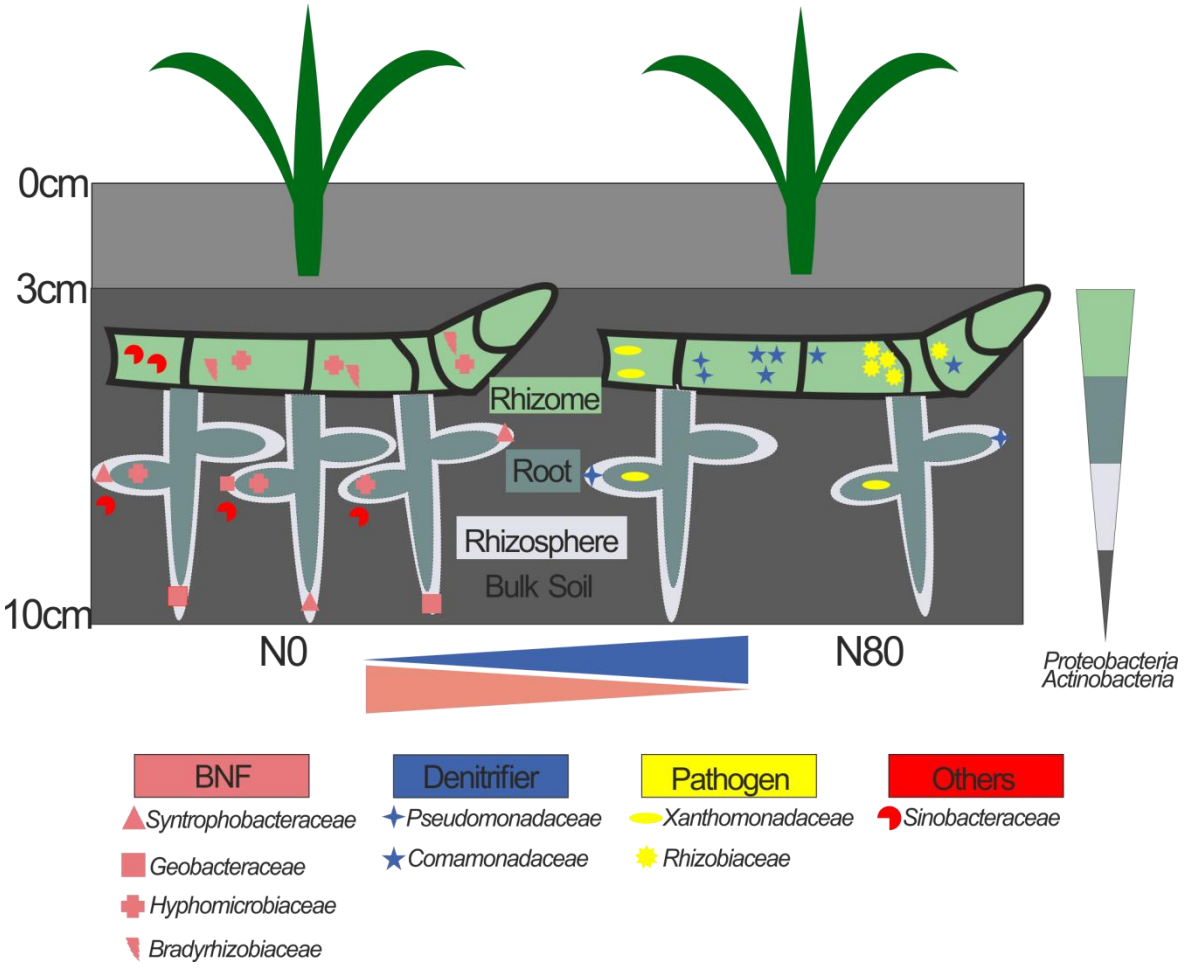

Figure 26: Summary of nitrogen-dependent enrichment and depletion of functional families in phylum *Proteobacteria*.

The no nitrogen fertilizer (N0) leads to higher diazotroph abundance, provided more biological fixed nitrogen to *Miscanthus*, while the perennial nitrogen fertilizer lead to higher denitrifier abundance and plant pathogen accumulation. The phenomena are widespread in *Miscanthus* field, from bulk soil to rhizome. Meanwhile, *Proteobacteria* and *Actinobacteria* composition are gradually enriched from bulk soil to rhizome, no matter in which nitrogen fertilizer regime. Pink triangle: enriched diazotroph composition from N80 to N0, blue triangle; enriched denitrifier composition from N0 to N80. N0, no nitrogen fertilizer application; N80, 80 kg N $ha^{-1}a^{-1}$ application; BNF, biological nitrogen fixing

bacteria.

6.2. Outlook

Admittedly, the successful field establishment trial of stem cuttings is limited by one field environment, one location, one soil type, and one establishment year. More field experiments are needed to confirm that direct stem cutting plantings in the field are a practical variant of propagation. Meanwhile, Meyer & Hong (2011) verified that two nodes stem cuttings established more successfully than single nodes in the vertical stem planting experiment, which may also help to improve the establishment success for the horizontal stem planting. The optimal planting depth and suitable environments must be studied to improve the stem propagation establishment.

The mechanism of how *Herbaspirillum frisingense* improved the establishment of stem nodes remains unclear. Exogenous plant hormones experiments may provide a key to uncover the improvement factors by *Herbaspirillum frisingense* inoculation. Other plant growth promoting bacteria might have similar effects and may serve as alternative to promote stem cutting plantings of *M.* × *giganteus* in the field. So further researches are needed to seek the ideal candidate bacterium (maybe a group of bacteria), which could dominate the inside niche for a long period, owning nitrogen-fixing or other growth promoting traits.

In this research, the plant associated microbiome is limited by the 16S rDNA sequencing and analysis, which only exhibit the distribution and composition of bacterial communities. To further investigate the functional bacterial communities and plant-microbe interactions, such as N-related bacterial communities, more metagomonics sequencing technologies and

deeper sequencing are needed. Meanwhile, arbuscular mycorrhizal fungi are important participants of *M.* × *giganteus* life cycle (Firmin *et al.*, 2015), so the related studies are also needed.

7 References

Alves, G. C., Videira, S. S., Urquiaga, S., and Reis, V. M. (2015). Differential plant growth promotion and nitrogen fixation in two genotypes of maize by several Herbaspirillum inoculants. *Plant and soil*, 387, 307-321.

Atkinson, C. J. (2009). Establishing perennial grass energy crops in the UK: A review of current propagation options for Miscanthus. *Biomass and bioenergy*, 33, 752-759.

Babin, D., Deubel, A., Jacquiod, S., Sørensen, S. J., Geistlinger, J., Grosch, R., Smalla, K. (2019). Impact of long-term agricultural management practices on soil prokaryotic communities. *Soil Biology and Biochemistry*, 129, 17-28.

Badri, D. V., Quintana, N., El Kassis, E. G., Kim, H. K., *et al.* (2009). An ABC transporter mutation alters root exudation of phytochemicals that provoke an overhaul of natural soil microbiota. *Plant Physiology*, 151, 2006-2017.

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.*, 57, 233–66

Baldani, V. D., Baldani, J. I., Döbereiner, J. (2000). Inoculation of rice plants with the endophytic diazotrophs *Herbaspirillum seropedicae* and *Burkholderia spp*. *Biology and Fertility of Soils*, 30, 485-491.

Baptista, R. B., de Morais, R. F., Leite, J. M., Schultz, N., Alves, B. J. R., Boddey, R. M., Urquiaga, S. (2014). Variations in the ^{15}N natural abundance of plant-available N with soil depth: their influence on estimates of contributions of biological N_2 fixation to sugar cane. *Applied soil ecology*, 73, 124-129.

Beale, C.V., Long, S.P. (1997). Seasonal dynamics of nutrient accumulation and partitioning in the perennial C4-grasses *Miscanthus* × *giganteus* and *Spartina cynosuroides*. *Biomass & Bioenergy*, 12, 419-428.

Berg, G., Smalla, K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS microbiology ecology*, 68, 1-13.

Bertin, C., Yang, X., Weston, L. A. (2003). The role of root exudates and allelochemicals in the rhizosphere. *Plant Soil*, 256, 67–83.

Bhattacharjee, R. B., Singh, A., Mukhopadhyay, S. N. (2008). Use of nitrogen-fixing bacteria as biofertiliser for non-legumes: prospects and challenges. *Applied microbiology and biotechnology*, 80, 199-209

Blaha, D., Prigent-Combaret, C., Mirza, M. S., Mo ënne-Loccoz, Y. (2006). Phylogeny of the 1-aminocyclopropane-1-carboxylic acid deaminase-encoding gene acdS in phytobeneficial and pathogenic Proteobacteria and relation with strain biogeography. *FEMS Microbiology Ecology*, *56*, 455-470.

Boehmel, C., Lewandowski, I., Claupein, W. (2008). Comparing annual and perennial energy cropping systems with different management intensities. *Agricultural systems*, 96, 224-236.

Boersma, N. N., Heaton, E. A. (2012). Effects of temperature, illumination and node position on stem propagation of *Miscanthus* × *giganteus*. *Gcb Bioenergy*, 4, 680-687.

Boersma, N. N., Heaton, E. A. (2014a). Does propagation method affect yield and survival? The potential of *Miscanthus* × *giganteus* in Iowa, USA. *Industrial crops and products*, 57, 43-51.

Boersma, N. N., Heaton, E. A. (2014b). Propagation method affects *Miscanthus* × *giganteus* developmental morphology. *Industrial crops and products*, 57, 59-68.

Bolhuis, H., Severin, I., Confurius-Guns, V., Wollenzien, U. I., Stal, L. J. (2010). Horizontal transfer of the nitrogen fixation gene cluster in the cyanobacterium Microcoleus chthonoplastes. *The ISME journal*, 4, 121.

Bothe, H., Schmitz, O., Yates, M. G., Newton, W. E. (2010). Nitrogen fixation and hydrogen metabolism in cyanobacteria. *Microbiol. Mol. Biol. Rev.*, 74, 529-551.

Broeckling, C. D., Broz, A. K., Bergelson, J., Manter, D. K., Vivanco, J. M. (2008). Root exudates regulate soil fungal community composition and diversity. *Appl Environ Microbiol*, 74, 738–744

Cadoux, S., Riche, A. B., Yates, N. E., Machet, J. M. (2012). Nutrient requirements of Miscanthus x giganteus: conclusions from a review of published studies. *Biomass and Bioenergy*, 38, 14-22.

Cankar, K., Kraigher, H., Ravnikar, M., Rupnik, M. (2005). Bacterial endophytes from seeds of Norway spruce (*Picea abies L.* Karst). *FEMS microbiology letters*, 244, 341-345.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., *et al.* (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature methods*, 7, 335.

Caporaso, J. G., Lauber, C. L., Walters, W. A., *et al.* (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal*, *6*, 1621-1624.

Chaparro, J. M., Sheflin, A. M., Manter, D. K., Vivanco, J. M. (2012). Manipulating the soil microbiome to increase soil health and plant fertility. *Biology and Fertility of Soils*, 48, 489-499.

Chapman, G. P., Peat, W. E. (1995). An introduction to the grasses. Acribia, SA,

Chatterjee, S., Hadi, A. S., Price, B. (2000). Regression analysis by example. John Wiley & Sons. Inc., New York.

Christian, D. G., Poulton, P. R., Riche, A. B., Yates, N. E. (1997). The recovery of ^{15}N-labelled fertilizer applied to *Miscanthus* × *giganteus*. *Biomass and Bioenergy*, 12, 21-24.

Christian, D. G., Riche, A. B., Yates, N. E. (2008). Growth, yield and mineral content of *Miscanthus* × *giganteus* grown as a biofuel for 14 successive harvests. *Industrial crops and products*, 28, 320-327.

Clifton-Brown, J. C., Lewandowski, I., Andersson, B., *et al.* (2001). Performance of 15 Miscanthus genotypes at five sites in Europe. *Agronomy Journal*, 93, 1013-1019.

Clifton‐Brown, J., Hastings, A., Mos, M., *et al.* (2017). Progress in upscaling Miscanthus biomass production for the European bio‐economy with seed‐based hybrids. *Gcb Bioenergy*, 9, 6-17.

Cole, J. R., Wang, Q., Cardenas, E., *et al.* (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic acids research*, 37, D141-D145.

Compant, S., Clément, C., Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo-and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biology and Biochemistry*, 42, 669-678.

Compant, S., Duffy, B., Nowak, J., Clément, C., Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and environmental microbiology*, 71, 4951-4959.

Contesto, C., Desbrosses, G., Lefoulon, C., *et al.* (2008). Effects of rhizobacterial ACC deaminase activity on Arabidopsis indicate that ethylene mediates local root responses to plant growth-promoting rhizobacteria. *Plant science*, 175, 178-189.

Cope‐Selby, N., Cookson, A., Squance, M., Donnison, I., Flavell, R., Farrar, K. (2017). Endophytic bacteria in *Miscanthus* seed: implications for germination, vertical inheritance of endophytes, plant evolution and breeding. *Gcb Bioenergy*, 9, 57-77.

Davis, S. C., Parton, W. J., Dohleman, F. G., Smith, C. M., Del Grosso, S., Kent, A. D., DeLucia, E. H. (2010). Comparative biogeochemical cycles of bioenergy crops reveal nitrogen-fixation and low greenhouse gas emissions in a *Miscanthus*× *giganteus* agro-ecosystem. *Ecosystems*, 13, 144-156.

de Aquino, G. S., Ventura, M. U., Alexandrino, R. P., *et al.* (2018). Plant-promoting rhizobacteria Methylobacterium komagatae increases crambe yields, root system and plant height. *Industrial crops and products*, 121, 277-281.

Defra. NF0415—investigation of stem rooting in Miscanthus [Internet]. Available from: ⟨http://randd.defra.gov.uk/Document.aspx?Document=NF0415_ 1199_FRP.doc⟩; 2002.

De-la-Pena, C., Badri, D. V., Lei, Z., *et al.* (2010). Root secretion of defense-related proteins is development-dependent and correlated with flowering time. *Journal of Biological Chemistry*, 285, 30654-30665.

de Oliveira, A. L. M., de Canuto, E. L., Urquiaga, S., Reis, V. M., Baldani, J. I. (2006). Yield of micropropagated sugarcane varieties in different soil types following inoculation with diazotrophic bacteria. *Plant and Soil*, 284, 23-32.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., *et al.* (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.*, 72, 5069-5072.

De Souza, R. S. C., Okura, V. K., Armanhi, J. S. L., *et al.* (2016). Unlocking the bacterial and fungal communities assemblages of sugarcane microbiome. *Scientific reports*, 6, 28774.

Dohleman, F. G., Heaton, E. A., Arundale, R. A., Long, S. P. (2012). Seasonal dynamics of above‐and below‐ground biomass and nitrogen partitioning in *Miscanthus*× *giganteus* and P anicum virgatum across three growing seasons. *Gcb Bioenergy*, 4, 534-544.

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature methods*, 10, 996.

Eyles, A., Bonello, P., Ganley, R., Mohammed, C. (2010). Induced resistance to pests and pathogens in trees. *New Phytologist*, 185, 893-908.

Fadrosh, D. W., Ma, B., Gajer, P., Sengamalay, N., Ott, S., Brotman, R. M., Ravel, J. (2014). An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome*, 2, 6.

Fang, R., Lin, J., Yao, S., *et al.* (2013). Promotion of plant growth, biological control and induced systemic resistance in maize by Pseudomonas aurantiaca JD37. *Annals of Microbiology*, 63, 1177-1185..

de Santi Ferrara, F. I., Oliveira, Z. M., Gonzales, H. H. S., Floh, E. I. S., Barbosa, H. R. (2012). Endophytic and rhizospheric enterobacteria isolated from sugar cane have different potentials for producing plant growth-promoting substances. *Plant and soil*, 353, 409-417.

Fierer, N., Jackson, R. B. (2006). The diversity and biogeography of soil bacterial communities. *Proceedings of the National Academy of Sciences*, 103, 626-631.

Firmin, S., Labidi, S., Fontaine, J., *et al.* (2015). Arbuscular mycorrhizal fungal inoculation protects *Miscanthus* × *giganteus* against trace element toxicity in a highly metal-contaminated site. *Science of the Total Environment*, 527, 91-99.

Fitzsimons, M. S., Miller, R. M. (2010). The importance of soil microorganisms for maintaining diverse plant communities in tallgrass prairie. *American Journal of Botany*, 97, 1937-1943.

Flores, H. E., Vivanco, J. M., Loyola-Vargas, V. M. (1999). 'Radicle'biochemistry: the biology of root-specific metabolism. *Trends in plant science*, 4, 220-226..

Garc á de Salamone, I. E., Hynes, R. K., Nelson, L. M. (2001). Cytokinin production by plant growth promoting rhizobacteria and selected mutants. *Canadian Journal of microbiology*, 47, 404-411.

Glick, B. R. (2004). Bacterial ACC deaminase and the alleviation of plant stress. *Advances in applied microbiology*, 56, 291-312.

Glick, B. R., Todorovic, B., Czarny, J., Cheng, Z., Duan, J., McConkey, B. (2007). Promotion of plant growth by bacterial ACC deaminase. *Critical Reviews in Plant Sciences*, 26, 227-242.

Glick, B. R. (2012). Plant growth-promoting bacteria: mechanisms and applications. *Scientifica*, 2012.

Gomez, J. D., Denef, K., Stewart, C. E., Zheng, J., Cotrufo, M. F. (2014). Biochar addition rate influences soil microbial abundance and activity in temperate soils. *European Journal of Soil Science*, 65, 28-39.

Goodwin, S., McPherson, J. D., McCombie, W. R. (2016). Coming of age: ten years of next-generation sequencing technologies. *Nature Reviews Genetics*, 17, 333.

Gottel, N. R., Castro, H. F., Kerley, *et al.* (2011). Distinct microbial communities within the endosphere and rhizosphere of Populus deltoides roots across contrasting soil types. *Appl. Environ. Microbiol.*, 77, 5934-5944.

Gubišová, M., Gubiš, J., Žofajová, A., Mih álik, D., Kraic, J. (2013). Enhanced in vitro propagation of *Miscanthus* × *giganteus*. *Industrial crops and products*, 41, 279-282

Gutjahr, C., Parniske, M. (2017). Cell biology: control of partner lifetime in a plant–fungus relationship. *Current Biology*, 27, R420-R423.

Gutjahr, C., Sawers, R. J., Marti, G., *et al.* (2015). Transcriptome diversity among rice root types during asymbiosis and interaction with arbuscular mycorrhizal fungi. *Proceedings of the National Academy of Sciences*, 112, 6754-6759.

Hardoim, P. R., Hardoim, C. C., Van Overbeek, L. S., Van Elsas, J. D. (2012). Dynamics of seed-borne rice endophytes on early plant growth stages. *PLoS One*, 7, e30438.

Hardoim, P. R., van Overbeek, L. S., van Elsas, J. D. (2008). Properties of bacterial endophytes and their proposed role in plant growth. *Trends in microbiology*, 16, 463-471.

Hayat, R., Ali, S., Amara, U., Khalid, R., Ahmed, I. (2010). Soil beneficial bacteria and their role in plant growth promotion: a review. *Annals of Microbiology*, 60, 579-598.

Heaton, E. A., Dohleman, F. G., Long, S. P. (2009). Seasonal nitrogen dynamics of *Miscanthus* × *giganteus* and *Panicum virgatum*. *Gcb Bioenergy*, 1, 297-307.

Heaton, E. A., Long, S. P., Voigt, T. B., Jones, M. B., Clifton-Brown, J. (2004). Miscanthus for renewable energy generation: European Union experience and projections for Illinois. *Mitigation and Adaptation Strategies for Global Change*, 9, 433-451.

Hirimburegama, K., Gamage, N. (1995). Propagation of Bambusa vulgaris (yellow bamboo) through nodal bud culture. *Journal of Horticultural Science*, 70, 469-475.

Ho, A., Di Lonardo, D. P., Bodelier, P. L. (2017). Revisiting life strategy concepts in environmental microbial ecology. *FEMS microbiology ecology*, 93, fix006.

Holmes, D. E., Nevin, K. P., Lovley, D. R. (2004). In situ expression of nifD in Geobacteraceae in subsurface sediments. *Applied and environmental microbiology*, 70, 7251-7259.

Hong, J., Meyer, M. (2007). Effect of medium, date, and node position on rooting of *Miscanthus* × *giganteus* stem cuttings. *Hortscience*, 42, 910-910.

Hurek, T., Reinhold-Hurek, B. (2003). Azoarcus sp. strain BH72 as a model for nitrogen-fixing grass endophytes. *Journal of Biotechnology*, 106, 169-178.

Itakura, M., Saeki, K., Omori, H., *et al.* (2009). Genomic comparison of Bradyrhizobium japonicum strains with different symbiotic nitrogen-fixing capabilities and other Bradyrhizobiaceae members. *The ISME journal*, 3, 326-339.

Iqbal, Y., Gauder, M., Claupein, W., Graeff-Hönninger, S., Lewandowski, I. (2015). Yield and quality development comparison between miscanthus and switchgrass over a period of 10 years. *Energy*, 89, 268-276.

James, E. K., Olivares, F. L. (1998). Infection and colonization of sugar cane and other graminaceous plants by endophytic diazotrophs. *Critical Reviews in Plant Sciences*, 17, 77-119.

Johnston-Monje, D., Lundberg, D. S., Lazarovits, G., Reis, V. M., Raizada, M. N. (2016). Bacterial populations in juvenile maize rhizospheres originate from both seed and soil. *Plant and Soil*, 405, 337-355.

Johnston-Monje, D., Raizada, M. N. (2011). Conservation and diversity of seed associated endophytes in Zea across boundaries of evolution, ethnography and ecology. *PLoS One*, 6, e20396.

Jones, M. B., Walsh, M. (Eds.). (2001). Miscanthus for energy and fibre. Earthscan.

Keymer, D. P., Kent, A. D. (2014). Contribution of nitrogen fixation to first year *Miscanthus* × *giganteus*. *Gcb Bioenergy*, 6, 577-586.

Kirchhof, G., Eckert, B., Stoffels, M., Baldani, J. I., Reis, V. M., Hartmann, A. (2001). *Herbaspirillum frisingense* sp. nov., a new nitrogen-fixing bacterial species that occurs in C4-fibre plants. *International journal of systematic and evolutionary microbiology*, 51, 157-168.

Landwirtschaftliches Technologiezentrum (LTZ) Augustenberg (2016). Agrarmeteorologie Baden-Württemberg, Station Hohenheim. Retrieved from http://www.wetter-bw.de/.

Lanzerstorfer, C. (2019). Combustion of Miscanthus: Composition of the Ash by Particle Size. *Energies*, 12, 178.

Lasudee, K., Tokuyama, S., Lumyong, S., Pathom-Aree, W. (2017). Mycorrhizal spores associated Lysobacter soli and its plant growth promoting activity. *Chiang Mai J. Sci*, 44, 94-101.

Lattin, J. M., Carroll, J. D., Green, P. E. (2003). Analyzing multivariate data.

Lau, J. A., Lennon, J. T. (2011). Evolutionary ecology of plant–microbe interactions: soil microbial structure alters selection on plant traits. *New Phytologist*, 192, 215-224.

Lavecchia, A., Curci, M., Jangid, K., Whitman, W. B., Ricciuti, P., Pascazio, S., Crecchio, C. (2015). Microbial 16S gene-based composition of a sorghum cropped rhizosphere soil under different fertilization managements. *Biology and fertility of soils*, 51, 661-672.

Lewandowski, I., Kicherer, A. (1997). Combustion quality of biomass: practical relevance and experiments to modify the biomass quality of *Miscanthus x giganteus*. *European Journal of Agronomy*, 6, 163-177.

Lewandowski, I. (1998). Propagation method as an important factor in the growth and development of *Miscanthus* × *giganteus*. *Industrial Crops and Products*, 8, 229-245.

Lewandowski, I., Clifton-Brown, J. C., Scurlock, J. M. O., Huisman, W. (2000). Miscanthus: European experience with a novel energy crop. *Biomass and Bioenergy*, 19, 209-227.

Lebeis, S. L., Paredes, S. H., Lundberg, D. S., *et al.* (2015). Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. *Science*, 349, 860-864.

Lewandowski, I., Clifton-Brown, J., Trindade, L. M., *et al.* (2016). Progress on optimizing miscanthus biomass production for the European bioeconomy: Results of the EU FP7 project OPTIMISC. *Frontiers in plant science*, 7, 1620.

Li, D., Voigt, T. B., Kent, A. D. (2016). Plant and soil effects on bacterial communities associated with *Miscanthus* × *giganteus* rhizosphere and rhizomes. *Gcb Bioenergy*, 8, 183-193.

Liu, Y., Yang, H., Ludewig, U. (2014). Dynamic Element Concentrations and Similar Proteome af the Rhizome and Root of *Miscanthus X Gigantheus*. *J Plant Biochem*

Physiol, 2, 2.

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., *et al.* (2012). Defining the core Arabidopsis thaliana root microbiome. *Nature*, 488, 86.

Magoč, T., Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, 27, 2957-2963.

Mangold, A., Lewandowski, I., Xue, S., Kiesel, A. (2018). 'Collar propagation'as an alternative propagation method for rhizomatous miscanthus. *GCB Bioenergy*, 10, 186-198.

Maughan, M., Bollero, G., Lee, D. K., *et al*. (2012). *Miscanthus* × *giganteus* productivity: the effects of management in different environments. *GCB Bioenergy*, 4, 253-265.

Meyer, M. H., Hong, J. (2011). *Miscanthus* × *giganteus* can be propagated from stem cuttings. *Journal of Environmental Horticulture*, 29, 193-196.

Micallef, S. A., Shiaris, M. P., Colón-Carmona, A. (2009). Influence of Arabidopsis thaliana accessions on rhizobacterial communities and natural variation in root exudates. *Journal of Experimental Botany*, 60, 1729-1742.

Momose, A., Ohtake, N., Sueyoshi, K., Sato, T., Nakanishi, Y., Akao, S., Ohyama, T. (2009). Nitrogen fixation and translocation in young sugarcane (Saccharum officinarum L.) plants associated with endophytic nitrogen-fixing bacteria. *Microbes and environments*, 0905180090-0905180090.

Monteiro, R. A., Balsanelli, E., Wassem, R., *et al.* (2012). Herbaspirillum-plant interactions: microscopical, histological and molecular aspects. *Plant and Soil*, 356, 175-196.

Morales, S. E., Holben, W. E. (2011). Linking bacterial identities and ecosystem processes: can 'omic'analyses be more than the sum of their parts?. *FEMS microbiology ecology*, 75, 2-16.

Moreira, L. M., Almeida, N. F., Potnis, N., *et al.* (2010). Novel insights into the genomic basis of citrus canker based on the genome sequences of two strains of Xanthomonas fuscans subsp. aurantifolii. *BMC genomics*, 11, 238.

Nautiyal, C. S., Srivastava, S., Chauhan, P. S., Seem, K., Mishra, A., Sopory, S. K. (2013). Plant growth-promoting bacteria Bacillus amyloliquefaciens NBRISN13 modulates gene expression profile of leaf and rhizosphere community in rice during salt stress. *Plant Physiology and Biochemistry*, 66, 1-9.

Naylor, D., DeGraaf, S., Purdom, E., Coleman-Derr, D. (2017). Drought and host selection influence bacterial community dynamics in the grass root microbiome. *The ISME journal*, 11, 2691.

Nishiwaki, A., Mizuguti, A., Kuwabara, S., *et al.* (2011). Discovery of natural Miscanthus (Poaceae) triploid plants in sympatric populations of *Miscanthus sacchariflorus* and Miscanthus sinensis in southern Japan. *American Journal of Botany*, 98, 154-159.

Ofek-Lalzar, M., Sela, N., Goldman-Voronov, M., Green, S. J., Hadar, Y., Minz, D. (2014). Niche and host-associated functional signatures of the root surface microbiome. *Nature communications*, 5, 4950.

Oliveira, A. D., Urquiaga, S., Döbereiner, J., Baldani, J. I. (2002). The effect of inoculating endophytic N_2-fixing bacteria on micropropagated sugarcane plants. *Plant and Soil*, 242, 205-215.

O'Loughlin, J., McDonnell, K., Finnan, J. (2017). Establishing *miscanthus x giganteus* crops in Ireland through nodal propagation by harvesting stems in autumn and sowing them immediately into a field. *Biomass and bioenergy*, 107, 345-352.

O'Loughlin, J., McDonnell, K., Finnan, J. (2018). Quantifying the economic and greenhouse gas balance advantages of establishing miscanthus from stem cuttings. *Biomass and bioenergy*, 109, 147-154.

Paszkowski, U., Gutjahr, C. (2013). Multiple control levels of root system remodeling in arbuscular mycorrhizal symbiosis. *Frontiers in plant science*, 4, 204.

Pieterse, C. M., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C., Bakker, P. A. (2014). Induced systemic resistance by beneficial microbes. *Annual review of phytopathology*, 52, 347-375.

Pitombo, L. M., do Carmo, J. B., De Hollander, M., Rossetto, R., López, M. V.,

Cantarella, H., Kuramae, E. E. (2016). Exploring soil microbial 16S rRNA sequence data to increase carbon yield and nitrogen efficiency of a bioenergy crop. *Gcb Bioenergy*, 8, 867-879.

Prakamhang, J., Minamisawa, K., Teamtaisong, K., Boonkerd, N., Teaumroong, N. (2009). The communities of endophytic diazotrophic bacteria in cultivated rice (Oryza sativa L.). *Applied soil ecology*, 42, 141-149.

Ramanayake, S. M. S. D., Meemaduma, V. N., Weerawardene, T. E. (2006). In vitro shoot proliferation and enhancement of rooting for the large-scale propagation of yellow bamboo (Bambusa vulgaris 'Striata'). *Scientia Horticulturae*, 110, 109-113.

Reinhold-Hurek, B., Bünger, W., Burbano, C. S., Sabale, M., Hurek, T. (2015). Roots shaping their microbiome: global hotspots for microbial activity. *Annual review of phytopathology*, 53, 403-424.

Reinhold-Hurek, B., Hurek, T., Claeyssens, M., Van Montagu, M. (1993). Cloning, expression in Escherichia coli, and characterization of cellulolytic enzymes of Azoarcus sp., a root-invading diazotroph. *Journal of bacteriology*, 175, 7056-7065.

Reinhold-Hurek, B., Hurek, T. (1998a). Interactions of gramineous plants with Azoarcus spp. and other diazotrophs: identification, localization, and perspectives to study their function. *Critical Reviews in Plant Sciences*, 17, 29-54

Reinhold-Hurek, B., Hurek, T. (1998b). Life in grasses: diazotrophic endophytes. *Trends in microbiology*, 6, 139-144.

Rousk, J., Bååth, E., Brookes, P. C., *et al.* (2010). Soil bacterial and fungal communities across a pH gradient in an arable soil. *The ISME journal*, 4, 1340-1351.

Schloss, P. D., Handelsman, J. (2006). Toward a census of bacteria in soil. *PLoS computational biology*, 2, e92.

Sevilla, M., Burris, R. H., Gunapala, N., Kennedy, C. (2001). Comparison of benefit to sugarcane plant growth and $^{15}N_2$ incorporation following inoculation of sterile plants with Acetobacter diazotrophicus wild-type and nif mutant strains. *Molecular plant-microbe interactions*, 14, 358-366.

Spaepen, S., Vanderleyden, J., Remans, R. (2007). Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS microbiology reviews*, 31, 425-448.

Rocha, F. R., Papini-Terzi, F. S., Nishiyama, M. Y., *et al.* (2007). Signal transduction-related responses to phytohormones and environmental challenges in sugarcane. *BMC genomics*, 8, 71.

Rodrigues Coelho, M. R., De Vos, M., Carneiro, N. P., Marriel, I. E., Paiva, E., Seldin, L. (2008). Diversity of nifH gene pools in the rhizosphere of two cultivars of sorghum (Sorghum bicolor) treated with contrasting levels of nitrogen fertilizer. *FEMS microbiology letters*, 279, 15-22.

Rothballer, M., Eckert, B., Schmid, M., *et al.* (2008). Endophytic root colonization of gramineous plants by Herbaspirillum frisingense. *FEMS microbiology ecology*, 66, 85-95.

Saharan, B. S., Nehra, V. (2011). Plant growth promoting rhizobacteria: a critical review. *Life Sci Med Res*, 21, 30.

Siddiqui, M. H., Al-Whaibi, M. H. (2014). Role of nano-SiO_2 in germination of tomato (Lycopersicum esculentum seeds Mill.). *Saudi journal of biological sciences*, 21, 13-17

Spaepen, S., Vanderleyden, J., Okon, Y. (2009). Plant growth-promoting actions of rhizobacteria. *Advances in botanical research*, 51, 283-320.

Straub, D., Yang, H., Liu, Y., Ludewig, U. (2013a). Transcriptomic and proteomic comparison of two *Miscanthus* genotypes: high biomass correlates with investment in primary carbon assimilation and decreased secondary metabolism. *Plant and soil*, 372, 151-165.

Straub, D., Rothballer, M., Hartmann, A., Ludewig, U. (2013b). The genome of the endophytic bacterium H. frisingense GSF^{30T} identifies diverse strategies in the Herbaspirillum genus to interact with plants. *Frontiers in microbiology*, 4, 168.

Straub, D., Yang, H., Liu, Y., Tsap, T., Ludewig, U. (2013c). Root ethylene signalling is involved in Miscanthus sinensis growth promotion by the bacterial endophyte Herbaspirillum frisingense GSF^{30T}. *Journal of experimental botany*, 64, 4603-4615.

Thaweenut, N., Hachisuka, Y., Ando, S., Yanagisawa, S., Yoneyama, T. (2011). Two

seasons' study on nifH gene expression and nitrogen fixation by diazotrophic endophytes in sugarcane (Saccharum spp. hybrids): expression of nifH genes similar to those of rhizobia. *Plant and soil*, 338, 435-449.

Thimann, K. V., Skoog, F. (1933). Studies on the growth hormone of plants: III. The inhibiting action of the growth substance on bud development. *Proceedings of the National Academy of Sciences of the United States of America*, 19, 714.

Urquiaga, S., Xavier, R. P., de Morais, R. F.,*et al*. (2012). Evidence from field nitrogen balance and ^{15}N natural abundance data for the contribution of biological N^2 fixation to Brazilian sugarcane varieties. *Plant and soil*, 356, 5-21.

Van Der Heijden, M. G., Bardgett, R. D., Van Straalen, N. M. (2008). The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology letters*, 11, 296-310.

Van Heerwaarden, L. M., Toet, S., Aerts, R. (2003). Current measures of nutrient resorption efficiency lead to a substantial underestimation of real resorption efficiency: facts and solutions. *Oikos*, 101, 664-669.

van Overbeek, L. S., Saikkonen, K. (2016). Impact of bacterial–fungal interactions on the colonization of the endosphere. *Trends in plant science*, 21, 230-242.

Williams, P. M., De Mallorca, M. S. (1982). Abscisic acid and gibberellin-like substances in roots and root nodules ofGlycine max. *Plant and Soil*, 65, 19-26.

Xue, S., Kalinina, O., Lewandowski, I. (2015). Present and future options for Miscanthus propagation and establishment. *Renewable and sustainable energy reviews*, 49, 1233-1246.

Xun, W., Li, W., Huang, T., Ren, Y., *et al*. (2018). Long-term agronomic practices alter the composition of asymbiotic diazotrophic bacterial community and their nitrogen fixation genes in an acidic red soil. *Biology and fertility of soils*, 54, 329-339.

Yeoh, Y. K., Paungfoo-Lonhienne, C., Dennis, P. G., Robinson, N., Ragan, M. A., Schmidt, S., Hugenholtz, P. (2016). The core root microbiome of sugarcanes cultivated under varying nitrogen fertilizer application. *Environmental microbiology*, 18, 1338-1351.

Yu, P., Wang, C., Baldauf, J. A., Tai, H., Gutjahr, C., Hochholdinger, F., Li, C. (2018).

Root type and soil phosphate determine the taxonomic landscape of colonizing fungi and the transcriptome of field-grown maize roots. *New Phytologist*, 217, 1240-1253.

Zarraonaindia, I., Owens, S. M., Weisenhorn, P., *et al.* (2015). The soil microbiome influences grapevine-associated microbiota. *MBio*, 6, e02527-14.

Zehr, J. P., Jenkins, B. D., Short, S. M., Steward, G. F. (2003). Nitrogenase gene diversity and microbial community structure: a cross‐system comparison. *Environmental microbiology*, 5, 539-554.

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Electronic data processing

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