Impact of glyphosate application to transgenic Roundup Ready® soybean on horizontal gene transfer of the *EPSPS* gene to *Bradyrhizobium japonicum* and on the root-associated bacterial community

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To my father, my mother and my Felix

Contents

Abbreviations

Aqua dest	Distilled water
bp	Base pairs
CaMV	Cauliflower mosaic virus
CIP	Calf intestinal phosphatase
CP4-EPSPS	EPSPS gene from *Agrobacterium tumefaciens* strain CP4
dH2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside-5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
et al	*et alteri*, and others
EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase
FAM	6-Carboxyfluorescein
Fig	Figure
G	Glyphosate- treated samples
GM	Genetically modified
Glyphosate	N-phosphonomethylglycine
HGT	Horizontal gene transfer
K	Contol camples
Kan	Kanamycin resistance
KB	Kilo base pairs
nifD	Nitrogen fixation gene

NOS	Nopaline synthase gene
nptII	Kanamycin resistance gene
oriT	Origin of transfer
oriV	Origin of replication
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PEP	Phosphoenol pyruvate
Pi	Inorganic phosphate
PSY	Peptone-salts-yeast extract medium
rDNA	Ribosomal deoxyribonucleic acid
RR	Roundup Ready
rRNA	Ribosomal RNA
RS	Repeated sequences
S3P	Shikimate 3-phosphate
Tet	Tetracycline resistance
Tn5	Transposon number 5
T-RF	Terminal Restriction Fragment
T-RFLP	Terminal Restriction Fragment Length Polymorphism
UV	Ultraviolet
YEM	Yeast extract-mannitol medium
YEX	Yeast extract-xylose medium
X-Gal	5-Bromo-4-chloro-3-indolyl- β-D-galactopyranoside

Abstract

In this study, two topics causing major public concern related to transgenic plants were investigated: The possibility of a horizontal gene transfer from plant to bacteria and the impact of transgenic plants after herbicide treatment on root associated bacteria. The transgenic plant chosen for this study was Roundup Ready® (RR) soybean, which is tolerant to the herbicide glyphosate and is the most commonly used genetically modified crop worldwide. Glyphosate, the active ingredient of Roundup Ready®, inhibits the EPSPS enzyme (5-enolpyruvylshikimate-3-phosphate synthase). EPSPS is an enzyme involved in the shikimic acid pathway leading to the aromatic amino acid biosynthesis and its inhibition leads to growth reduction of plants and microorganisms. RR crops are glyphosate tolerant due to the introduction of the *CP4-EPSPS* gene coding for a glyphosate insensitive EPSPS enzyme. The transgenic construct is under expression of a CaMV 35S promoter a *nos* transcriptional termination element from *Agrobacterium tumefaciens*. Horizontal gene transfer experiments with the *EPSPS* gene of the RR soybean were performed under controlled laboratory conditions and were targeted to the nitrogen fixing symbiont of soybean *Bradyrhizobium japonicum*. This bacterium comprises the requirements of a possible receptor for the glyphosate resistance trait, as it is sensitive to the herbicide and thus the acquirement of glyphosate resistance would signify a positive adaptation to glyphosate accumulated in the roots after herbicide application. Two key conditions for gene transfer from the *CP4-EPSPS* gene from the RR soybean to *B. japonicum* were evaluated in this study: The required specific conditions for *B. japonicum* to undergo natural transformation and the expression of the *CP4-EPSPS* gene in *B. japonicum*. For that purpose, the *CP4-EPSPS* gene was cloned into a *B. japonicum* chromosomal integration vector and was transferred by biparental mating into the *B. japonicum* genome. Subsequently, the expression of the *CP4-EPSPS* gene in *B. japonicum* was tested under increasing glyphosate selection pressure. Results of these experiments indicated that *B. japonicum* is not naturally transformable under any conditions known from

the more than 40 so far reported naturally transformable bacteria. Furthermore, the *CP4-EPSPS* genetic construct, as contained in RR soybean, has been shown in this study to be not active in *B. japonicum*. Consequently, if there would be a gene transfer of the plant *CP4-EPSPS* to *B. japonicum*, this genetic construct does not confer glyphosate resistance to *B. japonicum* and does not constitute any adaptive advantage to the bacterium under glyphosate selection pressure. As the genetic trait of glyphosate resistance has been found in several bacteria, it would be more probable that the common mating exchange between bacterial groups could disperse the glyphosate resistance within an environment. Moreover, in the specific case of *B. japonicum*, a high spontaneous mutation rate for glyphosate resistance was observed, suggesting that *B. japonicum* can also adapt to the glyphosate selection pressure by mutation under natural conditions.

The impact of transgenic plants with their respective herbicide treatments on root associated bacteria was investigated in a greenhouse experiment. The composition and diversity of bacterial communities of RR soybean rhizospheres were analyzed and compared between glyphosate-treated and untreated plants. Samples from five harvests with two glyphosate applications were analysed by 16S rRNA gene T-RFLP analysis complemented with the evaluation of three clone libraries. Multivariate statistical analysis of the data was used to visualize changes in the microbial populations in response to glyphosate applications and in order to find groups of organisms responsible for the observed community shifts. A comparison of the rhizosphere communities revealed that a *Burkholderia* related group was significantly inhibited by glyphosate application, while the abundance of a group of *Gemmatimonadetes* related sequences increased significantly after the herbicide treatment. The significant increment of *Gemmatimonadetes* abundance after glyphosate application could indicate that these organisms are able to metabolize the herbicide. Shannon diversity indices were calculated based on the T-RFLP results with the aim to compare bacterial diversity in the rhizosphere of glyphosate-treated and non treated RR soybeans. Interestingly, the bacterial community associated to RR soybean roots after glyphosate application not only demonstrated effective resilience after the disturbance but in addition the bacterial diversity also increased in comparison to the untreated control samples. It is possible, that in an environment with organisms which are able to metabolize glyphosate, the key for enhancing diversity could be the succession of metabolites, which can be further utilized by a diverse range of bacteria.

Zusammenfassung

In dieser Arbeit wurden zwei Fragestellungen bezüglich transgener Pflanzen, die von erheblichem öffentlichen Interesse sind, bearbeitet: Die Möglichkeit eines Gentransfers von diesen Pflanzen zu Bodenbakterien, sowie der Einfluss transgener Pflanzen in Kombination mit Herbizidapplikationen auf die bakterielle Gemeinschaft der Rhizosphäre. Als Versuchspflanze wurde eine transgene Roundup Ready® (RR) Sojabohne verwendet, welche tolerant gegenüber dem Herbizid Glyphosat ist. Diese Pflanze ist die weltweit am häufigsten angebaute genetisch veränderte Pflanze. Glyphosat, der aktive Wirkstoff von Roundup Ready®, inhibiert das Enzym EPSPS (5-Enolpyruvylshikimat-3-phosphate Synthase). EPSPS ist ein Enzym des Shikimatweges, der für die Bildung aromatischer Aminosäuren verantwortlich ist. Die Inhibierung dieses Enzyms führt zu einer Verringerung des Wachstums von Pflanzen und Mikroorganismen. Durch die Anwesenheit eines Glyphosate-toleranten EPSPS-Enzymes sind RR-Pflanzen gegenüber Glyphosat tolerant. RR Pflanzenlinien beinhalten das *CP4-EPSPS*-Gen, welches ursprünglich aus *Agrobacterium sp.* CP4 stammt, jedoch den CaMV 35S Promotor sowie den Nos Terminator enthält. Experimente über die Möglichkeit eines horizontalen Gentransfers des *EPSPS* Gens von RR Soja wurden unter kontrollierten Laborbedingungen durchgeführt. Das stickstofffixierende, symbiontische Bakterium, *Bradyrhizobium japonicum*, erfüllt wichtige Voraussetzungen eines möglichen Empfängers der Glyphosatresistenz, da es sensitiv gegenüber dem Herbizid ist und somit die Erlangung einer Glyphosatresistenz eine positive Anpassung an erhöhte toxische Mengen des Wirkstoffes in den Wurzeln nach Herbizidapplikation darstellt. Zwei wichtige Bedingungen für den Gentransfer des *CP4-EPSPS* Gens von RR Soja zu *B. japonicum* wurden in dieser Arbeit überprüft: Die von *B. japonicum* benötigten spezifischen Bedingungen für die Erlangung einer natürlichen Transformierbarkeit und die Expression des *CP4-EPSPS* Gens in *B. japonicum*. Ergebnisse dieser Experimente belegen, dass unter den bekannten Bedingungen für natürliche Transformation *B. japonicum* nicht transformierbar ist. Es ist jedoch nicht

auszuschließen, dass *B. japonicum* unter bisher unbekannten Bedingungen mit *CP4-EPSPS* transformiert werden könnte. Des Weiteren wurde untersucht, ob ein *CP4-EPSPS* Gen integriert in das Genom von *B. japonicum* von diesem Bakterium exprimiert wird und dadurch zu einer Glyphosattoleranz führen kann. Für diesen Zweck wurde *CP4-EPSPS* in einen chromosomalen Integrationsvektor für *B. japonicum* kloniert und durch biparental mating in das *B. japonicum* Genom transferiert. Anschließend wurde die Expression des Gens in *B. japonicum* unter ansteigendem Selektionsdruck durch Glyphosat getestet. Es konnte jedoch keine erhöhte Glyphosate Resistenz bei Anwesenheit dieses Genkonstruktes aus RR Soja in *B. japonicum* festgestellt werden. Folglich würde ein möglicher Gentransfer des pflanzlichen *CP4-EPSPS* zu *B. japonicum* zu keiner Glyphosatresistenz führen und somit keinen Anpassungsvorteil für das Bakterium unter Glyphosatselektionsdruck darstellen. Eine Übertragung der genetischen Eigenschaft der Glyphosatresistenz, welche in mehreren Bakterien gefunden wurde, würde sich daher wahrscheinlich eher durch herkömmliches Maiting zwischen den Bakteriengruppen in der Umwelt verbreiten, als durch eine Übertragung von Pflanze zu Bakterium. Die Auswirkung der Anpflanzung von RR Soja mit Glyphosatbehandlung auf die wurzelassoziierten Bakterien wurde in einem Gewächshausversuch untersucht. Die Zusammensetzung und Diversität der bakteriellen Gemeinschaft der Rhizosphäre von RR Soja wurde analysiert und zwischen Glyphosat behandelten und unbehandelten Pflanzen verglichen. Proben von fünf Zeitpunkten und zwei Glyphosatapplikationen wurden mittels 16S rRNA Gen T-RFLP Methode analysiert und ergänzt durch die Erstellung und Auswertung dreier Klonbanken. Multivariate statistische Datenanalysen wurden verwendet, um Veränderungen der bakteriellen Populationen durch die Glyphosatbehandlung zu visualisieren und um Indikatorgruppen von Organismen zu identifizieren, die von diesen Veränderungen betroffen sind. Die Analysen zeigten, dass eine *Burkholderia*-ähnliche Gruppe signifikant durch Glyphosatapplikation inhibiert wurde, während die Häufigkeit *Gemmatimonadetes* verwandter Sequenzen signifikant nach Herbizidanwendung anstieg. Der signifikante Anstieg dieser Gruppe könnte andeuten, dass diese Bakterien fähig sind das Herbizid zu metabolisieren.

Shannon Diversitätsindizes wurden anhand der T-RFLP Profile berechnet, mit dem Ziel, die Diversität der bakteriellen Rhizosphärengemeinschaften zwischen Glyphosat behandelten und unbehandelten RR Sojabohnen zu vergleichen. Interessanterweise zeigte die bakterielle

Gemeinschaft der RR Sojarhizosphäre nach Glyphosatapplikation nicht nur eine effektive Resilienz nach der Störung, sondern es konnte sogar eine Erhöhung der Diversität im Vergleich zu unbehandelten Proben festgestellt werden. Folglich ist es möglich, dass diverse Bakterien, die fähig sind über eine Suksession von Metaboliten des Glyphosates abzubauen, zur Erhöhung der Diversität der bakteriellen Gemeinschaft beitragen.

1 Introduction

1.1 Worldwide situation of genetically modified crops

The global area planted with genetically modified (GM) crops has consistently increased each year since GM crops were first commercially cultivated in 1996. Actually, approximately 125 million hectares were planted with GM crops in 25 biotech crop countries in 2008 (James, 2008) (Figure 1.1). The United States of America, followed by Argentina, Brazil, Canada, India and China, continue to be the principal adopters of biotech crops globally; with the USA retaining its world leading position with 62.5 million hectares. Herbicide tolerance has consistently been the dominant transgenic trait, accompanied by increasing application of herbicides (James, 2008). Since its commercial introduction in 1974, glyphosate has become the dominant herbicide worldwide (Woodburn, 2000). Nevertheless, the most important aspect of the success of glyphosate has been the introduction of transgenic, glyphosate-tolerant crops. Nowadays almost 90% of all transgenic crops grown worldwide are glyphosate tolerant, and the adoption of these crops is steadily increasing at a steady pace (Duke and Powles, 2008). Glyphosate products are manufactured by Monsanto Company worldwide marketed under the trade name Roundup®. The most abundant genetic modified crop worldwide continues to be Roundup Ready® (RR) soybean (*Glycine max* L. Merr.), occupying 65.8 million hectares globally (53 % of total biotech area) (James, 2008) (Figure 1.1).

Despite the potential benefits of this new technology to improve the reliability and quality of the world food supply , the possibility of non target effects of transgenic plants on native flora and fauna, including soil microorganisms, is one cause of concern in environmental risk assessments of transgenic plants (Wolfenbarger and Phifer, 2000). Lack of evidence about safety assessments have limited the acceptance of genetically modified plants and food products, particularly in Europe and Japan (Knight et al., 2007; Nishiura et al., 2002; Ronteltap et al., 2007).

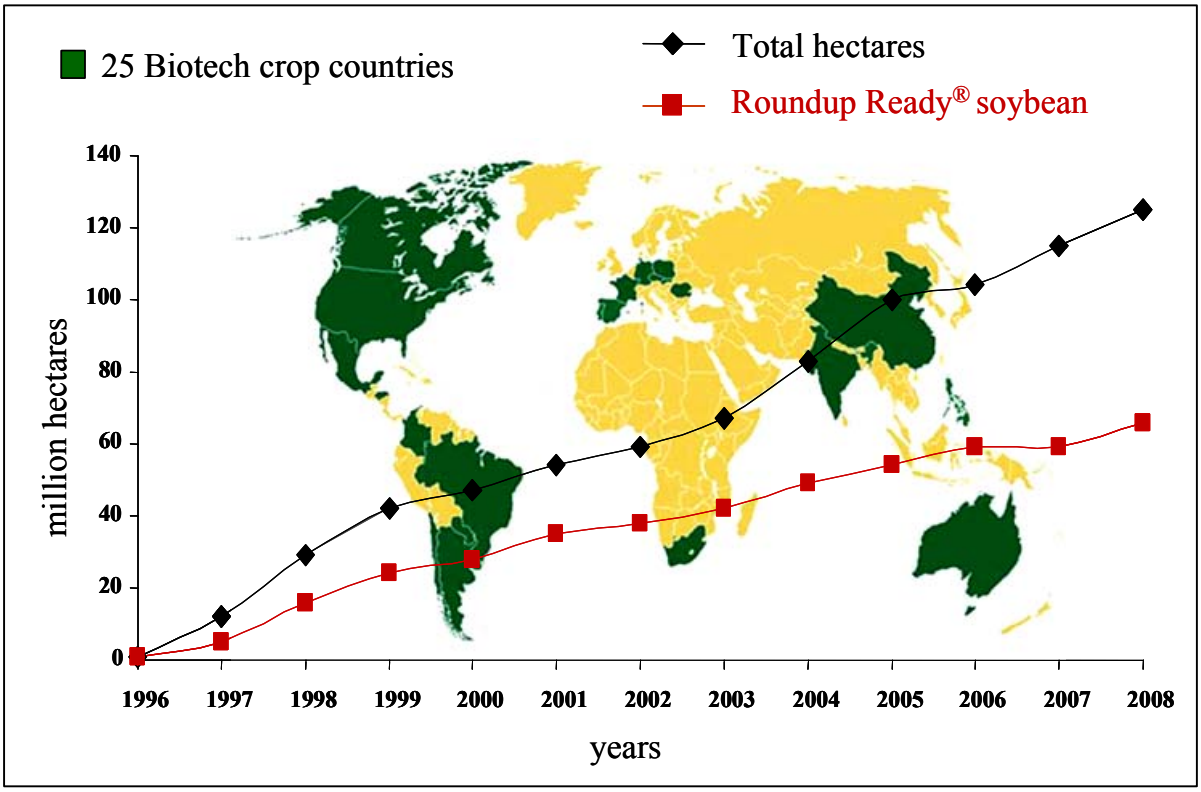

Fig. 1.1. Global area of transgenic crops in the years 1996 to 2008 (modified from James (2008). Area in green indicates the 25 countries where transgenic crops are planted. The black rhombus connected with a black line designate the area (in millions of hectares) planted with transgenic crops per year. The red squares linked with a red line show the area (in millions of hectares) cultivated with RR soybean.

1.2 Glyphosate mode of action and transgenic resistance mechanism

Glyphosate (N-phosphonomethylglycine) is a broad-spectrum, nonselective herbicide that controls a wide range of weeds (Duke and Powles, 2008). Furthermore, glyphosate is the most widely used herbicide in the world, largely due to the increasing acceptance of Roundup Ready® crops (Yu et al., 2007). Glyphosate, active ingredient of Roundup Ready®, inhibits the EPSPS enzyme (5-enolpyruvylshikimate-3-phosphate synthase) in plants and several micro-organisms (Amrhein et al., 1980). EPSPS is an enzyme involved in the shikimic acid pathway leading also to the aromatic amino acid biosynthesis. Bacteria, fungi and plants are capable of synthesizing the three aromatic amino acids phenylalanine, tyrosine, and tryptophan *de novo* (Braus, 1991).

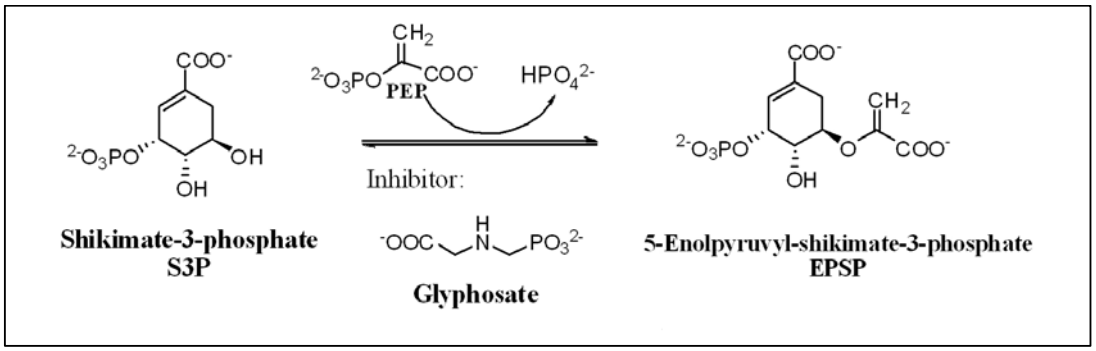

Fig. 1.2. Reaction catalyzed by the enzyme EPSPS (Funke et al., 2006).

Inhibition of the shikimate pathway leads to general metabolic disruption and several associated metabolic disturbances, including the arrest of protein production and prevention of subsequent product formation like vitamins, lignins, alkaloids, and phenolics (Dill, 2005). EPSPS catalyzes the transfer of the enolpyruvyl group from phosphoenol pyruvate (PEP) to the 5-hydroxy position of shikimate 3-phosphate (S3P) to form the products 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate (Pi). The mechanism of inhibition is also unique in that the binding site for glyphosate is reported to overlap closely with the binding site of PEP (Funke et al., 2006) (Figure 1.2).

Glyphosate is competitive with respect to the PEP binding to EPSPS and the resulting glyphosate:EPSPS:S3P complex is very stable, has a very slow reversal rate and therefore inhibits the EPSPS catalyzed reaction (Kishore and Shah, 1988).

The strategy behind commercial glyphosate resistance, marketed in multiple crops under the Roundup Ready® brand name (RR), is the introduction of a glyphosate insensitive EPSPS. RR crop lines contain a gene derived from *Agrobacterium* sp. strain CP4, encoding a glyphosate-tolerant enzyme, the so-called CP4-EPSPS (Padgette et al., 1995). The RR soybean was produced by inserting the glyphosate resistant *CP4-EPSPS* under expression of an enhanced 35S promoter from cauliflower mosaic virus (CaMV) and a nopaline synthase (*nos*) transcriptional termination element from *Agrobacterium tumefaciens* (Padgette et al., 1995). The result is the ability to overcome the endogenous EPSPS system with the CP4-EPSPS insertion that allows the shikimate pathway to function normally in the presence of glyphosate (Fig. 1.3) (Dill, 2005).

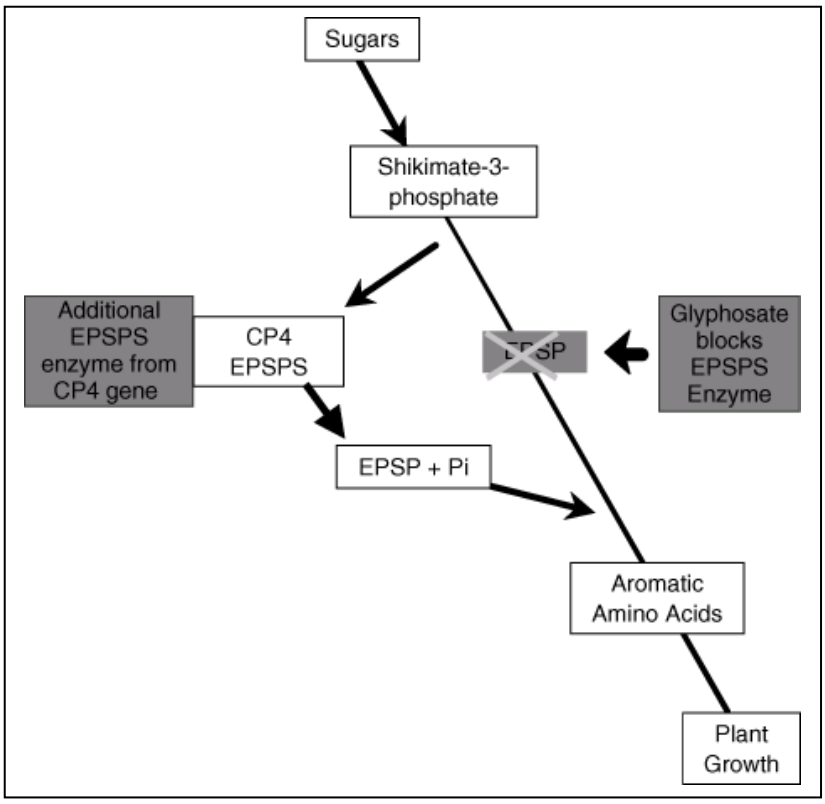

Fig. 1.3. Strategy for the development of glyphosate resistant crops (Dill, 2005). Glyphosate resistant crops overcome glyphosate inhibition of the endogenous EPSPS system through the CP4-EPSPS insertion that allows the shikimate pathway to function normally.

1.3 Horizontal gene transfer in the rhizosphere

One of the major concerns raised by the use of genetically modified plants in agriculture is the ecological impact of engineered genes if they are disseminated into the environment. Besides pollen hybridization between related species, plants do not have any identified mechanism to facilitate gene transfer, so that the possibilities and barriers of genetic transfer from plants to bacteria have been approached based on known genetic mechanisms between microorganisms (Nielsen et al., 1998). The exchange of DNA among organisms of different species is known as horizontal gene transfer (HGT). Genome sequence comparisons have already demonstrated that a high percentage of the genes of prokaryotes results from HGT (Narra and Ochman, 2006), which has motivated a broad discussion about concepts of bacterial adaptation, evolution and speciation (de la Cruz and Davies, 2000; Gogarten et al., 2002; Gogarten and Townsend, 2005; Sorensen et al., 2005).

Three well-studied processes are responsible for most naturally occurring DNA transfer in microorganism: Transduction by bacterial viruses, conjugation by bacterial plasmids and natural transformation (Lorenz and Wackernagel, 1994b). In natural systems, most of the studies have focused on the conjugal transfer of plasmids (Langergraber et al., 2003; Rosewich and Kistler, 2000), with the benefit that a gene is moved within a broad-host-range plasmid that might be spread without the need of recombination (Thomas and Nielsen, 2005). The rise and spread of multiple antibiotic resistance plasmids in medically important bacteria is a consequence of bacterial conjugation coupled to selective pressures achieved by the increased use of antibiotics in medicine (Beaber et al., 2004). Similarly, the evolution of plasmids conferring xenobiotic degradative function is a response to the increasing presence of xenobiotic pollutants in soil and water (Davison, 1999).

In the case of plant DNA transfer to bacteria, the expected transfer mechanism is the natural transformation, which implies the active uptake of free plant DNA by bacterial cells and the heritable incorporation of its genetic information (Lorenz and Wackernagel, 1994a). Nucleotide sequences in databases indicate that some genes in soil bacteria genomes were originated from integration of plant genetic material (Doolittle et al., 1990; Smith et al., 1992; Wakabayashi et al., 1986). Bacteria are the only organisms capable of natural transformation (Lorenz and Wackernagel, 1994b). However the DNA uptake by natural transformation depends on the development of a physiological state of competence and on the similarity of the exchangeable sequences (Bertolla and Simonet, 1999; Lorenz and Wackernagel, 1994b). The transient conditions to reach the natural competent state are unique for each bacterium and can be reached at early growth stages, during the late exponential growth phase or in the stationary phase (Lorenz and Wackernagel, 1994b). Once the single-stranded DNA was taken up by the bacteria, it can either be stably integrated into the bacterial genome by homologous recombination (de Vries and Wackernagel, 2002), or form an autonomous replicating element (Bertolla and Simonet, 1999). Most bacterial recombination is homologous; that means that two recombining segments have identical or almost identical sequences and base pairing between their strands replaces one with the other (Meier and Wackernagel, 2003). The decline in recombination between organisms with increasing sequence divergence maintains the separation of species (Fraser et al., 2007). Consequently, non-homologous or illegitimate recombination is a less-common process (Meier and Wackernagel, 2003), in which two

unrelated sequences become connected either by incorrect rejoining of broken ends or by insertion of one DNA segment into another (Redfield, 2001). According to the model of homology-facilitated illegitimate recombination, homologous DNA serves as a recombination anchor for homologous exchange and facilitates an illegitimate recombination event on the same DNA molecule leading to its integration (de Vries and Wackernagel, 2002). The stable maintenance of DNA in bacteria requires ultimately that the DNA in the cytoplasm is linked to an origin of replication, such as via integration into the chromosome or into a plasmid (Nielsen et al., 1997). In the end, gene transfer events from plants to bacteria have remained rare and difficult to detect, since after the novel DNA is introduced into the bacterial cell and integrated into the bacterial chromosome, the plant gene must be able to be expressed by the bacterium (Lorenz and Wackernagel, 1994b).

The experimental demonstration of HGT from plant DNA to bacteria has been performed by several groups using a marker rescue system. The strategy requires the presence of the same marker gene in donor and recipient organisms that generates the required homologous sequences to improve recombination in the bacterial receptor genome (Gebhard and Smalla, 1998). Accordingly, the transgenic plant and the recipient bacterium were engineered with the same marker gene, but the bacterial target gene carried a deletion. Using homologous recombination, the deletion in the bacterial marker gene was complemented with the plant donor DNA. This strategy has been successfully applied in high natural transformable strains of *Acinetobacter* sp. and *Pseudomonas* sp under *in vitro* conditions. (de Vries and Wackernagel, 1998; Gebhard and Smalla, 1998 ; Kay et al., 2002; Meier and Wackernagel, 2003; Nielsen et al., 1997).

Limiting factors in nature for a HGT have been broadly reviewed, concluding that essential conditions for genetic transfer are available in soil (Thomas and Nielsen, 2005). These essential circumstances for a HGT comprise the availability of free DNA over years in soil (Wackernagel, 2006), the development of bacterial competence under natural conditions (Chen and Dubnau, 2004) and the stable integration of the captured DNA (Nielsen et al., 2001). On the other hand, other authors supported that the soil is an unlikely environment for transformation-mediated gene transfers (Bertolla and Simonet, 1999; Stotzky, 1989), while much more favorable conditions could be encountered in association with plant tissues in which saprophyte, symbiotic or pathogenic bacteria multiply actively (Bertolla et al., 2000).

Additionally plant roots provide good conditions for bacterial growth, colonization and mating, resulting in the occurrence of locally enhanced densities of active cells. These sites are often conducive to horizontal gene transfer processes and have been identified as hot spots for bacterial gene transfer activity (van Elsas et al., 2002).

Besides the important role of the bacterial competent state and the sequence homology in HGT, it is interesting that not all genes are equally likely to be transferred, as the transfer of genes seems to be strongly correlated with the gene function (Brown and Doolittle, 1997; Feng et al., 1997; Koonin et al., 1997; Smith et al., 1993). A consequence of gene transfer is that bacterial genomes can be regarded as core and accessory genomes (Young et al., 2006). The core genome includes genes participating in transcription, translation, and most essential processes for bacteria survival. The core genome might be the basis for a species that maintain coherence through homologous recombination (Prosser et al., 2007). Core genome genes are far less likely to be horizontally transferred than genes participating in special ecological adaptations that can be gained and lost, described as operational genes (Jain et al., 1999; Rivera et al., 1998). The role of horizontal gene transfer in introducing operational genes into bacterial chromosomes lead to reconsider the adaptation benefits that those operational genes can provide to bacteria in its ecological niche(Lawrence, 2002; Offre et al., 2007) . Finally, the most critical factor required for the establishment of adaptation benefits in fitness-dependent environments is the selection pressure (Rainey et al., 2000).

1.4 *Bradyrhizobium* as candidate for horizontal gene transfer of the *CP4-EPSPS* from RR soybean

With the annual increment of RR soybean plantations accomplished by glyphosate applications, optimal spatial and selective conditions for HGT to glyphosate sensitive bacteria associated to soybean are achieved (Zablotowicz and Reddy, 2004). Glyphosate restrains the symbiosis between soybean plants and the nitrogen fixing bacterium *Bradyrhizobium japonicum*. This bacterium possesses a glyphosate sensitive EPSPS, which resulted in growth inhibition and death at high glyphosate concentrations (Padgette et al., 1995). Symbiotic nitrogen fixation in soybeans represents about 40% to 70% of the plant nitrogen requirement

(Klubek et al., 1988), which can be important for economically sustainable soybean yields, especially in soils containing low available soil nitrogen (Moorman et al., 1992).

Close physical contact between donor DNA and receptor competent cells is required for transformation (Lorenz and Wackernagel, 1994b). This prerequisite for gene transfer in nature is best met by bacteria, that have specific symbiotic or pathogenic relationships with plants (Kay et al., 2002), as *Bradyrhizobium*. The required RR-soybean DNA availability in soil for a HGT has been demonstrated given that recombinant *CP4-EPSPS* genes were detected in soil for at least 30 days during RR soybean leaf biomass decomposition (Levy-Booth et al., 2008a) and aggregate formation facilitated the persistence of recombinant RR-soybean DNA (Levy-Booth et al., 2008b). Although the selection pressure in soil seems to be much lower than inside the plant, since a concentration of 400 μM glyphosate has been measured in RR nodules, what is 100 times higher than the concentration of glyphosate in the first two centimetres of soil (Grundmann et al., 2008). Initial inhibition of *B. japonicum* growth induced by glyphosate has been observed at 200 μM (Wagner et al., 2008) and complete inhibition at 5 mM glyphosate (Moorman et al., 1992; Zablotowicz and Reddy, 2004), meaning that the glyphosate concentration in the nodules (400 μM) constitutes a selection pressure for this bacterium. Several autors support that glyphosate can reduce nodulation and nitrogen fixation activity in glyphosate-resistant soybean (King et al., 2001; Motavalli et al., 2004), although these effects on the *Bradryhizobium japonicum*-soybean symbiosis have not been consistently observed (Weaver et al., 2007; Zablotowicz and Reddy, 2004).

A homologous recombination between the transgene *CP4-EPSPS* and the bacterial gene is also facilitated due to the fact that sequence homologies among the bacterial, fungal, and plant EPSP synthases are substantial (Gasser et al., 1988; Stallings et al., 1991). Additionally, high similarity between regions of the *CP4-EPSPS* inserted into the RR soybean and the *EPSPS* gene of *B. japonicum* has been reported (Wagner et al., 2008). Thus, the genetic transfer of *CP4-EPSPS* could provide the transformed bacteria a selective advantage in an environment under glyphosate selection pressure; nevertheless under field conditions several studies monitoring over years the horizontal gene transfer from transgenic plant DNA to soil bacteria did not confirmed any genetic transfer. No HGT to soil bacteria could be evidenced from the *EPSPS* gene from RR soybean (Wagner et al., 2008), the ampicillin resistance gene from

Bt176 transgenic corn (Badosa et al., 2004) and the gentamicin resistance gene from transgenic tobacco (Paget et al., 1998).

1.5 Rhizosphere and rhizodeposition of translocated glyphosate

The definition of the rhizosphere, as stated by Lorenz Hiltner (Hiltner, 1904), describes the soil portion surrounding plant roots that is influenced by the living root (Hartmann et al., 2008a). The release of carbon compounds from living plant roots (rhizodeposition) leads to a proliferation of microorganisms within the roots (endorhizosphere), on their surface (rhizoplane) and outside the root in its close vicinity (ectorhizosphere) (Jones et al., 2004). Between 40% and 90% of the carbon transferred to the root is lost as rhizodeposition into the soil and thus represents a considerable factor, which controls microbial activity and diversity associated to plant roots (Lynch and Whipps, 1990).

The chemical components released from roots can be classified into the following four groups, depending on their mode of exudation: 1) water soluble exudates, such as sugars, amino acids, organic acids, hormones and vitamins, which leak from roots without involvement of metabolic energy; 2) secretions, like polymeric carbohydrates and enzymes depend upon metabolic processes for their release; 3) lysates are released when cells autolyse including cell walls, and with time whole root segments; 4) gases such as ethylene and CO_2 (Lynch and Whipps, 1990). These chemical components are released during all stages of plant growth and development with the balance of these various processes changing dependent on the age of the plant (Jones et al., 2004). It is a common fact, that the rhizosphere soil has much higher numbers of metabolically active microorganisms than the surrounding bulk soil. Studies have demonstrated increases greater than 100-fold in microbial counts (Macek et al., 2000). Microbes colonizing root surfaces are exposed to the high concentrations of products released from the roots (Hartmann et al., 2008b). Since bacteria respond differently to these compounds, differences in the composition of root exudates influence the types of bacteria present in the rhizosphere community (Bardgett et al., 1999; Dunfield and Germida, 2001). Plant species differ in both, the quantity and quality of root exudates (Uren, 2007). Furthermore, root zone and soil type (Fang et al., 2005) as well as the interactions between

these variables induce significant effects on community structure (Hartmann et al., 2008b; Marschner et al., 2001).

Glyphosate, as foliar-applied herbicide, is symplastically translocated to the meristems of growing plants (Rubin et al., 1982; Zablotowicz and Reddy, 2004). After foliar application, glyphosate is taken up relatively rapidly through plant surfaces (Kirkwood et al., 2000). Due to its physicochemical properties, glyphosate is translocated from the leaf via the phloem to the same tissues that are metabolic sinks for sucrose (Duke and Powles, 2008). After glyphosate is translocated to young growing tissues of roots and shoots, the herbicide accumulates in millimolar concentrations in the plant tissue (Hetherington et al., 1999; Preston and Wakelin, 2008). Significant amounts of applied glyphosate has been reported to be released into the rhizosphere of soybean (Kremer et al., 2005). Similarly, high accumulation of glyphosate and its degradation product aminomethylphosphonic acid (AMPA) have been found in soybean root nodules (Grundmann et al., 2008). AMPA is the principal product of glyphosate degradation in soils (Börjesson and Torstensson, 2000; Heinonen-Tanski et al., 1985) followed by sarcosine, glycine and CO_2 (Gimsing et al., 2004). Yet, leaf uptake, translocation rates and glyphosate accumulation can vary considerably between plant species. Maximum translocation rates were obtained in beggarweed three days after glyphosate application (Sharma and Singh, 2001). In *Agropyron repens,* a perennial grass weed, glyphosate absorption did not increase beyond three days after application and translocation to the rhizomes continued up to seven days after application (Devine and Bandeen, 1983). In RR soybean two weeks after herbicide application, the ratio between glyphosate and AMPA was balanced indicating a fast degradation of glyphosate (Grundmann et al., 2008). A study about glyphosate translocation in transgenic maize expressing the *CP4-EPSPS* determined that younger shoot tissues and roots were major sinks for translocated glyphosate, accumulating 25 to 40% of the foliar applied dose (Hetherington et al., 1999). Similar rates of absorption and accumulation to those in maize have been reported for barnyardgrass (Kirkwood et al., 2000).

After glyphosate application, the highest concentration of glyphosate in soils is located in the upper soil layer (0-2 cm) (Grundmann et al., 2008) and the transport of ^{14}C-residues through the soil is so low that glyphosate or AMPA could not be detected in soil leachates (Klier et al., 2008). Therefore, it can be hypothesized that glyphosate applied directly to the soil is less important for root associated microbes than the effect of foliar applied glyphosate. It has been

also shown that translocated glyphosate is acummulated in roots and nodules of RR soybean and is a component of the rhizodeposition (Grundmann et al., 2008; Kremer et al., 2005).

1.6 Effects of glyphosate on non target organisms

Intensive cultivation of glyphosate-resistant crops and frequent use of glyphosate to control weeds in these crops could lead to undesirable ecological side effects (Freckman and Ettema, 1993). Additionally, the alertness regarding weeds resistant to glyphosate due to herbicide selection pressure is increasing (Powles et al., 1998) and some weeds, such as redvine, *Brunnichia ovata*, are becoming limiting production factors. Others such as *Conyza canadensis*, *Abutilon theophrasti* (Owen and Zelaya, 2005), *Lolium rigidum* (Powles et al., 1998), *Convolvulus arvensis*, *Amaranthus* spp., *Ipomoea* spp., *Chenopodium album*, *Commelina communis*, *Dicliptera chinensis* and *Eleusine indica* (Sandermann, 2006) have developed glyphosate resistance and will require new management strategies.

Additionally it is accepted knowledge that anthropogenic influences such as agricultural management, including herbicide applications, can have a profound impact on bacterial communities leading to reduced bacterial diversity (Borneman, 2004; Torsvik et al., 1996). As soil microorganisms are typically the first organisms to react to changes in the environment (Paul, 1984), effects of agricultural management on the ecosystem are assessed by monitoring key ecological indicators, such as soil microbial activity, biomass, respiration and the abundance of bacteria and fungi (Chen and Edwards, 2001; Johnsen et al., 2001; Schutter et al., 2001). Most studies have shown that the use of herbicides at recommended application rates does not adversely affect soil microbial communities (Biederbeck et al., 1987; Busse et al., 2001; Ernst et al., 2008; Li et al., 2004; Liphadzi et al., 2005; Wardle and Parkinson, 1990). Some experiments confirmed no significant effects of glyphosate on soil microbial community when applied at suggested rates (Busse et al., 2001; Locke et al., 2008; Olson and W., 1991; Santos and Flores, 1995). Contrasting results indicated that glyphosate application can increase soil microbial biomass, respiration and carbon and nitrogen mineralization (Haney et al., 2002a; Haney et al., 2002b; Haney et al., 2000) and can stimulate individual organisms in the endophytic bacterial community of RR soybean plants (Kuklinsky-Sobral et al., 2005).

The growing awareness of non-target effects of glyphosate (Heatherly et al., 2002) is sustained by case studies demonstrating different reactions of microorganisms to this herbicide. Although glyphosate inhibits the EPSPS of many bacteria (Padgette et al., 1995), it is moderately biodegradable by some soil bacteria (Peterson and Shama, 2005). *Agrobacterium* CP4, the source of the transgene *CP4-EPSPS* contained in glyphosate-resistant plants (Padgette et al., 1995), is the first example of such a bacterium. Several *Agrobacterium* sp. are able to degradate glyphosate and can utilize it as sole source of phosphorus in the presence of the aromatic amino acids (Liu et al., 1991). Glyphosate is representative of a broad class of compounds, known as phosphonic acids, which contain a direct carbon-to-phosphorus (C-P) bond. Although the C-P bond is chemically and thermally very stable, several bacteria have the ability of enzymatically cleaving the bond and thereby liberating inorganic phosphate (Ermakova et al., 2008).

Increased colonization of roots by pathogenic organisms belonging to the fungal genus *Fusarium* and the oomycetous genera *Phytophthora* (Lévesque and Rahe, 1992) and *Pythium* (Descalzo et al., 1998) have been observed after glyphosate treatments. This is possibly due to the fact that glyphosate blocks the production of phenolics involved in disease resistance of plants to these pathogens (Lévesque and Rahe, 1992). On the other hand, root infection may be due to decrease in the production of antifungal compounds like phytoalexins, which are synthesized by the plant via the shikimate pathway (Sharon et al., 1992). Moreover, for several *Fusarium* strains it has already been elucidated that they are able to biodegrade glyphosate and use the herbicide as a phosphorous source (Castro et al., 2007). On the contrary, other pathogenic fungi, like *Phakopsora pachyrhizi*, *Puccinia striiformis* and *Puccinia triticina*, which are causal agents of rust diseases in RR soybean, can be controlled by glyphosate application (Feng et al., 1997).

1.7 Methodological approach for analysing bacterial diversity

In recent years, microbial diversity has turned out to be an important issue due to the importance of microorganisms in energy and organic matter transformation (Hunter-Cevera, 1998). Studies about the bacterial community structure and function are fundamental to the understanding of relationships between environmental factors and the functioning of ecosystems. Such knowledge can be used to assess the effect of environmental stress and perturbations like pollution, agricultural exploitation and global change on ecosystems (Torsvik et al., 1990). In microbial terms, biodiversity describes the number of different phylotypes (species) and their relative abundance in a given community of a given habitat (Garbeva et al., 2004). Yet, the concept of species for prokaryotes is still controversial since asexual organisms without isolation by the barrier of recombination violate genetic assumptions for the species term (Kassen and Rainey, 2004). The ecological species concept defines a species as a set of organisms that are considered identical in all relevant ecological properties (Cohan, 2002). The molecular phylogenetic approach, combined with a polyphasic taxonomy using physiological and molecular genetic data, uses the similarity of the genetic structure of microbes, which is most often determined in phylogenetic marker genes like the ribosomal genes (Amann et al., 1995). Molecular phylogenetic methods have also provided means for identifying the types of organisms that occur in microbial communities avoiding the limitations of cultivation (Hugenholtz and Pace, 1996). Estimations of microbial diversity are mostly based on 16S rRNA sequences, which provide an operational measure of species (Prosser et al., 2007). Thus, the bacterial diversity can be defined as the number and distribution of different sequence types present in the DNA extracted from the community in the given habitat (Garbeva et al., 2004). Molecular community analysis techniques have already proven to be powerful tools for comparing microbial community structures (Kent and Triplett, 2002; Olsen et al., 1994; Winderl et al., 2008). The initial steps of these methods include the extraction of the total microbial DNA followed by the PCR amplification of the 16S rRNA genes from the community DNA using universal or group specific primers (Griffiths et al., 2000). The terminal restriction length polymorphism (T-RFLP) analysis is a method that yields community profiles with end-labeled PCR amplified products that are cut with restriction enzymes producing end-labeled terminal restriction fragments (T-RF) (Liu et

al., 1997). Restriction fragment lengths can be determined for the entire ribosomal database and, therefore, provide a logical phylogenetic starting point (Hugenholtz et al., 1998). Experimentally, restriction fragment lengths can be determined down to ±1.5 bases using sequencing gel technology (Jones and Thies, 2007). Moreover, the measure of each terminal restriction fragment of every 16S rRNA gene represents a single operational taxonomic unit (OTU). This, in turn, provides a quantitative basis for contrasting bacterial diversity, since the identified OTUs allow comparison of the following three elements of diversity in a sample: the types of bacteria present (composition), the number of types (richness), and the frequency distribution or relative abundance of types (structure) (Dunbar et al., 1999; Hedrick et al., 2000). For diversity estimation based on OTUs, the Shannon index of diversity is recognised as a sensitive indicator of perturbation when comparing bacterial biodiversity in rhizosphere environments (De Leij et al., 1994; Lynch, 2002). The T-RFLP method is recommended as a comparative analysis (Brown et al., 2005), however one has to keep in mind that it is not a tool to describe the absolute richness of a microbial community, since it captures only the dominant members of complex assemblages. It therefore always underestimates actual diversity in a habitat (Bent et al., 2007). Nonetheless, T-RFLP analyses can be an effective tool for analyzing microbial communities when used in conjunction with gene sequence information. This allows the prediction of T-RF sizes and therefore the assignment of identity to individual peaks in a profile (Clement et al., 1998; Osborne et al., 2006; Thies, 2007).

1.8 Problem statement and objectives of this work

1.8.1 Horizontal gene transfer of *CP4-EPSPS* to *Bradyrhizobium japonicum*

The increase in planting of transgenic crops resistant to glyphosate accompanied by the extensive use of this herbicide generates agricultural environments with more or less severe glyphosate selection pressure (James, 2008). A gene transfer from the transgene *CP4-EPSPS*, which confers glyphosate resistance, to herbicide sensitive bacteria may result in a beneficial adaptation for survival in environments under these environmental conditions. *Bradyrhizobium japonicum*, the nitrogen fixing symbiont of soybean, comprises the requirements of a possible recipient for the glyphosate resistance trait. This bacterium is sensitive to the herbicide, thus the acquirement of glyphosate resistance signifies a positive adaptation to accumulated glyphosate present in the nodules after foliar herbicide application (Grundmann et al., 2008). The high sequence homology required for a horizontal gene transfer (Lorenz and Wackernagel, 1994b) is also fulfilled by the presence of donor and receptor *EPSPS* gene similarity of the RR soybean and *Bradyrhizobium*. There are high homologies between the glyphosate resistant *CP4-EPSPS* donor gene, originated from *Agrobacterium* CP4 (Padgette et al., 1995), and the sensitive *EPSPS* receptor gene from *Bradyrhizobium* (Wagner et al., 2008). The nodules formed by *Bradyrhizobium* constitute a space of close physical contact between donor DNA and receptor competent cells, as required for transformation (Kay et al., 2002; Lorenz and Wackernagel, 1994b). Although there has been no evidence of HGT in nature (Wagner et al., 2008), gene transfer from plant DNA to natural competent bacteria has been shown to occur under optimized laboratory conditions (de Vries and Wackernagel, 1998; Gebhard and Smalla, 1998 ; Kay et al., 2002; Meier and Wackernagel, 2003; Nielsen et al., 1997). The final condition required for a successful HGT is the expression of the transferred gene by the recipient bacterium (Gebhard and Smalla, 1998) in order to achieved a selective advantage that will maintain the novel gene under selection pressure. In the case of a possible HGT of the *CP4-EPSPS* gene from the RR soybean to *Bradyrhizobium*, it is not known if this gene can be expressed in *Bradyrhizobium*.

Therefore, the first objective of this thesis was to analyse the probability of a HGT of the *CP4-EPSPS* from RR soybean plants to its symbiont *B. japonicum* under optimized laboratory

conditions and glyphosate selection pressure. To fulfil this objective, different approaches to induce natural competence of the bacterium and to facilitate cross-over by homologous recombination were implemented. Secondly, the growth of *B. japonicum* harbouring the integrated *CP4-EPSPS* should be monitored at increasing glyphosate concentration to determine the resistance level conferred by the *CP4-EPSPS* gene in this bacterium.

1.8.2 Effect of glyphosate application on the microbial rhizosphere community

As glyphosate inhibits growth of several microorganisms (Amrhein et al., 1980) yet there are several bacterial species resistant to the herbicide (Padgette et al., 1995). It should be tested if the application of glyphosate can alter the soil microbial communities. Shifts in the bacterial community after pre-planting application of glyphosate has been demonstrated (Kuklinsky-Sobral et al., 2005). Nevertheless, as glyphosate remains in the upper soil layer (Grundmann et al., 2008) and no glyphosate has been detected in leachates or lower soil layers (Klier et al., 2008), the effect of glyphosate applied directly to the soil does not reach the rhizosphere community. On the contrary, it has been shown that glyphosate is translocated and exudated through the roots after foliar application (Grundmann et al., 2008; Kremer et al., 2005). Therefore, investigation of the effect of foliar applied glyphosate on the rhizosphere is required, since the glyphosate exudated through the roots can have relevant ecological effects on the rhizosphere. Consequently, it is expected that after glyphosate foliar application there will be significant alterations in the bacterial community associated to RR soybean roots.

Therefore, the second objective of this work was to examine the impact of glyphosate on the composition and diversity of RR soybean root associated bacteria. For this purpose, the bacterial communities of RR soybean roots were analyzed and compared between glyphosate-treated and untreated soybean plants in a greenhouse experiment. After two glyphosate-applications, samples from five harvests were analysed by T-RFLP accomplished by clone bank analysis. Multivariate statistical analysis of data was used in order to find groups of organisms responsible for the observed community shifts in response to glyphosate applications. Shannon diversity indices were calculated based on the T-RFLP results with the aim of comparing bacterial diversity in the rhizosphere of glyphosate-treated and non treated RR soybeans.

2 Materials and Methods

2.1 Buffers and solutions

50x TAE buffer

Tris base	242 g
EDTA	18.6 g
Acetic acid	57.1 mL

Volume was adjusted to 1 L adding distilled water and pH was adjusted to 8.0.

100x trace element stock solution (for PSY medium)

H_3BO_3	1 g
$ZnSO_4 * 7H_2O$	0.1 g
$CuSO_4 * 5H_2O$	0.05 g
$Na_2MoO_4 * 2H_2O$	0.01 g
$MnCl_2 * 4H_2O$	0.01 g
$FeCl_3$	0.019 g
Aqua dest.	*ad* 1000 mL

1000x vitamin stock (for PSY medium)

Thiamin-HCl	1 g
Biotin	1 g
Na-Pantothenat	1 g
Aqua dest.	*ad* 1000 mL

2.2 Media

Unless differently indicated, growth media were adjusted to pH 7.0 and autoclaved for 20 min at 121 °C.

LB (Luria-Bertani)-Medium
(Bertani, 1951)

Peptone	10 g
NaCl	10 g
Yeast extract	5 g
Aqua dest.	*ad* 1000 mL

Solid medium was prepared by adding 15 g/L agar.
YEM Medium (Yeast Extract-Mannitol)
(Vincent, 1970)

Yeast extract	1 g
Mannitol	5 g
NaCl	0.1 g
K_2HPO_4	0,5 g
$MgSO_4 * 7H_2O$	0,2 g
Aqua dest.	*ad* 1000 mL

Solid medium was prepared by adding 15g/L agar.

YEX Medium (Yeast extract-Xylose)
(Adams et al., 1984)

Yeast extract	0.04 g
Xylose	0.3 g
NaCl	0,1 g
K_2HPO_4	0,5 g
$MgSO_4 * 7H_2O$	0,2 g
Aqua dest.	*ad* 1000 mL

SOC medium
(Sambrook et al., 1989)

Bacto-tryptone	20 g
Yeast extract	5 g
NaCl	0.5 g
KCl	2,5 g
$MgCl_2$	1,02 g
$MgSO_4 * 7H_2O$	1,23 g
Aqua dest.	*ad* 1000 mL

After the SOC medium was autoclaved and cooled down, 20 mL of a sterile 1 M solution of glucose (prepared by dissolving 18 g glucose in 100 mL deionised H_2O and sterilised by filtration through a 0.2 µM filter (Millipore GMBH, Schwalbach, Germany) were added.

PSY Medium (Peptone-salts-yeast extract)
(Regensburger and Hennecke, 1983)

KH_2PO_4	0.3 g
Na_2HPO_4	0.3 g
1 M $CaCl_2$	34 µL
$MgSO_4$ * $7H_2O$	0.1 g
Bacteriological peptone	3 g
Yeast extract	1 g
100x trace element solution (section 2.2)	10 mL
1000x vitamin solution (section 2.2)	1 mL
Aqua dest.	*ad* 990 mL

After autoclaving the medium, 10 mL of 10% L-arabinose solution (steril filtered; final concentration: 0.1%) were added. Solid medium was prepared by adding 15 g/L agar.

2.3 Plant material and glyphosate source

Transgenic RR soybean (GTS 40-3-2) seeds expressing the *EPSPS* gene derived from *Agrobacterium* sp. strain *CP4* were provided by Monsanto Europe (Brussels, Belgium). This genetically engineered soybean, developed from the A5403 soybean variety (Asgrow Seed Co., USA Ort, Land), has been developed to be tolerant to broad-spectrum glyphosate-containing herbicides, specifically Roundup® (Monsanto Europe, Brussels, Belgium) which was used as glyphosate source throughout this study.

2.4 Reference strains

Table. 2.1. Bacterial strains and genotype description

Strains used in this study	Relevant genotype or description	Source or reference
E. coli K12 strain One Shot® TOP10F´ electrocompetent *E. coli*	Strain used for general cloning F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	(Invitrogen, Karlsruhe, Germany)
E. coli K12 strain S17-1	Mobilizing donor strain *thi pro recA hsdR hsdM* RP4-2-Tc::Mu-Km::Tn7	(Simon et al., 1983)
B. japonicum 110spc4	110 derivate, *Spc*^{r}	(Acuña et al., 1987)

2.5 Growth of bacterial cultures

2.5.1 Cultivation of *E. coli*

One Shot® TOP10F´ electrocompetent *E. coli* (Invitrogen) was used as host in standard cloning procedures and *E. coli* S17-1 (Simon et al., 1983) served as donor in conjugative plasmid transfer. *E. coli* was cultivated at 37 °C on LB agar plates or in liquid cultures (200 rpm) according to standard methods (Sambrook et al., 1989). For long-term storage, bacterial cultures were mixed with an equal amount of glycerol and stored at -80 °C. After transformation with TA Cloning® system vectors positive clones were identified on kanamycin selective LB agar plates by standard blue-white screening in media containing X-Gal (40µg/mL) (Sambrook et al., 1989). *E. coli* was grown under kanamycin (50µ/mL) and tetracyclin (10µg/mL) selection in order to verify the plasmid insertion of the pRJ1035 and pRJ1042 vectors (Table 2.2).

Table 2.2. Antibiotic and additives concentrations used for *E. coli*.

Antibiotics and additives	Solvent	Stock solution (mg/mL)	Final concentration (μg/mL)
Kanamycin	dH_2O	50	50
Tetracycline	70% Ethanol	10	10
X-Gal	Dimethylformamide	40	40

2.5.2 Cultivation of *B. japonicum*

B. japonicum strain 110spc4 was used in this study. PSY medium (Regensburger and Hennecke, 1983) and YEM medium (Vincent, 1970) were used for mating and natural transformation experiments. YEX medium was employed for electroporation experiments. Cultures were grown at 28 °C to 30 °C with shaking at 200 rpm. For long-term storage, mixtures of equal volumes of bacterial cultures and glycerol were stored at -80 °C. For the selection of the *B. japonicum* strain 110spc4 spectinomycin and chloramphenicol were added to the media (Loh et al., 2002). *B. japonicum* strain 110spc4 is naturally resistant to spectinomycin (100μg/mL), which was applied to every culture as a precaution against bacterial contaminations. Chloramphenicol (20μg/mL) was added as a counter selection for *E. coli* strains. Additionally kanamycin (100μg/mL) and tetracyclin (50μg/mL) were added during biparental mating experiments for selection of the pRJ1035 and pRJ1042 plasmids (Table 2.3).

Table 2.3. Antibiotic concentrations used for *B. japonicum*.

Antibiotics	Solvent	Stock solution (mg/mL)	Final concentration (μg/mL)
Kanamycin	dH_2O	100	100
Tetracycline	70% Ethanol	10	25 (liquid), 50 (solid)
Spectinomycin	dH_2O	100	100
Chloramphenicol	100% Ethanol	20	20

2.6 Growth measurements: Optical density

Growth in liquid culture was monitored by measuring the optical density at 600 nm with an Ultrospec 3100 Pro spectrophotometer (BioChrom, Ltd., Cambridge, United Kingdom).

2.7 Oligonucleotides

Primers for the target sequences were designed for a G+C content of about 50-60%, a melting temperature of 57 °C to 60 °C and a length of 18 – 20 base pairs. Oligonucleotides were purchased from Microsynth (Balgach, Switzerland).

Table. 2.4. List of primers and description of PCR products

Primer	Sequence (5`-3`)	Product / position	Source or reference
35S-F	TGGAAAAGGAAG GTGGCTC	*35S-EPSPS* construct. Positions 1-19 and 2167-2185.	This work
NOS-ter-R	GGGATCGATCCC CGATCT	*Glycine max* transgenic *cp4epsps* expression cassette (NCBI, AB209952).	This work
ATG-EPSPS	ATGCTTCACGGT GCAAGC	*EPSPS* gene. Positions 514-532 and 1865-1884.	This work
EPSPS-R	TCATCAGGCAGC CTTCGTA	*Glycine max* transgenic *cp4epsps* expression cassette (NCBI, AB209952).	This work
Intern-F	TTACCGTCGAGA CGGATGC	Internal fragment of the *EPSPS* gene. Positions 1154-1173 and 1847-1866.	This work
Intern-R	ATCGGAGAGTTC GATCTTC	*Glycine max* transgenic *cp4epsps* expression cassette (NCBI, AB209952).	This work
nptII-F	TATCTGGACAAGG GAAAAC	Kanamycin promoter gene (*nptII*). Positions 1310-1329 and 1531-1549	This work
nptII-R	GCGAAACGATCCT CATCC	*E. coli* transposon Tn5, complete sequence (NCBI, U00004.1).	This work
RSα9-F	ATAAAAAGAACG AGGACTGGC	RSα9-RSβ3-internal-region. Position 1905742-1905763 and 1906604-1906684.	This work
RSβ3-R	ATTGAAGATCGA GCGGCCCG	*B. japonicum* USDA 110 DNA, complete genome. (NCBI, BA000040.2).	This work
Ba27f-5´FAM	AGAGTTTGATCM TGGCTCAG	16S-rDNA fragment. Positions 27-47 and 887-907. Universal 16S primers.	(Edwards et al., 1989)
907r	CCGTCAATTCCTT TGAGTTT		(Muyzer et al., 1995)
M13 forward	GTAAAACGACGG CCAG	Insert in pCR®2.1-TOPO® vector. Positions 205-221 and 391-406. Universal primers for DNA sequencing.	pCR®2.1-TOPO® Cloning® Kit (Invitrogen)
M13 reverse	CAGGAAACAGCT ATGAC		

2.8 Vectors

In this study, TA Cloning® system vectors (Invitrogen) were used for standard cloning into *E. coli*. Genetic constructs were amplified by PCR and then directly cloned into *E. coli* TA Cloning® system vectors. For this cloning procedure, the insert amplification using PCR requires an AccuPrime™ *Taq* DNA Polymerase high fidelity kit (Invitrogen), which does not contain extensive 3′ to 5′ exonuclease activity. The Taq polymerase adds a single deoxyadenosine (A) to the 3′ ends of PCR products. As the linearized TA Cloning® vectors have single 3′ deoxythymidine (T) residues, the PCR inserts can ligate efficiently to the vector. pCR®2.1-TOPO® and pCR®-XL-TOPO® were the two vectors used for PCR products cloning into *E. coli*. Both vectors carry a kanamycin resistance gene, which facilitated screening of transformed *E.coli*.

B. japonicum transformations were performed with the integration plasmid pRJ1035 (Figure 2.1) and its derivate pRJ1042, which are mobilizable vectors, designed for chromosomal integration of foreign DNA into the *B. japonicum* genome (Acuña et al., 1987). The vector can be mobilized from *E. coli* to *B. japonicum* due to the presence of *oriT* (*mob*). Its *oriV* site does not permit replication in *B. japonicum*. In consequence, the selection for any marker of pRJ1035 in *B. japonicum* exconjugants requires the rescue of these markers into the genome via recombination. The plasmid carries a *B. japonicum* non-essential DNA region with two different repeated sequences *RSα9* and *RSβ3* that are adjacent to a nitrogen fixation (*nifD*) gene cluster. These regions are the site for recombination of the plasmid with the cromosomal DNA.

The *B. japonicum* genome was previously shown to contain a total of 12 *RSα9* and 6 *RSβ3* copies (Kaluza et al., 1985). All of these could potentially serve as target sites for the integration of pRJ1035 and pRJ1042 by one single homologous recombination event. This arrangement (*RSα9-RSβ3-nifD*) is unique for the whole genome. Between the *RSα9-RSβ3* region, the vector possesses two useful cloning sites (*Eco*RI, *Sma*I) and a kanamycin resistance marker (*nptII*). Outside the *B. japonicum* homologous region, there is additionally a tetracycline resistance marker (*tet*) (Figure 2.1). This design allows that foreign DNA, integrated into the cloning sites, can be recombined into the *B. japonicum* genome by double

homologous crossover in the flanking *RSα9* and *RSβ3* sequences. DNA integration into the genome is found among *B. japonicum* exconjugants selected on kanamycin-containing plates that are tetracycline sensitive indicating loss of residual vector material. Regarding cloning procedures, the main difference between the two vectors is the plasmid size due to insertion of a 3.7 kb fragment inside the pRJ1042 vector.

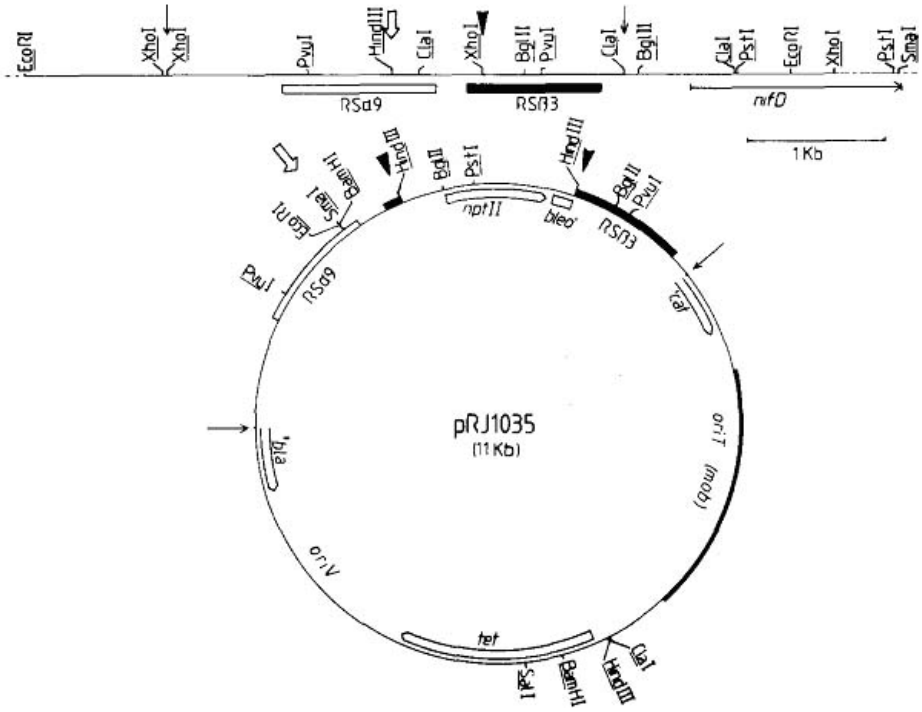

Fig. 2.1. Restriction enzyme cleavage maps of the *B. japonicum* genomic region harbouring *RSα9*, *RSβ3* and *nifD* (top), and of the integration vector plasmid pRJ1035 (Acuña et al., 1987) The filled arrowhead marks the position into which the kanamycin resistance gene (*nptII*) was inserted. The open arrow denotes the position that underwent modifications to result in new cloning sites. The other two arrows delimit the RS*α*9-RS*β*3-region that was cloned into the mobilizable vector pSUP202. Open bars stand for RS*α*9, filled bars for RS*β*3. Other genes are designated as follows: (*nifD*), structural gene a subunit of the nitrogenase MoFe protein ; (*nptII*), Tn5-derived neomycin/kanamycin phosphotransferase II gene; (*bleo'*), Tn5-derived bleomycin resistance gene (5' end); (*'cat*) chloramphenicol acetyltransferase gene (3' end); (*tet*), tetracycline resistance gene; (*oriV*), origin of replication; (*oriT/mob*), origin of conjugative transfer/mobilization.

Table 2.5. Genetic constructs and plasmids

Genetic constructs and plasmids	Relevant genotype or description	Source or reference
pCR®-XL-TOPO®	Cloning vector, *Zeo^r, Kan^r*	Invitrogen
pCR®2.1-TOPO®	Cloning vector, *Amp^r, Kan^r*	Invitrogen
Construct: *35S-EPSPS*	*CaMV* 35S promoter, CTP, *EPSPS* gene	This work
EPSPS	*EPSPS* gene	This work
Construct: nptII-EPS	*nptII* promoter, 242 bp of the *EPSPS* gene	This work
Construct: nptII-EPSPS	*nptII* promoter, *EPSPS* gene	This work
pT-35S-EPSPS	*CaMV* 35S promoter, CTP, *EPSPS* in pCR®-XL-TOPO®	This work
pT-EPSPS	*EPSPS* from transgenic soybean in pCR®2.1-TOPO®	This work
pT-nptII-EPS	*nptII* promoter + 242 bp of *EPSPS* in pCR®2.1-TOPO®	This work
pT-nptII-EPSPS	*nptII* promoter + *EPSPS* in pCR®2.1-TOPO®	This work
pRJ1042	*Tc^r Km^r Bj-(nifD-lacZ)* in pRJ1035	(Acuña et al., 1987)
pRJ-35S-EPSPS	*CaMV* 35S promoter, CTP, *EPSPS* in pRJ1042	This work
pRJ1035	*Tc^r Km^r Bj-RSα9-nptII-RSβ3*	(Acuña et al., 1987)
pRJ-nptII-EPSPS	*nptII* promoter + *EPSPS* in pRJ1035	This work

2.9 DNA extraction from RR-Soybean

Total genomic DNA was extracted from fresh leaf tissue using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Leaf material was first mechanically disrupted by grinding deep frozen leaves under liquid nitrogen to a fine powder using a mortar and pestle. Then, 100 mg of plant tissue was placed in 1.5 mL reaction tubes and processed according to the protocols supplied with the kit. The DNeasy Plant Mini Kit is based on silica gel membrane technology and allows an extraction of complete DNA from plant tissues. In these procedures the cellular components of the sample are first lysed mechanically and chemically. Subsequently, the DNA is bound to a membrane gel matrix, washed thoroughly, and then eluted with water.

2.10 DNA quantification

After each DNA extraction (total DNA or plasmid), DNA solutions were quantified using the Nanodrop ND-100 spectometer (Peqlab, Erlangen, Germany).

2.11 PCR amplification of the *EPSPS* construct from RR-Soybean

DNA fragments of the *EPSPS* construct for cloning into plasmid vectors and sequencing were generated by PCR. The AccuPrime™ *Taq* DNA Polymerase high fidelity kit (Invitrogen) was used for amplification of the full length transgene *EPSPS*. After testing numerous enzymes and buffers, this kit proved to be the only product, which was able to amplify the *EPSPS* gene from RR-Soybean. The AccuPrime™ system includes an enzyme mixture composed of recombinant *Taq* DNA polymerase, *Pyrococcus* species polymerase and monoclonal antibodies. These antibodies inhibit the *Taq* polymerase at ambient temperatures and restore the activity at 94 °C during the first PCR denaturation step. The PCR buffer mixture includes Mg^{2+} and dNTPs. The buffer also contains a thermostable Accuprime™ protein, which has DNA binding activity and provides priming specificity by interacting with the active primer-template-polymerase complex.

The PCR reactions were prepared in a total volume of 50 µL of reaction mixture following the manufacturer's recommended protocol:

10x AccuPrime PCR Buffer	5 µL
Sense Primer (10 µM)	1 µL
Anti-sense Primer (10 µM)	1 µL
Template DNA	10-30 ng
AccuPrime *Taq* high fidelity (5U/µL)	0.2 µL
Autoclaved, distilled water	to 50 µL

PCR amplifications were performed in a PTC 200 thermocycler (Biozym, Oldenburg, Germany). The optimized PCR program for the *EPSPS* construct was performed as follows:

Initial denaturation	94 °C for 3 min
33 cycles of:	
Denaturation	94 °C for 30 s
Annealing	58 °C for 45s
Elongation	68 °C for 2 min, 20 s
Terminal elongation	68 °C for 10 min
Cooling	4 °C
PCR products were store at – 20 °C	

2.12 DNA gel electrophoresis

The separation of DNA fragments according to length was done in gels with 1% agarose in TAE buffer. Samples were mixed with 6x loading dye solution (MBI Fermentas, St Leon-Rot, Germany) before loading and gels were run at voltage of 100 V. For the detection of DNA fragments, 0.05 µg/mL ethidium bromide was added to the liquid agarose. After separation, the fragments were visualised by UV light illumination (302 nm).

2.13 DNA size markers

Following DNA size markers were used to determine DNA fragment length.

1. DNA-Marker pBR328 Mix I. Range: 12 fragments (in bp): 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220 and 154 (MBBL Dr. Bartling GmbH, Bielefeld, Germany).
2. Lambda DNA/HindIII Marker 2: 8 fragments (in bp): 23130, 9416, 6557, 4361, 2322, 2027, 564, 125 (MBI Fermentas).
3. Lambda, DNA/ Eco91I (BstEII) Marker, 15: 14 fragments (in bp): 8453, 7242, 6369, 5687, 4822, 4324, 3675, 2323, 1929, 1371, 1264, 702, 224, 117 (MBI Fermentas).

2.14 Plasmid DNA extraction from *E. coli* cells

Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. This procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto a silica membrane in the presence of high salt-binding conditions. For extraction of pRJ1035 vector derivates, having sizes greater than 10 kb, it was necessary to preheat the dilution water to 70 °C prior to eluting DNA from the QIAprep membrane.

2.15 Sequencing of cloned DNA constructs

PCR amplified products were sequenced prior to cloning into the TA Cloning® system vectors. Additionally, cloned inserts (*EPSPS, 35S-EPSPS* and *nptII-EPSPS*) were sequenced after being cloned into the TA Cloning® system vectors and into the *B. japonicum* vectors pRJ1035 and pRJ1042. Sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany).

2.16 Restriction enzyme cleavage

Restriction digests were performed with restriction enzymes from New England Biolabs (Frankfurt am Main, Germany), with the appropriate 1x buffer, at a temperature that was recommended for a particular enzyme by the manufacturer. Incubations were carried out in a thermoblock or waterbath, and bovine serum albumin (BSA) was added if recommended by the manufacturer. Enzyme concentration was chosen in agreement with the recommendations of New England Biolabs depending on DNA and enzyme stock concentration, adding 1 µL of enzyme and 300 ng to 500 ng plasmid DNA for a 20 µL restriction digest mix. Three restriction enzymes were used during this study: *Hind*III, *Cla*I and *EcoR*I. *Hind*III and *Cla*I were required for construction of the vector pT-nptII-EPSPS. *EcoR*I was used for subcloning the *EPSPS* inserts from the TA Cloning® system vectors to the *B. japonicum* plasmids pRJ1035 and pRJ1042. *EcoR*I restriction reactions were incubated for no longer than three hours at 37 °C. Reactions with *Hind*III and *Cla*I were incubated overnight.

2.17 Extraction and purification of DNA fragments from agarose gels

DNA bands (*EPSPS* construct and pRJ1035 vector) were cut out of the gel with a scalpel and transferred into a sterile micro tube. The isolation procedure was performed with the DNA extraction kit (Qiagen) according to the manufacturer's manual.

2.18 Enzyme removal from DNA samples

DNA was purified using Micropure EZ restriction enzyme removal columns (Millipore GMBH, Schwalbach, Germany) after enzymatic reactions with restriction enzymes and dephosphatases. This procedure was necessary when the DNA was required for further digestion reactions or for ligation. After restriction with *Hind*III the vectors pT-EPSPS and pT-nptII-EPS, were purified with the Micropure EZ restriction enzyme removal columns, prior to subsequent restriction with *Cla*I. The Micropure EZ columns were also used after dephosphorilation of plasmids pRJ1035 and pRJ1042.

2.19 Plasmid dephosphorylation

To avoid re-ligation, vectors pRJ1035 and pRJ1042 were dephosphorylated with alkaline phosphatase (CIP, Calf Intestinal Phosphatase), prior to ligation with the *EPSPS* constructs. The reaction was performed according to manufacturer's instructions (New England Biolabs). For a 20 µL reaction, 200 ng DNA with 10 units of alkaline phosphatase and 2 µL of 10 x buffer were incubated for 3 hours at 37 °C.

2.20 Ligation

Ligation was done by mixing purified vectors and inserts in molar ratio of 1:5 respectively. The 10 µL reaction volume was made up of 1 µL of 10 x ATP-containing reaction buffer, 1 µL of T4 DNA ligase (Invitrogen), between 5 and 15 ng of plasmid vector and between 45 and 100 ng of insert. If the total volume of 10 µL was not reached, the corresponding amount of distilled H_2O was also added to the mixture. The ligation mixture was incubated over night at 14 °C and plasmids were used for transformation the next day.

2.21 Colony PCR for cloned insert screening

Colony PCR was used after transformation to screen colonies for the desired plasmid. Each selected bacterial colony (of *E.coli* or *B. japonicum*) was picked with a sterile pipette tip from the plate and diluted into 50 µL autoclaved distilled water. The colony dilution was then heated for 10 minutes at 95 °C. Finally 1µL of the heated solution was applied to the tube containing the PCR reaction mixture.

2.22 Cloning *EPSPS* constructs into TA Cloning® system vectors in *E. coli*

Inserts *35S-EPSPS* and *EPSPS* were ligated according to the instructions of the manufacturer to the vectors pCR®-XL-TOPO® and pCR®2.1-TOPO® respectively. The ligations were transformed into One Shot® TOP10F´ electrocompetent *E. coli* cells, which were provided with the manufacture kit.

2.23 Cloning the *EPSPS* construct under the control of the *nptII* promoter

In order to generate a construct with the *EPSPS* gene under the control of the *nptII* promoter (*nptII-EPSPS*), two vectors were combined: The pT-EPSPS, carrying the single *EPSPS* gene, and the vector pT-nptII-EPS, which contained a synthetic *nptII* promoter linked to a fragment of the *EPSPS* gene (*nptII-EPS*). The synthetic fragment *nptII-EPS* includes the *nptII* promoter sequence linked to 224 bp of the *EPSPS* gene (Figure 2.2). This construct was synthesized by Eurofins MWG Operon and was harboured into the vector pT-nptII-EPS.

TATCTGGACAAGGGAAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTA
CATGGCGATAGCTAGACTGGGCGGTTTTATGGACAGCAAGCGAACCGGAAT*TGCCAGCTG*
*GGGCGCCCTCTGGTAAGGTT*GGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTTGCCGCC
AAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTC
GC**ATGCTTCACGGTGCAAGCAGCCGGCCCGCAACCGCCCGCAAATCCTCTGGCCTTT**
CCGGAACCGTCCGCATTCCCGGCGACAAGTCGATCTCCCACCGGTCCTTCATGTTCG
GCGGTCTCGCGAGCGGTGAAACGCGCATCACCGGCCTTCTGGAAGGCGAGGACGTC
ATCAATACGGGCAAGGCCATGCAGGCCATGGGCGCCAGGATCCGTAAGGAAGGCGA

Fig. 2.2. Synthetic sequence containg the *nptII* promoter joined to 224 bp of the *EPSPS* gene. Standard capital letters represent the *nptII* promoter sequence. Italic letters show the polymerase recognition site inside the *nptII* promoter. The *EPSPS* gene fragment is denoted in bold letters. Underlined letters indicate the restriction site for *Cla*I.

The *EPSPS* fragment includes a recognition site for the *Cla*I restriction enzyme, suitable for further subcloning experiments. Both vectors pT-EPSPS and pT-nptII-EPSPS were digested with the enzymes *Hind*III and *Cla*I. These restriction enzymes liberate the initial fragment of the *EPSPS* gene from the pT-EPSPS vector and excise the *nptII-EPS* construct from the pT-nptII-EPS vector. The released *nptII-EPS* construct was ligated to the corresponding adjacent fragment of the pT-EPSPS vector to raise the vector pT-nptII-EPSPS. The new vector pT-nptII-EPSPS carried the *EPSPS* gene under control of the *nptII* promoter. For subcloning the *nptII-EPSPS* construct from the pT-nptII-EPSPS vector into the *B. japonicum* vector pRJ1035, both plasmids were digested with *Eco*RI. After pRJ1035 linearization by *Eco*RI, the vector was dephosphorylated, purified and ligated with the insert *nptII-EPSPS*. Ligations were used for *E.coli* transformation by electroporation.

2.24 Generation of electrocompetent cells

2.24.1 *E. coli* S17-1 electrocompetent cells

To obtain electrocompetent cells of *E. coli* S17-1, overnight cultures were diluted 1:100 and were grown to OD_{600}= 0.8 - 1.0 (ca. 3.5 h). Bacterial cultures were chilled on ice for 30 min before harvest (10 min, 4 °C, 4000 x g, Sorvall RC-5B centrifuge, DuPont, Wilmington, USA). The resulting pellet was washed three times in 600 mL of ice cold distilled water. One further wash was carried out with 600 mL of ice cold 10% glycerol and the cells were then re-suspended in 1.2 mL of 10% glycerol before being shock frozen with liquid nitrogen in 100 µL aliquots and stored at –80 °C.

2.24.2 *B. japonicum* electrocompetent cells

B. japonicum cells were prepared for electroporation by modifications of two protocols (Guerinot et al., 1990; Hattermann and Stacey, 1990). Different media were tested for developing *B. japonicum* electrocompetent state. *B. japonicum* 110spc4 cells were cultivated in YEX (Adams et al., 1984), YEM (Vincent, 1970) and PSY media (Regensburger and Hennecke, 1983). Cell cultures (500 mL) were grown to an OD_{600} of 0.4 to 0.6, then chilled on ice and harvested by centrifugation for 15 min at 4 °C (Sorvall RC-5B centrifuge). Two different centrifugation speeds were tested: 4000 x g (Guerinot et al., 1990) and at 9820 x g (Hattermann and Stacey, 1990). Cells and solutions were maintained at 4 °C for every following step. Cells were suspended in 500 mL sterile distilled water and washed by alternate centrifugation and resuspention in 250 mL distilled water and then in 10 mL of sterile 10% glycerol. Cells were finally centrifuged and resuspended in 1 mL of glycerol. The concentrated cell suspention was distributed in 40 µL aliquots, frozen in liquid nitrogen and stored at -80 °C.

2.25 Transformation experiments

2.25.1 Electroporation of *E. coli*

Bacterial transformation was carried out by electroporation using a Gene Pulser (Bio Rad, München, Germany). For electroporation, *E. coli* DH5*α* electrocompetent cells (Invitrogen) and *E. coli* S17-1 were thawed on ice, mixed with plasmid DNA (10-100 ng) and transferred to a 1 mm gap cuvette. A pulse with a 25 µF of capacity, 1.7 kV and a resistance of 200 Ω was applied. Afterwards, cells were immediately suspended in 1 mL SOC medium and incubated at 37 °C for 1 h. Colony PCR was used to screen positive colonies harbouring the insert. Plasmids in selected positive colonies were purified with the QIAPREP spin miniprep kit (Qiagen), amplified and sequenced (Eurofins MWG Operon).

2.25.2 Electroporation of *B. japonicum*

For electroporation a Gene Pulser (Bio Rad) was used with a 0.2 cm cuvette supplied by the manufacturer. The DNA used for *B. japonicum* electroporation was from the *B. japonicum* plasmids pRJ1035 and pRJ1042. Following DNA concentrations were tested for electroporation: 10 ng/mL, 125 ng/mL, 250 ng/mL, 500 ng/mL, 1µg/mL. Electrocompetent cells were thawn on ice and 1 µL to 2 µL plasmid DNA was then mixed thoroughly with 40 µL of a cell suspension, placed on ice for 1 minute and transferred to a chilled cuvette. A pulse with 25 µF of capacity, 2.5 kV (12.5 kV/cm) and resistances of either 200 Ω or 400 Ω were tested. The pulse was applied and cells were immediatelly suspended in 1 mL of YEX or PSY medium. The cells were transferred to a tube and incubated on a horizontal shaker (200 rpm at 30 °C for 20 h; New Brunswick Scientific Co, New Brunswick, NJ, USA). Dilutions were plated on selective and non selective media. Controls consisted of cells mixed with plasmid DNA, which were not subjected to a pulse prior to incubation in liquid medium.

2.26 Conjugation – biparental mating

Conjugation is a common and naturally occurring mechanism of horizontal gene transfer in bacteria and is most easily achieved in laboratories via plasmids. For this study conjugation between two different bacterial species was attempted by applying the shuttle vector pRJ-35S-EPSPS, harbouring the *35S-EPSPS* construct, from *E. coli* S17-1 to *B. japonicum*. Bacterial mating was performed as described by (Pérez-Mendoza et al., 2006). Mating suspensions and selective growth on agar plates was performed in PSY liquid and solid medium. The vector pRJ1035-35S-EPSPS was transformed into the *E. coli* strain S17-1 by electroporation to be subsequently mobilized to *B. japonicum* 110spc4.

E. coli S17-1 donor and *B. japonicum* recipient cells were grown under standard conditions with addition of appropriate antibiotics (section 2.5). Overnight culture of *E. coli* was diluted 1:10 with fresh LB medium and incubated at the same conditions to an optical density of 0.5 at 600 nm and the recipient strain *B. japonicum* was grown to the late exponential phase (OD_{600} = 1). Afterwards, 1 mL of each culture was pelleted, washed twice with 20 mL PSY medium and finally diluted in 50 µL of PSY medium. The resulting suspension was spread on a sterile 0.45 µM nitrocellulose membrane filter (Millipore GmbH, Schwalbach, Germany). Filter mating mixtures were deposited on agar plates and incubated for 48 h at 30 °C. After conjugation, mating cells contained on the filters were resuspended in liquid PSY medium by vortexing vigorously. Serial dilutions of the liquid culture were prepared and nine successive dilutions (up to 10^{-9}) were plated to validate the results. The dilutions were plated on selective and non selective PSY agar plates and incubated at 30 °C for two weeks. The appearing colonies were counted. Transconjugants were selected on plates supplemented with appropriate antibiotics for *B. japonicum* (section 2.5) and with kanamycin for the pRJ1042 plasmid. Kanamycin resistant colonies were picked and streaked onto tetracycline selective agar plates. Kanamycin resistant colonies, yet sensitive to tetracycline are supposed to have integrated the *35S-EPSPS* construct into the *B. japonicum* chromosome (section 2.8). These *B. japonicum* transformants were screened afterwards on glyphosate (Roundup®**;** Monsanto Co, Brussels, Belgium) containing PSY agar plates under different herbicide concentrations: 5

mM, 10 mM and 25 mM. Each transformation experiment was done in triplicates and the transfer frequency was expressed as the number of transconjugants per output recipient.

2.27 *B. japonicum* natural transformation on agar plates and in liquid cultures

The development of a natural competence state in *B. japonicum* was tested on agar plates (Lorenz and Wackernagel, 1991) and under liquid medium conditions (Demaneche et al., 2001). Experiments were performed on PSY medium. Purified plasmid DNA (pRJ-35S-EPSPS) was added to exponentially grown *B. japonicum* cultures (OD_{600} = 1) at different concentrations (10 ng mL^{-1}, 100 ng mL^{-1}, 250 ng mL^{-1}, 500 ng mL^{-1}, and 1 µg mL^{-1}). Equivalent plasmid DNA concentrations were mixed with cultures for plate and liquid transformations. For plate transformation, 15 µL of *B. japonicum* suspensions mixed with plasmid DNA were spotted on PSY agar. In parallel, 10-fold concentrated mixtures were also spotted. After incubation for 48 h and 62 h at 30 °C, spots were resuspended in 1 mL of PSY medium and plated out on PSY medium under the appropriate selective conditions (kanamycin for selection of the pRJ-35S-EPSPS vector). Liquid conditions were tested adding plasmid DNA to *B. japonicum* shaking cultures (200 rpm, 30 °C) at increasing stages of the exponential growths phase (OD_{600} = 0.5, 0.65 and 0.8). Every 12 h aliquots of 100 µL culture were plated out under appropriate selective conditions and incubated at 30 °C. All experiments were done in triplicates.

2.28 Spontaneous mutations in *B. japonicum* under glyphosate selection pressure

B. japonicum cells were grown in 250 mL flasks containing 50 mL of PSY medium to a density of OD_{600}= 0.8-0.9. A volume of 100 µL of growing culture was spread to dryness on 100 PSY selective plates with 25 mM glyphosate. *B. japonicum* mutants were scored after 12 days incubation at 30 °C. The frequency of resistant mutants was expressed as the mean number of resistant colonies divided by the mean of the total number of viable cells

(Bjorkholm et al., 2001; Wang et al., 2001). Spectinomycin was applied to every culture to avoid bacterial contamination. Spontaneous mutation experiments were performed in triplicates.

2.29 Greenhouse experiment

2.29.1 Seed sterilization

RR soybean seeds were surface sterilized by the following procedure: Seeds were soaked in 70% ethanol for 20 seconds and then rinsed five times with sterile distilled water. Afterwards, they were incubated for 3 minutes in a 2% solution of sodium hypochlorite and rinsed again five times with sterile distilled water.

2.29.2 Seed germination

Sterilized seeds were germinated in the dark at 25 °C in petri dishes layered with 0.5 cm thick moist filter paper. The autoclaved filter paper was saturated with autoclaved water and dishes were laid inclined at an angle of 30 ° from the horizontal, with the lower end of the filter immersed in the water. After three days seedlings were planted in pots and cultivated in the greenhouse (as described below).

2.29.3 RR soybean cultivation conditions on the greenhouse

Cultivation of glyphosate-treated and non treated RR soybean plants were carried out in a climate controlled greenhouse chamber at the Helmholtz Zentrum München, Neuherberg. RR Soybean plants were grown in pots (18 cm in diameter) with an approximate soil volume of 400 cm^3 or 2.5 L. The growth medium was an agricultural soil characterized as haplic arenosol from Weichselstein near Neumarkt (Bavaria) which was sieved (< 2 mm) prior to planting. Day and night temperatures in the chamber were approximately 28 °C and 21 °C, respectively, and relative humidity was kept at 65% to 75 %. Sunlight was supplemented with metal halide lamps (HQI-TS 400W/D; Osram) for a day length of 14 h. Pots were placed in trays as water

reservoirs and water was supplied directly to the trays, allowing the plants to be watered from the base of the pot through capillary action. Irrigation was adjusted to changing water demands during the plants development.

2.29.4 Growth conditions in the greenhouse

Experiments were conducted to evaluate changes in the rhizoplane of RR soybean in response to postemergence glyphosate applications. Pregerminated seeds were sown in each pot. The seedlings were thinned 7-10 days after emergence to leave 4 plants per pot. Glyphosate was applied in the greenhouse at proportional rates as recommended for the fields (Figure 2.3). Each application was performed as a postemergence over-the-top spray to soybean leaves. A dilution of 0.3 mL glyphosate (360 g/L) in 40 mL distilled water was prepared in a container connected to a micro-sprayer (Ecospray, Labo Chimie, France) with a disposable gas pressure reservoir, allowing the application of fluids with high mist quality (fineness, even coating and distribution of particles). Control plants were sprayed with distilled water. RR soybean plants were arranged in 1 m^2 to be sprayed with glyphosate. For each area of 1 m^2 covered with plants, 0.3 mL glyphosate diluted in 40 mL water were sprayed (Figure 2.3). Two glyphosate applications were employed in this study at the physiologically stages recommended by Kremer and coworkers (Kremer et al., 2005). The first treatment was applied at around two weeks of growth (at the first trifoliate leaf stage) and the second application was 14 days after the first one (at the third trifoliate leaf stage) (Figure 2.3).

	Glyphosate (360 g/L) Field application 3 L in 400 L water per ha
	Glyphosate (360 g/L) Greenhouse application 0.3 mL in 40 mL water per m^2

2 Fig. 2.3. Glyphosate application in the greenhouse. Glyphosate aspersion over the RR soybean leaves. A dilution of 0.3 mL glyphosate (360g/L) in 40 mL water was applied per 1 m^2.

Following the first and second glyphosate applications, root samples were harvested after 3, 7 and 14 days respectively (Figure 2.4).

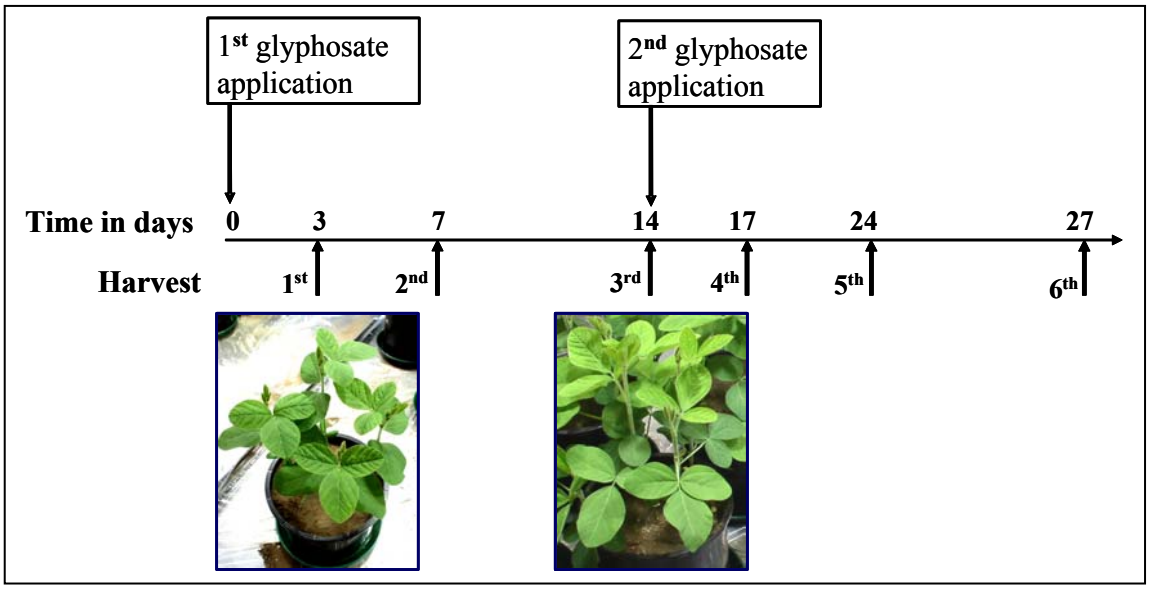

Fig. 2.4. Glyphosate applications and root harvesting. The first glyphosate application occurred when the plants where on the first trifoliate stage (day 0). Roots were harvested after 3, 7 and 14 days. At the day 14th glyphosate was applied for 2nd time. The next two harvests took place on the days 17 and 24.

At each of the six harvests, three replicate pots of glyphosate-treated plants were sampled, as well as three replicates of control non-treated plants. Roots of four plants from each pot were pooled and treated as one sample, to minimize the effect of genetic variation between different plants. Root samples were labelled according to the treatments: G for glyphosate-treated plants and K for control plants. The six different harvests were differentiated as G1 to G6 and K1 to K6 corresponding to the six sampling times of glyphosate-treated plants and control plants respectively (Figure. 2.5).

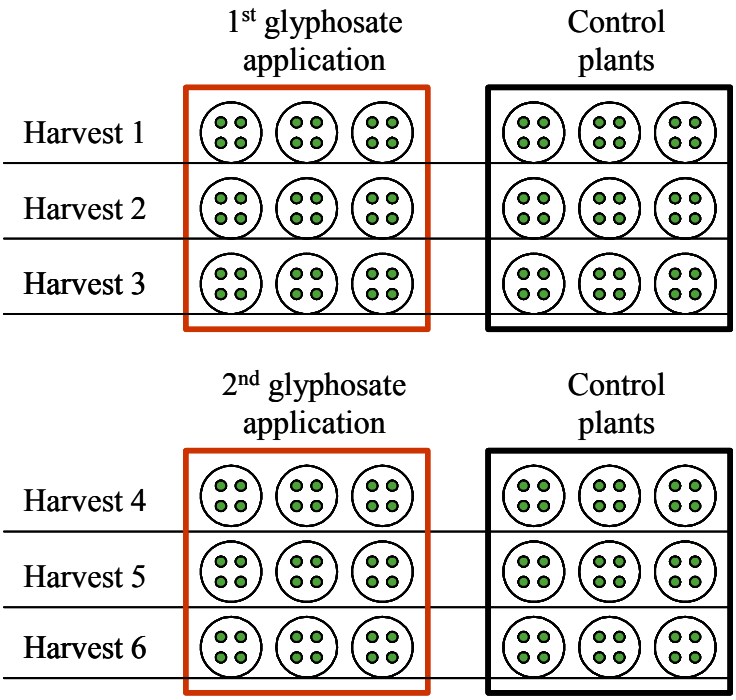

Fig. 2.5. Experimental design. At each harvesting time point, three glyphosate-treated pots and three control pots were sampled. Each pot contained four plants. Glyphosate-treated samples are indicated in red, control samples in black.

2.30 Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis of RR soybean root-associated bacteria

2.30.1 T-RFLP analysis

Terminal Restriction Fragment Length Polymorphism (T-RFLP) method was applied in this study in order to monitore the influence of foliar glyphosate applications on the bacterial community of the RR soybean rhisosphere. This technique combines restriction fragment analysis of PCR-amplified marker genes with automated sequencing gel technology (Clement et al., 1998).

After DNA is extracted from the soil, genes of interest are amplified by PCR. In many cases marker genes like 16S rRNA genes are chosen for analysis of microbial communities (Hartmann and Widmer, 2008; Liu et al., 2006; Muyzer, 1999), based on the fact that these genes contain different regions that are either highly heterogeneous or well-conserved between different species during evolution (Gupta and Griffiths, 2002).

The RNA genes are recomended biomarkers for studies of microbial structural diversity, since they are ubiquitous in living organisms. Thus, variations in rRNA gene sequences are correlated to the evolutionary relationship between organisms (Woese, 1987), making it possible to design specific primers or probes for different taxonomic levels (Kämpfer et al., 1996).

One of the primers used for amplification of these marker genes is labelled with a fluorescent dye, which allows detection of the terminal restriction fragments (T-RFs). The digested fragments are than mixed with a fragment size marker and detected using high-resolution electrophoresis in a DNA sequencer (Liu et al., 1997). Due to the sequence variation between different microbial taxa different T-RFs are observed from digested amplicons from environmental DNA. This results in characteristic fingerprints of the microbial communities, which are visualized as an electropherogram with peaks at different fragment sizes. In addition to fragment size information the laser detector also records the intensity of the fluorescence for each peak which can be used as an indication of the relative abundance of fragments of that size.

The T-RFLP analysis is recognised as a robust and reproducible methodology for the rapid analysis of microbial community structure in different samples (Osborn et al., 2000).

2.30.2 Total DNA extraction from the microbial community of washed RR soybean roots

In order to obtain rhizosphere soil from RR soybean roots, loosely attached soil was removed by gently tapping the root pieces. This soil was discarded and root samples (5 g) were washed successively two times in 25 mL distilled H_2O using a stomacher (Tekmar Stomacher 80, Laboratory Blender, Cincinnati) for 1 min (Wieland et al., 2001). Dilutions of each sample were centrifuged for 5 min at 500 x g and 4 °C (Sorvall RC-5B centrifuge) for removing soil particles and plant residues. Afterwards the supernatant was pelleted for 20 min at 10000 x g. The liquid was discarded and the pellet was resuspended in 1 mL of H_2O and divided in two microcentrifuge tubes in aliquots of 500 µL per tube. The samples were centrifuged for 10 min at 13000 x g (Sigma K312, Osterode, Germany) and the supernatant was removed by pipetting. Soil pellets were stored at -80 °C. Total DNA was extracted from 0.5 g of the soil pellets using the Fast Spin DNA Extraction Kit for Soil (MP Biomedicals, Eschwege, Germany) according to the manufacturer's instructions. The purified DNA was stored at -20 °C and was used as template for PCR amplification.

2.30.3 PCR for T-RFLP analysis

The primers used for PCR of the T-RFLP analysis were Ba27f-5´FAM (Edwards et al., 1989) and 907R (Muyzer et al., 1995), which give a 880 bp product of the 16S rRNA gene. Ba27f-5´FAM was 5′ end labeled with phosphoramidite fluorochrome 5-carboxyfluorescein. This label has its excitation wavelength at 488 nm and fluorescence emission maxima at 518 nm. Primers were purchased by Microsynth (table 2.4). The PCR reactions for the T-RFLP analysis were performed using a 2x PCR Master Mix (Promega, Mannheim, Germany) which contains: 50 units/ml of Taq DNA polymerase supplied in a proprietary reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$. Approximately 10 ng template DNA were used.

The 16S rRNA gene was amplified using the following protocol:

2x PCR Master Mix	25µL
Ba27f-5´FAM primer (10 µM)	1 µL
907R primer (10 µM)	1 µL
Template DNA	10 ng

The optimized PCR program for the 16S rRNA gene amplification was performed in a PTC 200 thermocycler (Biozym) as follows:

Initial denaturation	94°C for 3 min
23 cycles of:	
Denaturation	94°C for 1 min
Annealing	55°C for 30 s
Elongation	72°C for 1 min
Terminal elongation	72°C for 10 min
Cooling	4°C
PCR products were store at – 20°C	

Alternatively to the standard 16S rRNA gene PCR program, screening for T-RFs of individual clones from the 16S rRNA gene clone library was done by colony PCR using 1 µL of crude cell suspension as template in a 10-cycle PCR.

2.30.4 Restriction enzyme digestion of labelled PCR products

The PCR products were purified with a Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and were digested with the endonuclease *MspI* (NEB, Frankfurt am Main, Germany). *MspI* was chosen, since it seems to have the highest frequency of resolving single populations in model communities (Engebretson and Moyer, 2003; Wang et al., 2004). For complete restriction, 100 ng DNA was mixed with 10 units of the enzyme, 10 x restriction

buffer and distilled water to a final volume of 20 µL. The mixture was incubated at 37 °C for two hours.

2.30.5 DyeEx Spin purification

Desalting of digested DNA fragments was done using the DyeEx 2.0 Spin Kit (Qiagen, Hilden, Germany) according to instructions of the manufacturer. The resin contained in the spin columns was gently vortexed, the cap opened a quarter turn and then centrifuged for 3 min at 20 °C and 2000 x g. Columns were placed in a new 1.5 mL microcentrifuge tube and digested DNA (100 ng) was carefully pipeted onto the resin-bed. Subsequently, the tubes were centrifuged under the same conditions as mentioned above. The flow-through was transferred into a fresh tube and stored at -20 °C until further analysis.

2.30.6 Detection of end-labelled T-RFs

For capillary electrophoresis, 96-well sequencing plates were loaded with 1 µL of the desalted, digested amplicons and 13 µL of HiDi Formamide containing 1:400 dilution of MapMarker® 1000 ROX size Standard (BioVentures, Inc., Murfreesboro, TN, USA). Samples were consecutively loaded onto a 96-well microplate. Each reaction was loaded three times on different capillaries to minimize capillary effects. All empty wells were filled with 50 mL of LiChrosolv™ water (Merck, Darmstadt, Germany). Subsequently sequencing plates were denatured for 5 min at 95 °C in a PTC 200 thermocycler (Biozym) and immediately placed on ice. Sizes up to 880 base pairs) and intensities, measured in fluorescence units, of the labelled T-RFs were determined by means of capillary electrophoresis on an ABI 3730 Capillary Sequencer (Applied Biosystems, Foster City, USA) analyzed using GeneScan software (Applied Biosystems).

2.30.7 16S rRNA gene clone library

Amplification of the 16S rRNA gene of the bacterial community was performed with non labelled PCR primers Ba27f and 907R (table 2.4). Based on the profiles of the T-RFLP analysis, rhizosphere DNA from RR soybean extracted at three different sampling time points was chosen to be amplified by PCR, cloned and sequenced. After amplification of the 16S rRNA gene, the amplicons were ligated to the pCR®2.1-TOPO® vector (Invitrogen, Karlsruhe, Germany) and cloned into *E. coli* (as described in section 2.22). A clone library of 192 clones was generated and plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen).The purified plasmids of the 192 clones were sequenced using standard primers M13F, M13R for the multiple cloning site of pCR®2.1-TOPO® vector. Sequencing reactions were performed by Eurofins MWG Operon.

2.30.8 Phylogenetic analysis of the 16S rRNA gene clone library

Electropherograms obtained from the GeneScan software were edited and aligned using the SeqMan software (Lasergene® v7.1, DNASTAR, Inc., Madison, USA). All assembled sequences were examined for chimeric artifacts using the software Bellerophon (Huber et al., 2004). Potentially chimeric sequences were not given further consideration. T-RFs of cloned sequences were predicted using SeqMan software. The *in silico* predicted T-RFs were verified by direct T-RFLP analysis of clones amplicons to precisely assign observed environmental T-RFs to cloned lineages. The 16S rDNA sequences were compared to known sequences in GenBank with the advanced gapped BLAST (basic local alignment search tool) from the National Centre for Biotechnology Information (NCBI). All obtained sequences and their close relatives were aligned using the fast aligner tool of the ARB software package (Ludwig et al., 2004). The ARB package is built around an extensive and up-to-date ribosomal DNA-sequence library and contains special tools for alignment and analysis (tree reconstruction) of these structures. For phylogenetic affiliation, sequences were integrated into an ARB database (release January 2003) and aligned using alignment editor ARB-EDIT4. A phylogenetic tree, including selected clones and closely related representative sequences (>1400 bp) of cultivated and uncultivated species, was reconstructed using quartet puzzling

(Schmidt et al., 2002) as implemented in ARB using 10000 puzzling steps. The quartet puzzling reconstructs phylogenetic trees from molecular sequence data by maximum likelihood analysis. It provides a means to analyze and reconstruct evolutionary relationships and trees based on quartets (groups of four sequences) (Schmidt and von Haeseler, 2007). Tree topology was confirmed using maximum-likelihood, maximum-parsimony, and neighbour-joining algorithms as implemented in the ARB software package. In addition, sequences were divided into phylogenetic groups that were consistent with taxonomically related phyla and orders. These groups were assigned by determining the taxonomic class of the nearest database relative. Sequences that could not be linked to previously identified bacterial taxonomic groups were listed as uncultured bacterium.

2.30.9 Evaluation of the T-RFLP data

Only peaks at positions between 50 and 500 bp were considered in order to avoid T-RFs caused by primer-dimers and to obtain fragments within the linear range of the internal size standard (Singh et al., 2006). The relative abundance of a T-RF in a profile was calculated by dividing the peak height of the T-RF by the total peak height of all T-RFs above the threshold in the profile. Peaks smaller than 100 fluorescence units in height and T-RFs that did not represent more than 1% abundance (values below 0.01) per sample peak were not included in further analysis (Winderl et al., 2008).

The statistical evaluation of the T-RFLP data was done with the SYSTAT 10 Software (SPSS INC., Chicago, Illinois) and PCR-ORD version 5.0 (MjM Software, Gleneden Beach, USA). Shannon–Weaver index of diversity and standard deviation were calculated for all T-RFLP samples.

To visualize community dynamics and convergence, the extended data set of the T-RFLP was reduced and described by a principal component analysis (PCA) (Dollhopf et al., 2001). PCA is a multivariate projection method and has been used in microbial ecology for comparison of complex communities (Kaplan et al., 2001; Klamer et al., 2002). The object of PCA is to reduce a data set to a smaller number of synthetic variables that represent most of the

information from the original data set. The most interesting and strongest covariation emerges in the first few axes, hence "principal components" (Gauch, 1982).

Complementary to the PCA, an indicator species analysis was used in order to detect and describe in terms of significance the value of different species (T-RFs) for indicating environmental conditions. This value combines information about the concentration of species abundance in a particular group and the faithfulness of occurrence of a species in a particular group (McCune and Grace, 2002).

3 Results

3.1 Amplification of the full length transgene *EPSPS*

The *35S-EPSPS* construct in RR soybean includes the *EPSPS* gene under control of the CaMV 35S promoter and the nopaline synthase gene (*NOS*) terminator (Padgette, et al., 1995) (Figure 3.1).

Fig. 3.1: Original 35S-EPSPS construct as contained in Roundup Ready® soybean.

DNA from transgenic soybean was used as template to amplify the full length transgenic cassette by PCR including the *CaMV 35S* promoter up to the *NOS* terminator (Figure 3.2). The primer sequences used 35S-F and NOS-ter-R correspond to positions 1-19 and 2167-2185 of the *Glycine max* transgenic cp4epsps expression cassette, respectively (NCBI accession number AB209952).

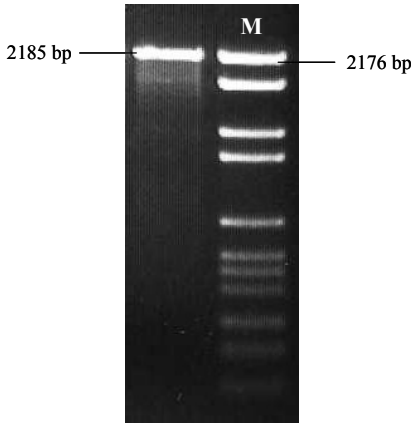

Fig. 3.2: Full length *35S-EPSPS* construct, amplified via PCR (2185 bp). The sequence includes the *CaMV 35S* promoter, the *EPSPS* gene and the *nopaline synthase* (*NOS*) terminator. M: DNA-Marker pBR328 Mix I.

The AccuPrime *Taq* DNA polymerase high fidelity kit was required for the successful amplification of the full length transgene *EPSPS*. Optimized PCR reactions with other polymerases in different buffers and diverse BSA and DMSO concentrations did not yield any PCR product. The AccuPrime buffer contains a trademarked AccuPrime binding protein, which seems to facilitate the amplification of the complete *EPSPS* gene from RR soybean. The fragment could also be amplified with other polymerases, but only if combined with AccuPrime buffer.

3.2 Cloning into the *B. japonicum* integration vectors pRJ1035 and pRJ1042

In this work, two *EPSPS* constructs were cloned into *B. japonicum* integration vectors. Initially, for cloning the original plant construct *35S-EPSPS*, the vector pRJ1042 was used. After experienced the disadvantage of the pRJ1042 vector, the vector pRJ1035 was chosen for cloning the *nptII-EPSPS* construct (see below).

The vector pRJ1042 (14.7 kb), is a derivate of the pRJ1035 (11 kb) containing a 3.7 kb *nifD::lacZ* fusion. Both vectors contain two convenient restriction sites (*EcoR*I and *Sma*I) for cloning. Initial cloning experiments into *B. japonicum* via this vector system were performed with the pRJ1042. Restriction of the pRJ1035 with two enzymes does not release any DNA fragment visible on an agarose gel. The advantage of the pRJ1042 vector was the visualisation of the insert (3.7 kb) released by two different enzymes. This excision proves the correct action of both restriction enzymes, leaving suitable cloning sites for the *EPSPS* insertion.

The pRJ1042 vector was linearized by *EcoR*I, demonstrating the correct activity of the enzyme. However, *Sma*I did not linearize the pRJ1042 vector. Several *Sma*I enzymes, purchased from different companies were tested, as well as different concentrations of this enzyme, yet the vector could not be cut with *Sma*I. For this reason, cloning into the pRJ1042 could only be done using one restriction enzyme (*EcoR*I) and the advantage of a visible released insert could not be used. As pRJ1035 has a reduced and more convenient size (11 kb) compared to the pRJ1042, the pRJ1035 was chosen for subsequent cloning of the *EPSPS* gene under the control of the *nptII* gene promoter.

3.3 Cloning the *35S-EPSPS* construct into a pCR2.1-TOPO® vector in *E. coli*

The DNA fragment, carrying the glyphosate resistance conferring *35S-EPSPS* cassette, was used for cloning experiments in order to analyse the potential effects of gene transfer to bacteria. The amplified *35S-EPSPS* construct, including the *CaMV 35S* promoter (2185 bp) was initially ligated directly into a pCR®2.1-TOPO® vector (pT-35S-EPSPS) and used for *E. coli* transformation by electroporation. Positive clones were identified on selective plates by standard blue-white screening in media containing X-Gal (Sambrook*, et al.*, 1989). Correct insertion of the complete *35S-EPSPS* construct into the vector pT-35S-EPSPS was verified by PCR amplification and DNA sequencing. Alignment of the sequenced PCR product displayed 100% nucleotide similarity to the expected *35S-EPSPS* construct contained in *Glycine max* transgenic cp4epsps (NCBI accession number AB209952).

3.4 Cloning the *35S-EPSPS* construct into the *B. japonicum* integration vector pRJ1042

The *35S-EPSPS* cassette, including the *CaMV 35S* promoter, was excised from the vector pT-35S-EPSPS by digestion with *Eco*RI and was site-specifically integrated into the same restriction site of the vector pRJ1042 (Figure 3.3).

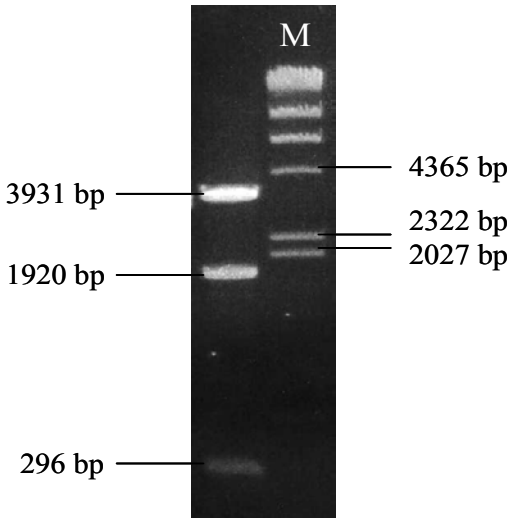

Fig. 3.3: Restriction analysis of the vector pT-*35S-EPSPS* excised with *Eco*RI. The *EPSPS* gene (1920 bp) and the *nopaline synthase* (*NOS*) terminator (296 bp) are released by *Eco*RI from the pT-35S-EPSPS (3931 bp) vector. M: Lambda DNA/HindIII Marker, 2.

The *35S-EPSPS* construct possesses an *Eco*RI site between the *EPSPS* gene and the *NOS* terminator (Figure 3.4). This together with an *Eco*RI site present in pCR®2.1-TOPO® vector cloning site, resulted in loss of the *NOS* terminator sequence (296 bp) after digestion with *Eco*RI. The NOS terminator sequence was therefore absent in the following cloning procedures. Transcription of the *35S-EPSPS* construct lacking the *NOS* terminator region would finish at two stop codons at the 3'-end of the *EPSPS* gene (Figure 3.4), allowing correct protein expression in bacteria without requirement of an additional terminator sequence.

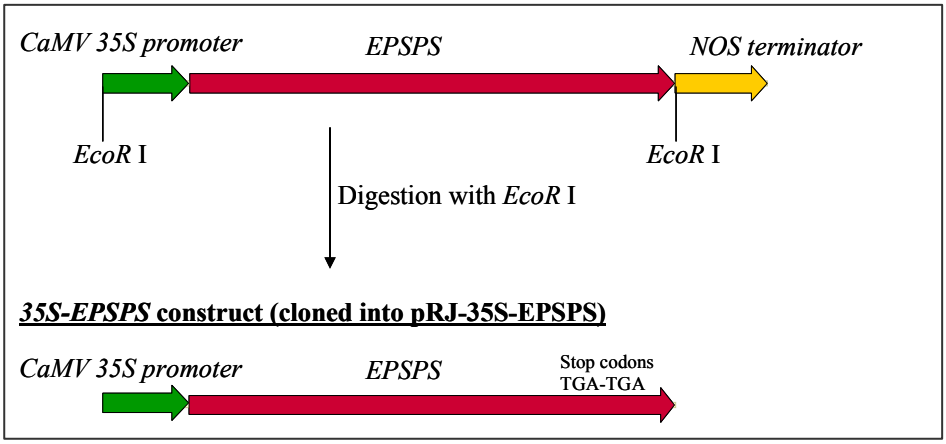

Fig. 3.4: Full length transgene *EPSPS* contained on the vector pT-35S-EPSPS, including the *CaMV 35S* promoter, the *EPSPS* gene and the nopaline synthase (*NOS*) terminator. After digestion with *EcoR* I the *NOS* terminator sequence was released, leading the *35S-EPSPS* construct finished with two termination codons TGA-TGA. The *35S-EPSPS* construct was cloned into the

Ligation of the *35S-EPSPS* cassette to complementary cohesive ends in pRJ1042 (14.7 kb) yielded the vector pRJ-35S-EPSPS, which was transformed into *E. coli* donor strain S17-1. Positive transformants were able to grow on kanamycin selective medium. Plasmids from 20 transformants were isolated and the integration of the *35S-EPSPS* (1929 bp) construct into the pRJ1042 (14.7 kb) was verified by enzymatic digestion (Figure 3.5), PCR amplification of the insert and DNA sequencing.

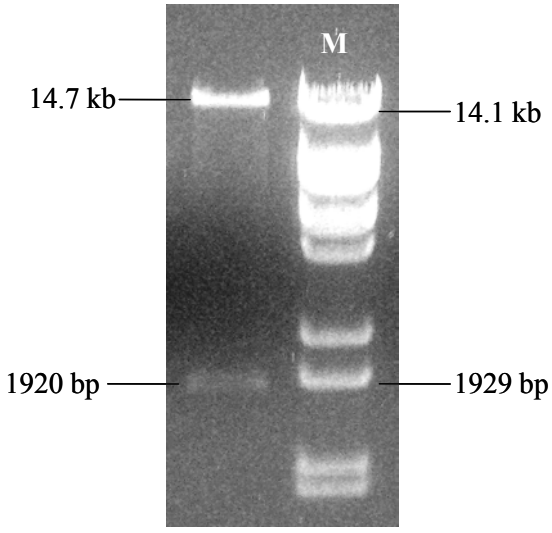

Fig. 3.5: Vector pRJ-35S-EPSPS digested with *EcoR* I for cloning verification. The *35S-EPSPS* construct (1920bp) was released from the vector pRJ-35S-EPSPS (14.7 kb). M: Lambda, DNA/ *Eco*91I (*Bst*EII) Marker, 15.

3.5 Mobilization of the pRJ-35S-EPSPS vector into *B. japonicum* by biparental mating

The vector pRJ-35S-EPSPS, carrying the *CaMV 35S* promoter, could be mobilized by biparental mating from the donor strain *E. coli* S17-1 to *B. japonicum* USDA110spc4 genome. Transfer frequencies of about 1.2 x 10^{-6} transfer plasmids per recipient cell (average of three experiments) were obtained (around 300 kanamycin resistant colonies per 2.5 x 10^{-8} *B. japonicum* cells/ml). Kanamycin resistant colonies of *B. japonicum* appeared after 14 days of incubation at 30 °C in PSY plates containing spectinomycin and chloramphenicol for selection of the *B. japonicum* strain 110*spc*4 and additionally kanamycin for plasmid integration selection.

B. japonicum kanamycin resistant colonies were also screened for sensitivity to tetracycline in order to evaluate the occurrence of the double recombination event. As the pRJ-35S-EPSPS vector (derived from the suicide vector pRJ1042) is not able to self-replicate in *B. japonicum*, expression of the plasmid-encoded kanamycin resistance will only occur if the vector has been integrated into the chromosome via homologous recombination. Colonies resistant to both antibiotics (kanamycin and tetracycline) were expected to have the complete pRJ-35S-EPSPS plasmid integrated into the *B. japonicum* genome after a single crossover, whereas kanamycin resistant and tetracyclin sensitive colonies were candidate to carry the *35S-EPSPS* construct integrated within the *B. japonicum* region RSα9 - RSβ3 via double crossover. In this

experiment, a total of 500 kanamycin resistant colonies were tested in tetracycline selective medium. 6.6% of the kanamycin resistant exconjugants (1.2×10^{-6}) were sensitive to tetracycline, which represent the proportion of *B. japonicum* transformants carrying the *35S-EPSPS* without residual vector material (7.2×10^{-8}). These colonies were assessed to be free of *E. coli* donor cell contaminants after picking and streaking 5 successive times onto fresh selective agar plates. The presence of pure *B. japonicum* clones and absence of any potential *E. coli* donor contaminations was assessed by 16S rRNA gene amplification and sequencing. The *35S-EPSPS* genomic integration into *B. japonicum* was confirmed by PCR amplification and DNA sequencing from these pure *B. japonicum* colonies. The identity of the amplified insert sequence was confirmed using the NCBI blastn tool and 100% similarity was observed to the *35S-EPSPS* construct (*Glycine max* transgenic cp4epsps expression cassette; NCBI accession number AB209952).

3.6 Monitoring expression of the *35S-EPSPS* construct in *B. japonicum*

The 33 colonies from *B. japonicum* harbouring the *35S-EPSPS* insert were picked with a sterile pipette tip and streaked out on sterile fresh agar plates to monitor growth on glyphosate selective plates at different concentrations (5mM, 10 mM, 20 mM). No colony was able to grow in glyphosate selection medium. The *35S-EPSPS* construct, under control of the CaMV *35S* promoter, as enclosed on the RR soybean, did not confer glyphosate resistance to *B. japonicum*. Transconjugants, harbouring the *35S-EPSPS* insert, behave like control non-transformed *B. japonicum* strain 110*spc*4, unable to grow in the presence of 5mM glyphosate (Zablotowicz and Reddy, 2004).

3.7 Cloning the *EPSPS* gene under control of the *nptII* promoter

3.7.1 *nptII* promoter joined to the *EPSPS* gene inside a pCR®-XL-TOPO® vector

In order to test if the lack of expression of the *EPSPS* gene in *B. japonicum* was due to inactivity of the *CaMV 35S* promoter in this bacterium, this promoter was replaced with the sequence coding for the *nptII* promoter, which has been shown to initiate gene expression in *B. japonicum* (Acuña et al., 1987). The novel construct, containing the *EPSPS* gene under

expression of the *nptII* promoter (*nptII-EPSPS*) resulted from the combination of two vectors: The pT-*EPSPS*, harbouring the single *EPSPS* gene, and the vector pT-nptII-EPS, which contained the synthetic fragment with the *nptII* promoter sequence linked to 224 bp of the *EPSPS* gene. The cloning strategy consisted of releasing the initial fragment of the original *EPSPS* gene from the vector pT-*EPSPS*, to be replaced with the complementary synthetic sequence of the same gene joined to the new *nptII* promoter (Figures 3.6 and 3.7).

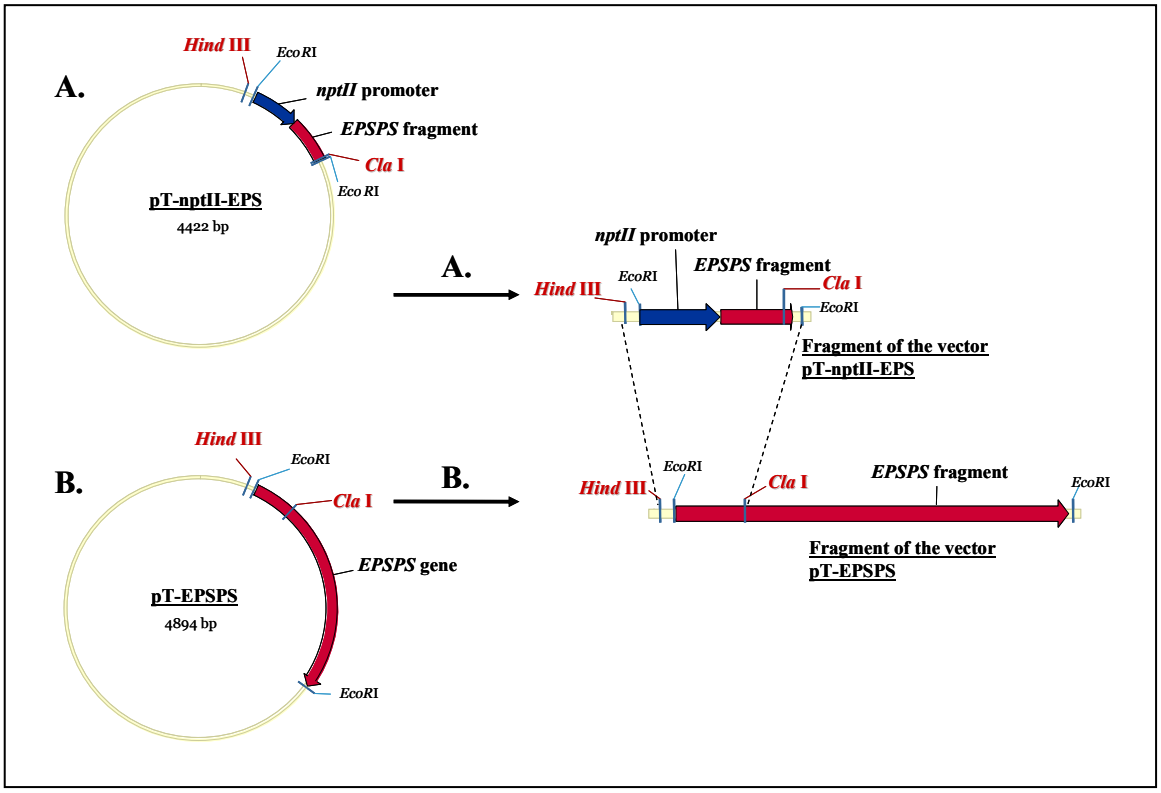

Fig. 3.6: Construction of the vector pT-nptII-EPSPS, carrying the *nptII* promoter ligated to the complete *EPSPS*. A. The vector pT-nptII-EPSPS carries the synthetic promoter joined to a fragment of the *EPSPS* gene. Excision with the restriction enzymes *Hind* III and *Cla* I released the synthetic construct *nptII-EPS* from the vector to be cloned into the same restriction sites into the vector pT-EPSPS. **B.** The vector pT-EPSPS liberated the initial fragment of the *EPSPS*, after restriction with *Hind* III and *Cla* I. The released *nptII* promoter joined to part of the *EPSPS* gene was ligated to the complementary sequence of the rest of the gene, contained in the vector pT-EPSPS.

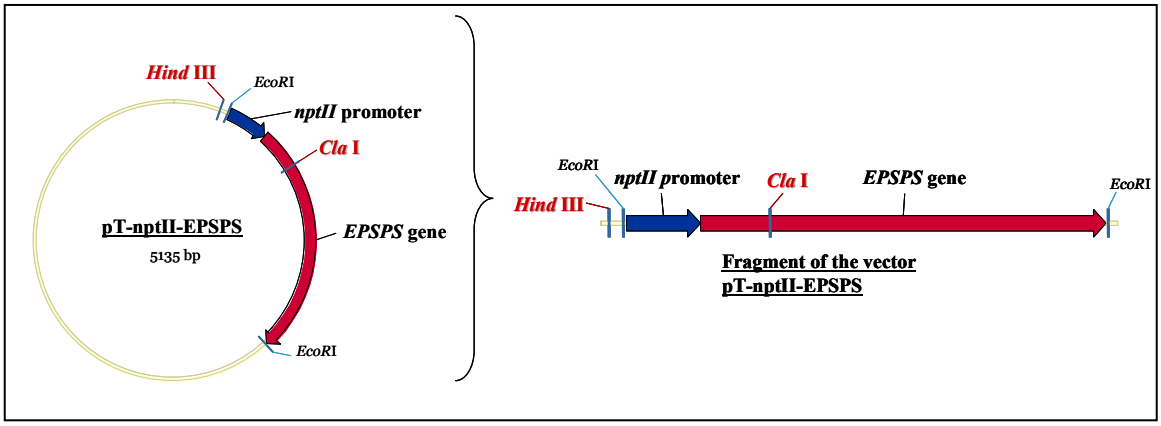

Fig. 3.7: The vector pT-nptII-EPSPS resulted from the arrangement of vectors pT-nptII-EPS and pT-EPSPS. The generated construct *nptII-EPSPS* was excised (*Eco*RI) and subcloned into the *B. japonicum* vector pRJ-nptII-EPSPS.

The new vector pT-nptII-EPSPS bears the *nptII* promoter immediately upstream of the promoterless *EPSPS* gene. Excision of the pT-nptII-EPSPS vector with *Eco*RI confirmed the correct size of the linearized pCR®-XL-TOPO® vector and the *nptII-EPSPS* insert (Figure 3.8). The insert was sequenced to verify its identity compared to the *nptII-EPSPS* construct. In an alignment of the two sequences using the NCBI bl2seq tool a 100% similarity was observed.

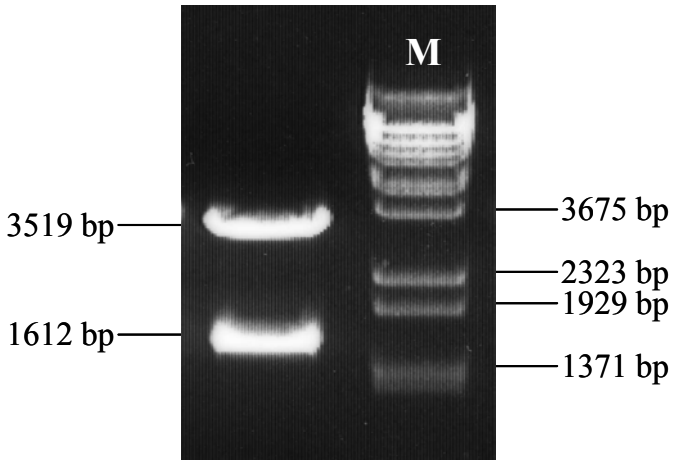

Fig. 3.8: Restriction analysis of the vector pT-nptII-EPSPS by *Eco*RI. The *nptII-EPSPS* construct (1612 bp), containing the *nptII* promoter joined to the full length *EPSPS* gene was released from the original pCR®-XL-TOPO® (3519 bp). M: Lambda, DNA/ Eco91I (BstEII) Marker, 15.

3.7.2 *nptII- EPSPS* construct cloned into the suicide vector pRJ1035

The insert containing the *nptII-EPSPS* was excised from the pT-nptII-EPSPS plasmid by *EcoR*I digestion and then cloned into the same restriction site of the pRJ1035 integration vector for *B. japonicum*, giving raise to plasmid pRJ-nptII-EPSPS. Plasmids from transformed *E. coli* were prepared and digested with *EcoR*I for initial verification of the *nptII-EPSP* plasmid construct. The digestion showed two bands on the agarose gel: One with linearized pRJ-nptII-EPSPS vector and a second corresponding to the insert *nptII-EPSPS*. For the *nptII-EPSPS* insert the expected size of 1612 bp was confirmed. Nevertheless every examined pRJ-nptII-EPSPS plasmid, cut with *EcoR*I, did not conserve the preliminary size (11 kb) of the linearized vector pRJ1035 (Figure 3.9). After ligation of the *nptII-EPSPS* insert with the pRJ1035 vector and transformation in *E. coli*, the resulted pRJ-nptII-EPSPS plasmid, carrying the insert *nptII-EPSPS*, showed a reduced size (less than 8 kb) compared to the initial vector pRJ1035 (11 kb).

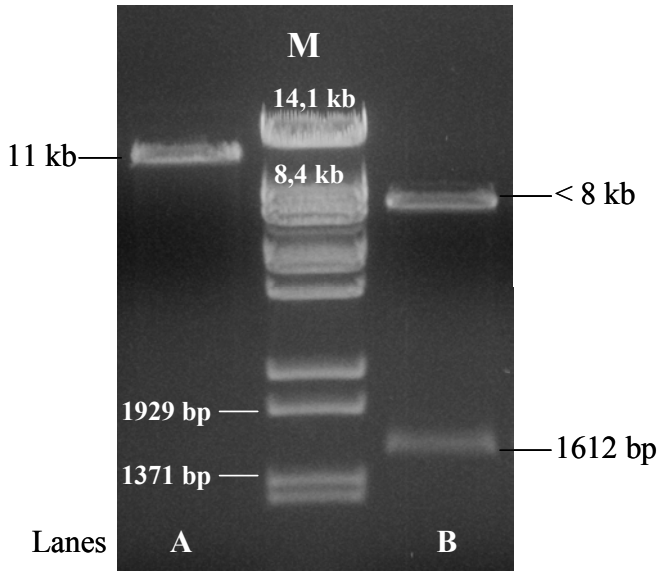

Fig. 3.9: Size comparison of vectors pRJ1035 and pRJ-nptII-EPSPS after restriction with *EcoR*I. Lane A: Vector pRJ1035 (11 kb). Lane B: Vector pRJ-nptII-EPSPS (< 8 kb) and released *nptII-EPSPS* insert (1612 bp).

Colony PCR was performed to verify the *nptII-EPSPS* insertion (Figure 3.10). Internal primers for *EPSPS* and primers flanking the complete *nptII-EPSPS* construct confirmed the presence of the insert. Prior to biparental mating for integration into *B. japonicum*, a last confirmation of the insert position inside the vector was done. PCR with primers amplifying between the RSα9 - RSβ3 region resulted in a band with the expected size of the cloning region in absence

of the insert. Further PCR analysis with internal primers and primers on the RS*α*9 and RS*β*3 regions, respectively, did not generate any amplified band. These PCR analyses proved the insertion of *nptII-EPSPS* into the pRJ-nptII-EPSPS vector and indicate that the *nptII-EPSPS* was not cloned between the integrative RSα9 - RS*β*3 sequences (Figure 3.10). Unknown reorganization of the pRJ-nptII-EPSPS vector forced to finish further experiments with the *nptII-EPSPS* construct inside the original pRJ1035 vector (see discusion chapter).

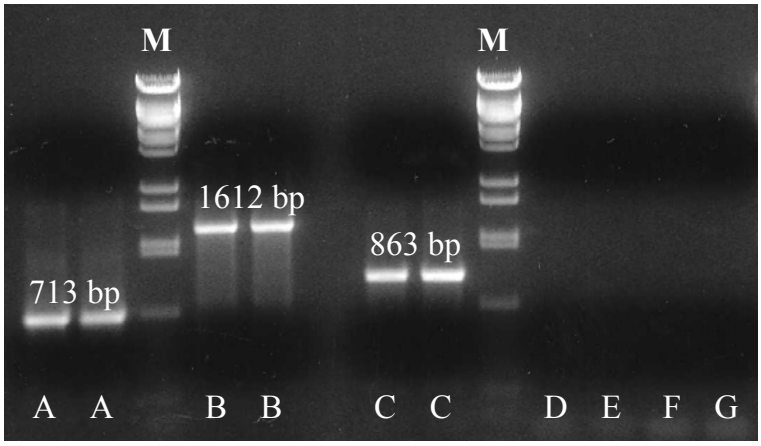

Fig. 3.10: PCR amplification of the *nptII-EPSPS* construct inserted into the pRJ-nptII-EPSPS vector. Lanes A to F designate PCR products originated from different primer combinations. Lanes A to C show products from two different clones. Lane A: *EPSPS* internal region (713 bp) amplified with primers intern-F and intern-R. Lane B: Full length *nptII-EPSPS* construct (1612 bp) amplified with primers nptII-F and EPSPS-R. Lane C: RS*α*9 - RS*β*3 intern region (863 bp) amplified with primers RS*α*9-F and RS*β*3-R. For samples contained on lanes D, E, F and G there was no PCR amplification signal. In these lanes primer combinations from the intern region of *EPSPS* to the RS*α*9 and RS*β*3 regions were used to test the *EPSPS* insertion place with respect to the cloning region RS*α*9 - RS*β*3. Following primer combinations were employed: Lane D: primers RS*α*9-F and intern-F. Lane E: primers RS*α*9-F and intern-R. Lane F: Primers RS*β*3-R and intern-F. Lane G: RS*β*3-R and intern-R.

3.8 *B. japonicum* transformation by electroporation

Various attempts to transform *B. japonicum* with recombinant plasmid pRJ-35S-EPSPS DNA, containing the transgenic *EPSPS* gene, were carried out in order to evaluate if the *EPSPS* can confer glyphosate resistance to *B. japonicum*.

Attempts to transform *B. japonicum* cells by electroporation were carried out including modifications of the described procedures (Guerinot et al., 1990; Hattermann and Stacey, 1990). Bacteria were grown under standard conditions to an OD_{600} of 0.4 to 0.6 in different

media (PSY, YEM and YEX). Transformation by electroporation was performed using several centrifugation steps at different speeds (4000 x g and 9820 x g) increasing plasmid DNA concentrations (10 ng/mL, 125 ng/mL, 250 ng/mL, 500 ng/mL, 1µg/mL), and two electroporation resistances (200Ω or 400 Ω). Under the tested conditions no positive transformants, carrying the pRJ-35S-EPSPS plasmid insertion, were obtained by this procedure.

3.9 *B. japonicum* natural transformation

The development of a natural competence state in *B. japonicum* was evaluated on agar PSY plates (Lorenz & Wackernagel, 1991) and under liquid medium conditions (Demaneche*, et al.*, 2001) using the *B. japonicum* integration plasmid pRJ-35S-EPSPS. Modifications of the methods regarding incubation times of the slow-growing *B. japonicum* were taken into account. Furthermore additional plasmid DNA concentrations were tested. Equivalent plasmid DNA concentrations were mixed with exponentially growing *B. japonicum* cultures for plate and liquid transformations (10 ng/mL, 100 ng/mL, 250 ng/mL, 500 ng/mL, and 1 µg/mL) in triplicates.

For plate transformation, mixtures of a bacterial culture (initial and 10-fold concentrated suspension) and plasmid DNA were spotted on non selective agar plates and incubated (48 h and 62 h) under standard conditions. After resuspension of the growing spots and incubation with kanamycin selective pressure, the obtained colonies were screened for pRJ-35S-EPSPS plasmid insertion. For each experiment 100 colonies were tested by PCR yet no insert (*35S-EPSPS*) was found.

The development of natural competence state under liquid conditions was assessed by adding plasmid DNA to *B. japonicum* shaking cultures at different time points of the exponential growth phase. Aliquots of the bacterial suspension were plated on kanamycin selective plates at successive time points. Obtained resistant colonies were screened by PCR for the *35S-EPSPS* insertion. No PCR amplification was observed from the tested colonies (100 colonies for each experiment). All evaluated colonies were spontaneously resistant to kanamycin.

Under the tested agar and liquid conditions, no natural transformation was detected in *B. japonicum* in the presence of different excess amounts of recombinant plasmid DNA.

3.10 Spontaneous mutations of *B. japonicum*

Cultures of *B. japonicum* were plated in triplicates on 100 glyphosate containing plates (25 mM) to estimate the frequency of appearance of naturally occurring glyphosate-resistant variants. The frequency of resistant mutants was expressed as the average number of resistant colonies divided by the total number of colony-forming units (CFU) (Bjorkholm*, et al.*, 2001, Wang*, et al.*, 2001).

Calculations were based on counting *B. japonicum* colonies grown on non-selective media. Cultures of *B. japonicum* with OD_{600}=1 contained an average of $2.5x10^8$ CFU per ml. *B. japonicum* glyphosate-resistant colonies appeared at a frequency of $3.2x10^{-7}$. Yet, the obtained spontaneous glyphosate resitant colonies were not stable, since the resistance was lost after cultivation on non selective medium.

3.11 Rhizosphere bacterial community patterns revealed by T-RFLP

The influence of glyphosate in the rhizosphere of transgenic soybean after foliar application was evaluated in an experiment under greenhouse conditions. Two post-emergence glyphosate treatments were applied to the foliage of the plants, as recommended by the manufacturer for regular the field application. Control plants were treated with the same volume of water as plants treated with glyphosate solution. The first application took place when the plants were in the first trifoliate stage and the second one, fourteen days later, when they reached the third trifolium stage. Five harvests were performed: three, seven and fourteen days after the first glyphosate treatment in addition to three and seven days after the second herbicide application. Plants grew in pots containing four plants per pot. At each harvest three pots, each with four plants, were sampled as three repetitions for the experiment. Total DNA from the bacterial rhizosphere communities associated with RR soybeans was monitored over time by T-RF fingerprint analysis of amplified 16S rRNA genes.

Fluorescent labelled amplicons were digested with the enzyme *MSP*I and separated on an automatic sequencer in order to generate a set of lengths from labelled terminal restriction fragments. Differences in the diversity and composition of bacterial communities between glyphosate-treated (G) and untreated control plants (K) were detected as changes in the T-RF fingerprints.

T-RFLP profiles of the five harvests and three sample repetitions per harvest were analysed statistically. Initially, the experimental design included two glyphosate applications and six sampling time points, however the sixth harvest (Figure 3.11) was not included for statistical analysis since the entire amplified community was composed of a single T-RF (149 bp). This T-RF 149 bp could be identified as *Bradyrhizobium* related species and was the dominant peak for control as for glyphosate-treated plants.

For all T-RFLP analyses, only peaks at positions between 50 and 500 bp were considered in order to avoid T-RFs caused by primer-dimers and to obtain fragments within the linear range of the internal size standard (Singh et al., 2006).

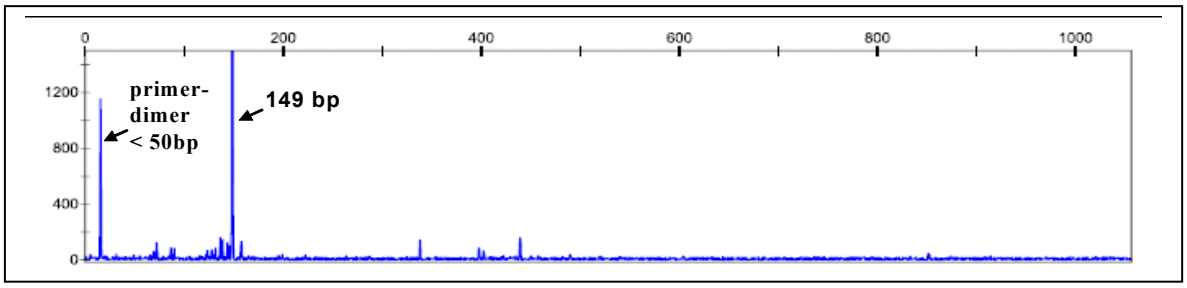

Fig. 3.11. Characteristic T-RFLP fingerprint retrieved from the sixth sampling time of glyphosate - treated plants. Two dominant T-RFs are present: One T-RF (<50 bp) corresponds to primer dimmers, not included in the analysis. The second T-RF (149 bp) represents the major part of the bacterial community.

3.12 Glyphosate influence on the bacterial community diversity

The Shannon diversity index (H') was calculated for the relative abundance of single T-RFs in every fingerprint. The index values comprised the mean for the three repetitions at each harvest time. In Figure 3.12 it is clearly recognisable that the diversity shifts within the community share a common pattern independent of the herbicide treatment.

The diversity indices from the first harvest varied the least throughout treated (H`=2.6) and untreated samples (H`=2.5). However, for this harvest, three days after glyphosate treatment, the bacterial rhizosphere diversity of glyphosate-treated plants was lower than the bacterial diversity associated to control plants.

The microbial diversity increased at the time of the second harvest independent from the treatments and for each treatment reached the maximum observed diversity level (2.9 for K2 and 3.3 for G2). Nevertheless, the diversity of root associated bacteria seven days after

glyphosate treatment (G2) was much higher than the diversity of the T-RFs retrieved from bacterial communities associated with roots of control plants (K2). At this time the beginning of the flowering period of the soybean plants took place, while glyphosate-treated plants started flowering three to four days earlier. Subsequently, the diversity of T-RFs decreased for both communities (K3 and G3) fourteen days after glyphosate application. However, for this third harvest the diversity of root associated bacteria from glyphosate-treated plants was still higher than those of untreated plant rhizosphere communities (G3=3.1; K3=2.6).

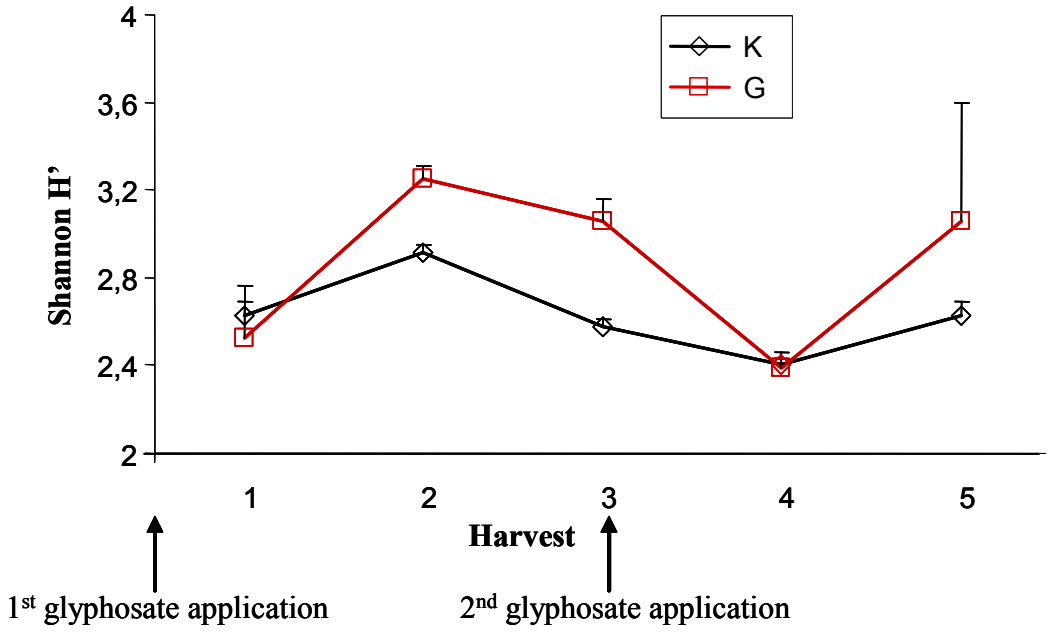

Fig. 3.12. The Shannon diversity index (H') calculated for the relative abundance of single T-RFs from every fingerprint. The diversity values comprise the mean of three replicates for each harvest time. Samples retrieved from glyphosate-treated plants (G) are shown in red and control samples (K) in black. Bars indicated standard deviation.

The fourth sampling time point occurred three days after the second glyphosate application. At this time, as observed from absolute T-RF numbers, the diversity indices dropped to the minima obtained (H`=2.4). The diversity of bacterial communities associated with glyphosate-treated soybean plants decreased to a similar level as after the first glyphosate application.

After the diversity decrement monitored at the fourth harvest, seven days after each glyphosate application, the bacterial diversity increased and was even higher than the rhizosphere diversity of untreated plants.

In general it can be stated that the Shannon diversity indices varied less within the untreated microbial community. Only at the second sampling time point there was a clear observable increase in the diversity of T-RFs retrieved from control plants. In contrast to the controls, 16S rDNA T-RFLP-based diversity observed from glyphosate-treated samples always indicated a higher H' with the exception of harvests directly following the application of the herbicide (G1 and G4). In these cases, the diversity was equal or lower than the corresponding controls. Therefore, the diversity index values based on measured T-RFs were more variable over time for the glyphosate-treated plants.

3.13 Principal component analysis on T-RFLP profiles

In addition to diversity, structural composition and relative abundance of detected T-RFs indicated microbial community shifts in the rhizosphere of glyphosate-treated soybean. This influence of glyphosate on the rhizosphere community structure over time was visualized by principal component analysis (PCA) performed on T-RFLP profiles. The community structural composition was evaluated based on the identity of T-RFs (in bp) and relative abundance of detected T-RFs within fingerprints (Winderl et al., 2008). With help of PCA, characteristic T-RFs were identified and were associated to specific treatments (with or without glyphosate) over the five sampling times.

The application of this method for reducing the multidimensional space of the sample/T-RF-matrix resulted in a two-dimensional coordinate system which describes the largest part of the variance between the samples. Into this coordinate system the five harvests time points, represented by the corresponding glyphosate-treated and untreated samples are projected and can be visualized in combination with the loadings of the single T-RFs (Figure 3.13). These loadings (displayed as arrows) indicate the correlation of a given species (T-RF) with an axis and therefore the importance of this T-RF for the separation of the samples. For visual clarity, PCA scores of averaged triplicate T-RFLP profiles were plotted. Therefore the standard

deviation is indicated for each sampling time per treatment (G or K), since each point represents the average of three sample repetitions.

PCA of T-RFLP profiles allowed the visualization of time-dependent community shifts within the microbial rhizosphere communities. The total variance of T-RFLP fingerprints was reduced to two hypothetical principal components, which explained 40.3% and 38.6% of the total variance, respectively. PCA showed a clear separation of 16S rRNA-derived profiles in the ordination space. Moreover, similarities between the five different sampling times within glyphosate-treated and untreated plants were revealed (Figure 3.13).

In the following, letters are assigned to glyphosate-treated plants (G) and control plants (K) and consecutive numbers from one to five indicate the five harvests performed. At the first harvest, occurring three days after the first glyphosate application, all samples were grouped in close space for both G1 and K1 samples indicating similar community compositions of glyphosate-treated and untreated plants. Furthermore, the samples corresponding to the second harvest (G2 and K2), which was performed seven days after glyphosate application, are again clustered close to each other but already separated from the first sampling date (G1 and K1). This distribution in the ordination space indicates similitude within the communities of the second harvest irrespective of the treatment, yet differences with respect to other sampling times.

Fourteen days after glyphosate application was the time of the third harvest. A clear shift in the community composition was observed at this sampling time point. The glyphosate-treated samples from the third harvest (G3) and untreated samples (K3) were not in close vicinity in the ordination space. G3 was grouped into a cluster with K4, while K3 and K5 clustered together in an opposing space. This indicates that the bacterial community composition of G3 was similar to the composition of K4, while the communities from K3 and K5 are more related to each other than to any other sample.

The microbial community of K4 and G4 was sampled three days after the second glyphosate application and K5 and G5 corresponds to the sampling seven days after this second treatment. G4 and G5 are located close to each other and are separated in the ordination space from the control treatments, showing a differentiation of the community composition. It is evident, that the samples provided from G4, G5 but also G3 are localized in the same direction, separated from other control samples.

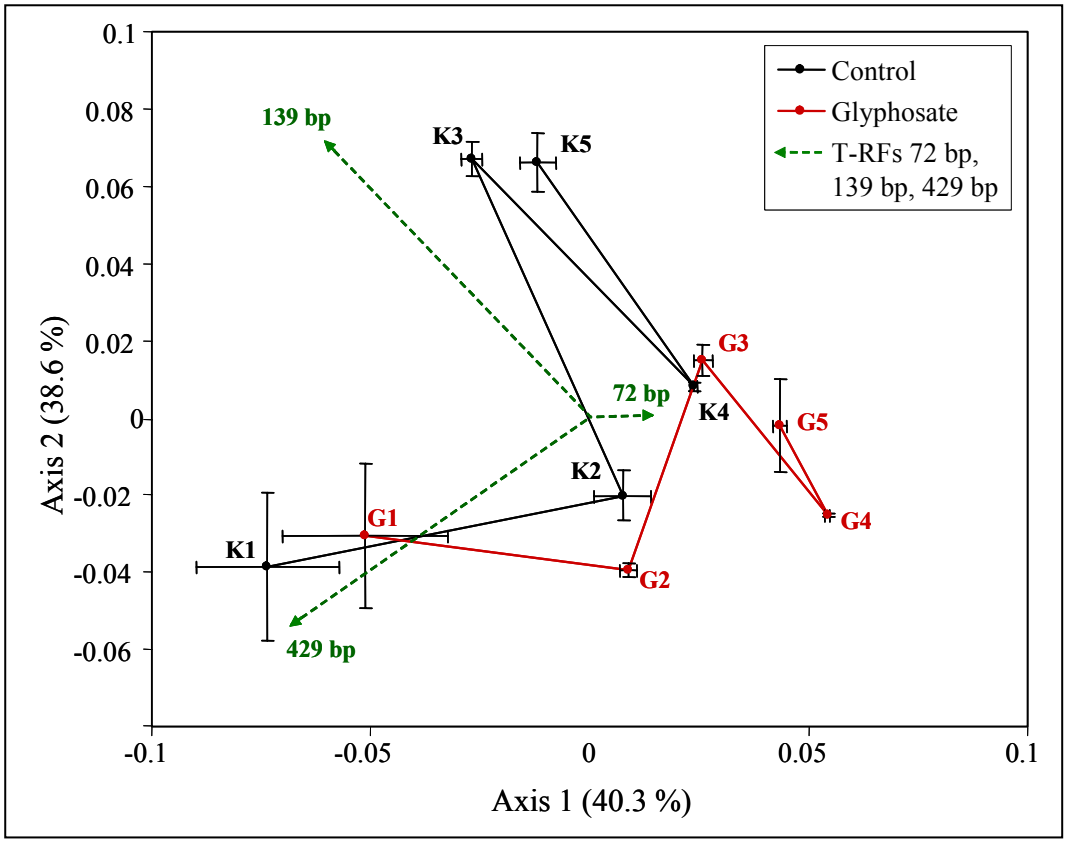

Fig. 3.13. Principal component analysis performed with T-RFLP data of all samples from control (K) and glyphosate (G) treated plant samples. Axis 1 and 2 accounted for 40.3% and 38.6% of the total variance, respectively. The identities (bp) of selected T-RFs with characteristic factoral loading are indicated with discontinues green arrows. The five harvests of control (K1 to K5) and glyphosate treatments (G1 to G5) are pointed out in black and red respectively and are joined with a line that follows the successive sampling times. For each harvest a centroid of the three replicates per treatment is shown and standard deviations for both axis are indicated by bars.

Only the bacterial community from the harvest K4, retrieved from a control treatment clustered together with the three last samplings of glyphosate-treated plants (G3, G4, and G5). In general, concerning the first two sampling times, samples retrieved from treated and untreated plants were grouped in clusters and the community shift was higher between sampling time points than between treatments. Up to the third harvest, fourteen days after the first glyphosate application, there was a tendency to separate glyphosate-treated from untreated samples.

With assistance of the loading plot of interfered PC factors, the most distinctive T-RFs responsible for the distinct PCA ordination of fingerprints were identified. The three most

characteristic T-RFs are illustrated in Figure 3.13 with discontinuous green arrows that indicate the loadings of fragments 72 bp, 139 bp and 429 bp. Fragment 429 bp was characteristic for the early sampling times (G1 and K1) as indicated by its strong negative correlation with both axis, while T-RF 139 bp was associated with the third and fifth sampling time of the control plants (strong positive correlation with axis 2). An indication for a positive correlation with axis 1, and therefore with the community shift after glyphosate application (G3, G4 and G5), could be seen for fragment 72 bp.

3.14 Indicator species analysis

Indicator species analysis (Dufrêne and Legendre, 1997) was used to evaluate the significance of T-RFs which were particularly associated with the treatment (glyphosate or control) and/or a sampling time. Indicator species analysis combines information about the frequency of a T-RF and the faithfulness of occurrence of that fragment to produce an indicator value for each T-RF in each treatment over time (harvest). The probability of this value arising by chance was then tested using a Monte Carlo procedure with 1000 iterations. Based on the obtained clustering of samples in the PCA, groups were chosen for a detailed analysis. The test calculates the proportional abundance of a particular T-RF in a particular group relative to that T-RF in all groups (McCune and Grace, 2002). T-RFs with *p*-values below 0.05 and indicator values above 55 were taken into account for the indication of the selected groups.

The first group was defined as “early samples”, which comprised the samples G1 and K1. In the statistical analysis, the T-RFs from this group were compared with the rest of the samples. The second group was defined as “control samples” and included the control samples K3 and K5 that were located in opposite direction to G3, G4, G5 and K4 in the PCA. This latter group formed the last cluster called “G + K4”. The most significant T-RFs for the community shifts were identified (Table 3.1).

Table. 3.1. Indicator species analysis contrasting significant T-RFs for selected groups.[a]

T-RF (bp)	Group	Mean relative peak height within groups (%)				Indicator value	*p* -value
		Indicated group	Standard deviation	Rest of the groups	Standard deviation		
429	Early samples	0.230	0.050	0.029	0.041	87.9	0.0002
487	Early samples	0.067	0.024	0.023	0.014	74	0.0024
490	Early samples	0.063	0.013	0.015	0.008	79.8	0.0004
139	Control samples	0.340	0.025	0.125	0.064	71.5	0.0008
72	G+K4	0.058	0.011	0.042	0.020	57.5	0.0348

[a] Selected indicator T-RFs responsible for the groups separation are shown. For each T-RF, the mean relative peak height within groups is compared with the same value for the remaining samples. Standard deviations are indicated. Indicator values were considered > 55. Significant *p*-values were < 0.05. G includes G3, G4 and G5 samples.

T-RFs 429 bp, 487 bp and 490 bp were positive indicators for the group of "early samples". As suspected from the factoral loadings of the PCA, T-RF 429 bp stands out as a dominant peak describing the early samples, with the highest abundance, the strongest indicator value and the most significant *p*-value for this indicator group. The most indicative peak for samples within the group "control samples" was T-RF 139 bp having the highest abundance within this indicated group (relative peak height 0.34). Additionally, as expected, peak 72 bp was characteristic for the cluster "G + K4", although the relative peak height was not as elevated as for the peaks 429 bp and 139 bp for their respective groups. This peak shows a tendency to be elevated in samples belonging to the "G + K4" cluster, yet the relatively low indicator value of 57.5 shows that the indication is not as strong as when compared to peaks 429 bp (indicator value 87.9) and 139 bp (indicator value 71.5).

3.15 Indicator peaks as revealed by T-RF fingerprints

The identified indicator peaks for the different groups can also be seen from the original T-RF fingerprints (Figure 3.14). The height of peak 72 bp increased in both treatments after the first harvest and in the last harvest this peak was higher in glyphosate-treated samples.

The behaviour of the peak 139 bp in the community was also recognisable in the fingerprints. The abundance of the T-RF 139 bp increased after the first harvest more in the control samples than in the glyphosate-treated ones. Finally, the most evident change was observed for the peak 429 bp. At the first harvest, for both treatments it is the highest peak in the fingerprints, while at later harvests this peak almost disappears.

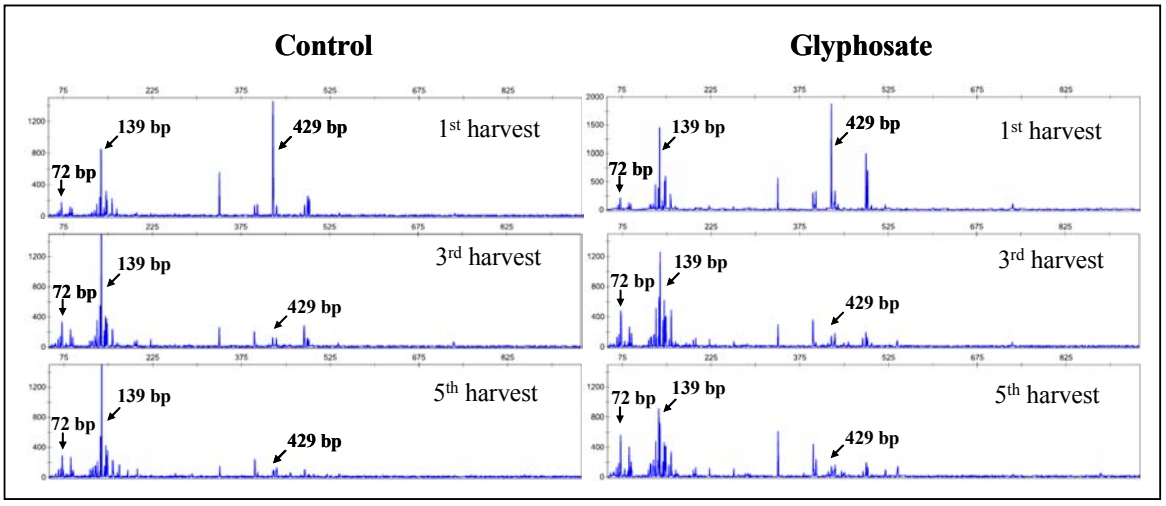

Fig. 3.14. Representative bacterial 16S rRNA T-RFLP fingerprint electropherograms derived from rhizosphere samples of control and glyphosate treated RR soybean. Selected characteristic T-RF fingerprints from the first, third and fifth harvests are shown. The three most significant peaks for the community shifts, 72 bp, 139 bp and 429 bp, are indicated with arrows.

3.16 Abundance variations over time of significant T-RFs

Assessment of the significant T-RFs relative abundances over time for each treatment was necessary in order to choose the samples to be used to establish clone libraries. Since peak 429 bp was characteristic for the early harvest times, DNA extracted from the rhizosphere provided by samples G1 or K1 was the most probable to contain high copy numbers of this 16S rDNA phylotype. Furthermore, the relative abundance of this peak was highest in control samples (Figure 3.15), thus DNA retrieved from the sampling K1 was used to establish one of the clone libraries. Although it is evident that at the first harvesting time point T-RF 149 bp is most abundant, the tendency of the abundance of this population over the time has a tendency to be equal, almost disappearing at the last harvests.

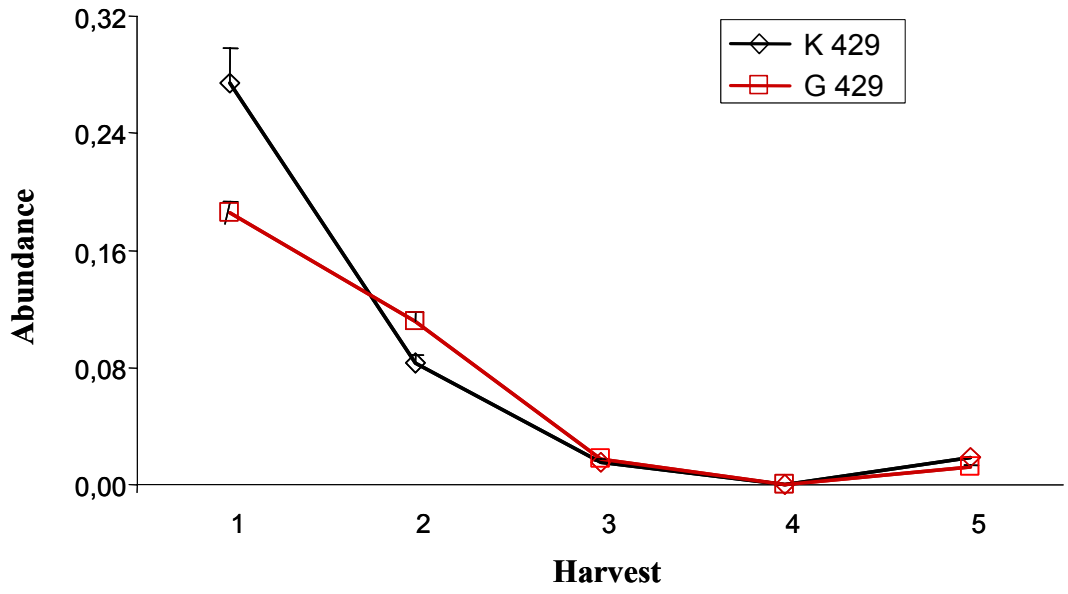

Figure 3.15. Abundances of the T-RF 429 bp at each harvesting time point. Abundance values retrieved from glyphosate-treated samples (G) are marked in red and from control samples (K) in black.

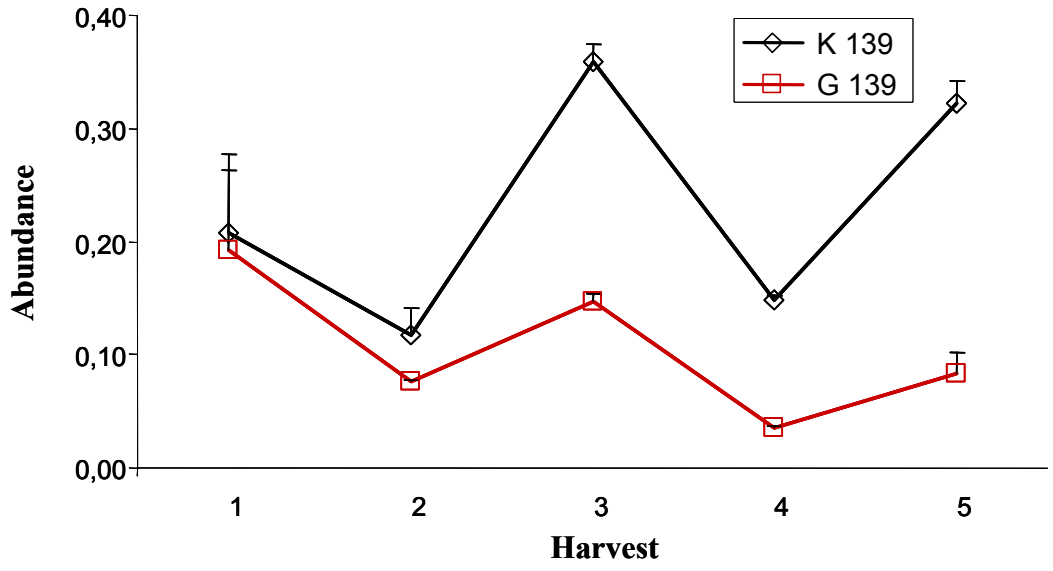

Fig. 3.16. Abundances of the T-RF 139 bp at each harvesting time point. Abundance values retrieved from glyphosate-treated samples (G) are marked in red and from control samples (K) in black.

The peak 139 bp was shown to be characteristic for the samples taken from the control plants, where it was constantly more abundant; however the abundance of this T-RF presented several noticeable variations over the time (Figure 3.16). Although for the T-RF 139 bp, as for peak 429 bp, the populations retrieved from both treatments showed the same tendency increasing or lowering the abundance over the time. In order to recover the organism associated with the peak 139 bp, the sampling time used for establishing a clone library was the K3, where the highest abundance was observed, based on the T-RFLP profiles.

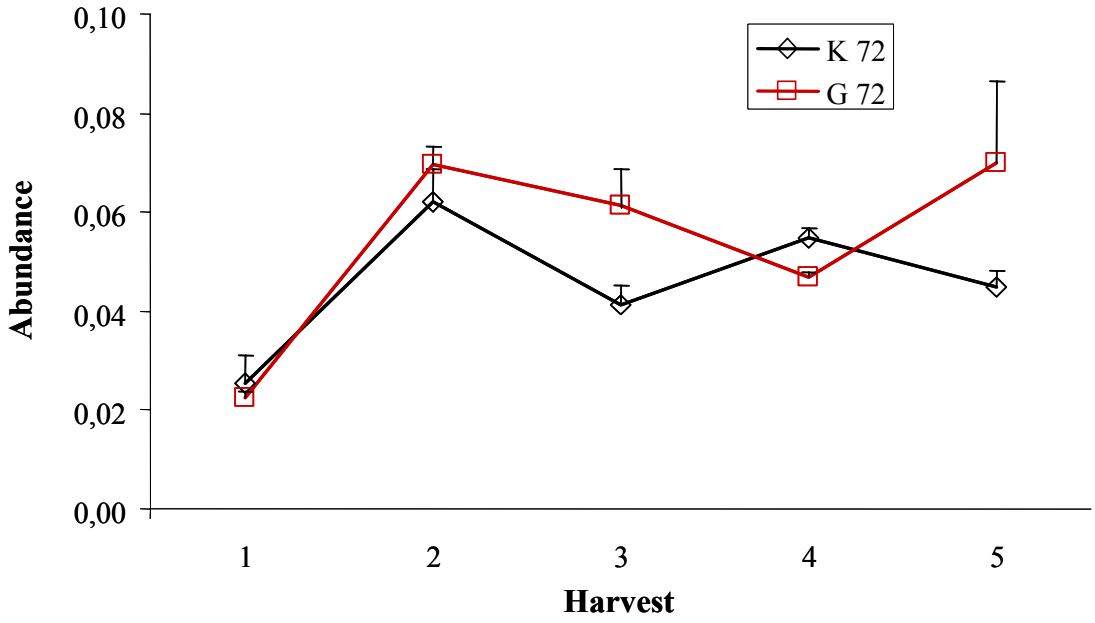

Fig. 3.17. Abundances of the T-RF 72 bp at each harvest time. Abundance values retrieved from glyphosate-treated samples (G) are marked in red and from control samples (K) in black.

The abundance of the peak 72 bp increased in control and glyphosate-treated samples to almost three times its original height from the first sampling time point to the second (Figure 3.17). The abundance changes in both treatments after the second harvest were not pronounced. In the second, the third and fifth harvests the T-RF 72 bp was more abundant in glyphosate treatments.

While at the first and the fourth harvest, which coincide with the earliest times points after glyphosate application (three days after), the abundance of the T-RF dropped below or equal to the abundance of the same T-RF for the control treatments. DNA from the fourth glyphosate-treated sample was used to create the final clone library, in order to identify the T-RF 72 bp.

3.17 Relative phylum-level compositions of the 16S rRNA gene clone libraries

In order to identify dominant rhizosphere bacteria, 16S rRNA gene clone libraries were established and partial insert sequences determined. Three clone libraries comprising 46, 90 and 48 nearly full-length 16S rRNA gene sequences were obtained from the libraries K116S, K316S and G416S, respectively. The K116S library were produced from amplicons of pooled sample DNA from the first root harvest of untreated RR soybean plants, the K316S library contained amplicons of pooled sample DNA of the third root harvest belonging to untreated plants and the G416S library corresponded to the fourth harvest from glyphosate-treated soybeans. The three libraries covered the dominant T-RFs allowing the description of the main root associated bacterial communities. In order to assign specific T-RFs to clones, digestions of obtained sequenced products with the enzyme MSPI were simulated *in silico* and individual T-RFLP analyses were performed for unique clones. Subsequently, community and individual fingerprints for each clone were compared to confirm the size and identity of each T-RF.

From 192 evaluated sequences, eight sequences were not used for further analysis, since they were identified as chimeras with assistance of the Bellerophon program. Most of the resulting nonchimaeric sequences showed at least 95% similarities to known sequences in the NCBI database.

3.17.1 Clone library K116S

The relative abundances of bacterial phyla, obtained from the first root sampling of untreated transgenic soybean plants (clone library K116S) are illustrated in Figure 3.18. For clone library K116S, almost half of the sequences fell into the division *Betaproteobacteria* (46 %). The second dominant phylum represented by a large proportion (amount) of clones was *Alphaproteobacteria* (22 %). The remaining more abundant sequences were distributed among uncultured bacteria (9 %) and Actinobacteria (7 %). The phyla *Gammaproteobacteria*, *Planctomycetes*, and *Gemmatimonadetes* were equally represented by 4 % of the clones. The clones with the lowest relative abundance in the library (2 %) belonged to the division of *Bacterioidetes*. Furthermore, plant derived sequences of *Glycine max* were also identified in the rhizosphere community, however with a relatively low abundance of 2 % (Figure 3.18).

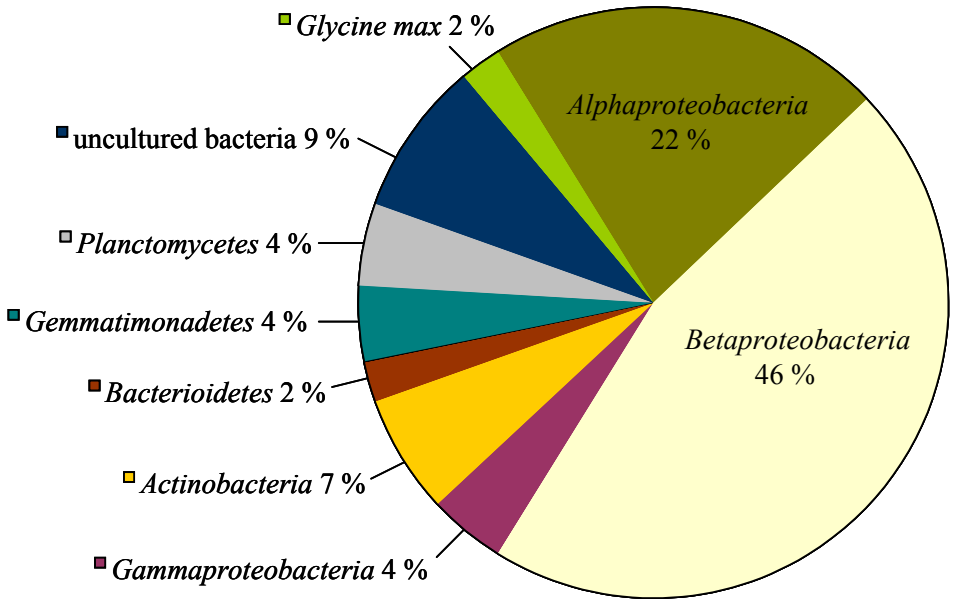

Fig. 3.18. Relative abundance of bacterial phyla obtained from 46 bacterial 16S rRNA sequences retrieved from the clone library K116S.

3.17.2 Clone library K316S

The clone library K316S contained 90 sequences of clones originating from the third harvest of control plants. This library was also dominated by *Betaproteobacteria*, yet exhibited a lower relative abundance (28 %) compared to the library K116S (46 %). The second most abundant group of clones belonged to *Alphaproteobacteria* (13 %). The remaining more abundant sequences were distributed among the groups *Actinobacteria* (8 %), *Gammaproteobacteria* (8 %), *Acidobacteria* (8 %), *Bacterioidetes* (7 %), *Gemmatimonadetes* (7 %) and uncultured bacteria (9 %). The minority of clones was associated to *Firmicutes* (4 %), *Chloroflexi* (3 %) and *Deltaproteobacteria* (2%). Moreover, plant sequences associated to *Glycine max* were represented by 3% of the clones in this library (Figure 3. 19).

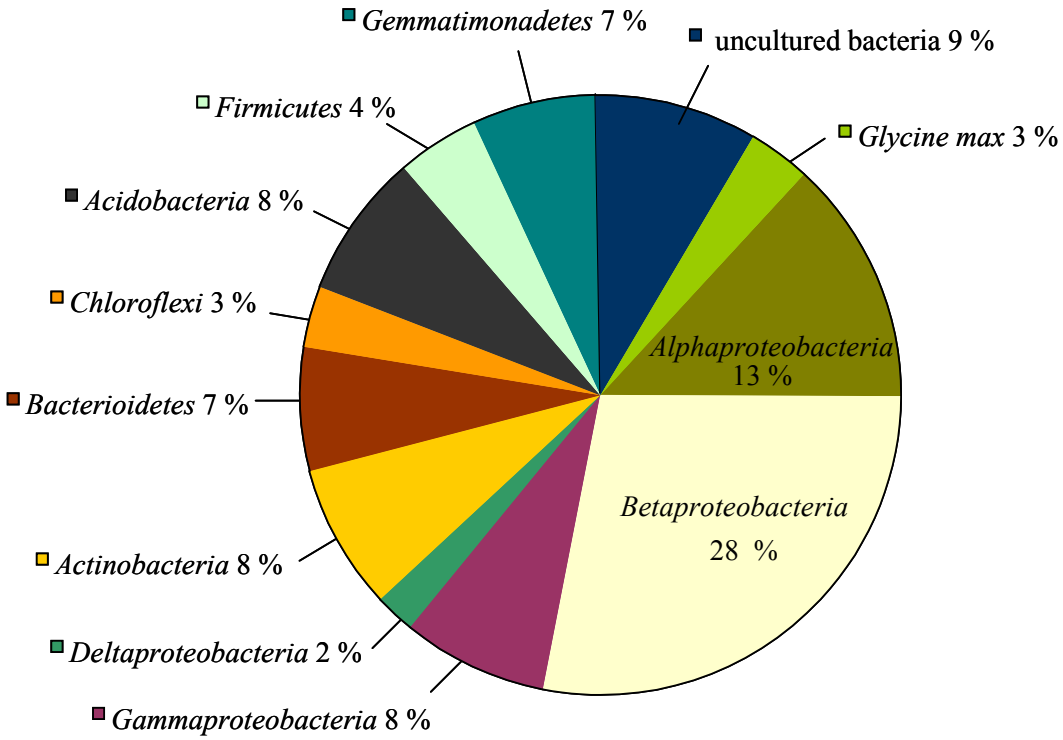

Figure 3.19. Relative abundance of bacterial phyla obtained from 90 bacterial 16S rRNA sequences retrieved from the clone library K316S.

3.17.3 Clone library G416S

Alphaproteobacteria (29 %) was the most abundant phyla from the clone library G416S, which resulted from the fourth root harvest of glyphosate-treated roots. *Betaproteobacteria* (15 %) and *Actinobacteria* (13 %) were the second most dominant classes within these glyphosate-treated samples. In this library there were medium amounts of uncultured bacteria sequences (6 %), Gammaproteobacteria (6%) and a higher number of *Gemmatimonadetes* related fragments (8 %). The remaining clones with lower abundances were distributed among *Bacterioidetes* (4 %), *Chloroflexi* (4 %), *Acidobacteria* (4 %), *Firmicutes* (2 %) and *Planctomycetes* (2 %). As for the other libraries, low abundance (4 %) of *Glycine max* sequences was detected apart from bacterial sequences (Figure 3.20).

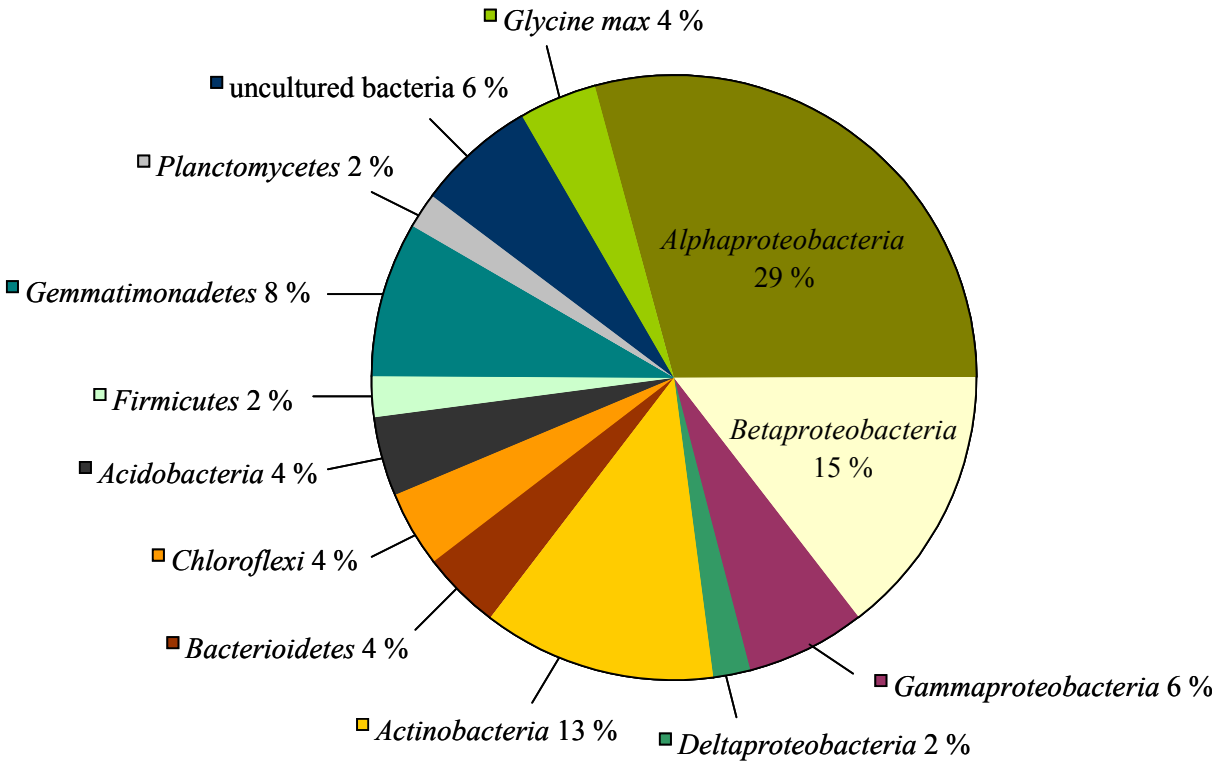

Fig. 3.20. Relative abundance of bacterial phyla obtained from 48 bacterial 16S rRNA sequences retrieved from the clone library G416S.

3.18 Clone identification of the most significant T-RFs investigated in the three clone libraries

Libraries K116S, K316S and G416S were established in order to search for clones related to significant dominant peaks identified by the PCA and the indicator species analysis as characteristic for the treatment and sample time points. A detailed description of the clone distribution inside each clone library is presented in table 3.2.

The T-RF 429 bp was characteristic for the "early samples", meaning samples retrieved from glyphosate-treated as well as from untreated plants. Therefore, as expected, T-RF 429 bp was the most abundant clone (22 %) in the K116S library composed of control samples from the first harvest. Clones represented by the T-RF 429 bp were associated to *Oxalobacteraceae* related species.

The results from the indicator species analysis revealed T-RFs 487 bp and 490 bp to be additional T-RFs characteristic for the early sampling time of both treatments. Clones related to these T-RFs were recovered from every clone library; however they could not be associated to a single phylogenetical lineage, since several clones yielded the same T-RF size.

Both peaks (487 and 490 bp) were associated with clones related to the genus *Duganella*, and T-RF 487 bp additionally coincided to *Ramlibacter*. Several uncultured *Betaproteobacteria* were associated to the T-RF 490 bp, as well as sequences of uncultured *Gammaproteobacteria*.

On the other hand, the library K316S was created in order to identify bacteria possibly associated with peak 139 bp, which was characteristic for the group "control samples" (K3 + 5). In this library, composed of samples retrieved from the third harvest of control plants, the T-RF 139 bp was the most abundant peak (11 %). Clones detected with this fragment size were associated to *Burkholderia* related species. *Burkholderia* clones were also found in the other libraries, but with lower abundances (2 %).

Characteristic for samples originated from the glyphosate-treated plants were sequences represented by the T-RF 72 bp. In order to identify these clones, the clone library G416S was established with DNA of root-associated bacteria retrieved from the fourth harvest of glyphosate-treated plants. Clones with a T-RF of 72 bp were associated with uncultured *Gemmatimonadetes* related species. As was expected, the abundance of *Gemmatimonatetes*

related clones (T-RF 72 bp) was higher in the G416S library (8 %), although clones associated to the T-RF 72 bp could also be retrieved from the libraries K116S (4 %) and K316S (7 %).

3.19 Selected genera or lineage-specific clone frequencies responsible for the phylum composition of the clone libraries

Each of the clone libraries K116S, K316S and G416S exposed different distribution of predominant phyla. Nevertheless, the predominance of specific phyla was occasionally caused by single genus or lineage specific clones as is reported in the Table 3.2. In this Table, measured T-RF values were compared with the predicted ones that were obtained from *in silico* cutings of every clone sequence. Differences between measured and predicted T-RFs were observed. Predicted values were mostly off by 2 to 3 bp higher than the measured ones. Nevertheless, there were cases, where the measured and predicted T-RFs were equal to each other, as in the cases of *Micromonospora* related clones (T-RF 274 bp) and *Sphingobacteriaceae* affiliated species (T-RF 540 bp). The maximum differences observed between peak sizes were for *Bacillus* related clones, identified under the measured T-RF 140 bp and the predicted T-RF 151 bp. Similarly, uncultured *Burkholderiales insertae sedis* associated clones were identified under the measured T-RF 421 and the predicted T-RF 431 bp. Characteristic T-RFs, associated to each clone are shown. Nevertheless in cases of T-RFs associated with uncultured organisms, there was no characteristic T-RF but several individual values (more than five).These cases were indicated as NA (not applicable).

3.19.1 *Alphaproteobacteria* related clones

The high abundance of *Alphaproteobacteria* for the different clone libraries was connected to characteristic T-RFs like the T-RF 149 bp associated to *Bradyrhizobium* and the T-RF 398 bp related to *Rhizobium*. For the G416S library *Alphaproteobacteria* were the most abundant group (29 %) represented mainly by 10 % of *Rhodoplanes* related clones (143 bp and 148 bp). The T-RFs 402 and 403 bp were characteristic for two genera belonging to *Caulobacteraceae*, *Brevudimonas* and *Erythrobacter*, respectively. These clones had a very low abundance and were just present in library K116S. Other *Caulobacteraceae* clones characterized by the T-RF 146 bp belonged to *Asticcaulis* related species.

3.19.2 *Betaproteobacteria* related clones

Betaproteobacteria were the most abundant phylogenetic group in the clone library K116S (46 %) and in the K316S (28 %). The high abundance of this lineage in the K116S library was correlated with elevated proportion (22 %) of *Oxalobacteriaceae* related clones (T-RF 149 bp), characteristic for the early sampling times. Additionally, *Burkholderia* related clones (T-RF 139 bp) were responsible for the higher abundance (11 %) of *Betaproteobacteria* in the K316S library.

Characteristic peaks present only in the *Betaproteobacteria* group were T-RF 491 bp associated to *Paucimonas, Thiobacillus* and *Methylobacillus* related clones. The family *Comamonadaceae* was represented mainly by the T-RFs 487 bp and 488 bp, which were associated to *Variovorax* and *Ramlibacter* clones respectively.

3.19.3 *Gammaproteobacteria* related clones

The *Gammaproteobacteria* group was characterized in all clone libraries mainly by members of the family *Xanthomonadaceae*. The relative highest clone abundances belonged to *Frateuria* related T-RFs (119 bp and 451 bp) that were found in the clone libraries K316S (3 %) and G416S (4 %). *Luteimonas* clones (T-RF 490 bp) and one clone of *Lysobacter* related species (T-RF 231 bp) were detected in the two clone libraries retrieved from control samples.

3.19.4 *Actinobacteria* related clones

Actinobacteria was the third most dominant phylum after *Alpha-* and *Betaproteobacteria* for the G416S library, comprising clones recovered from glyphosate-treated plants. The actinobacterial community for this library was characterized by *Streptomyces* (T-RF 157 bp), *Mycobacterium* (T-RF 159 bp) and *Nocardioides* (T-RF 156 bp) each represented by one related clone in the library. These clones were also present in library K316S. Apart from these known genera, uncultured *Actinobacteria* comprised the majority of clones related to this bacterial group and were characterized in different libraries by the T-RFs 61 bp, 127 bp, 150 bp, 297 bp and 497 bp.

3.19.5 *Bacterioidetes* related clones

The *Bacterioidetes* lineage was represented mostly by clones within the range from 87 bp to 91 bp. Uncultured *Bacterioidetes* clones were typically related to T-RF 87. Additionally, uncultured *Sphingobacteriales* were found with the T-RFs 89 bp and 91 bp. Moreover, there was one clone identified as *Cytophaga* species which was related to the T-RF 90 bp in library K116S. Furthermore, there were two clones related to *Sphingobacteriaceae* that were characterized by the T-RF 540 bp.

3.19.6 *Chloroflexi* and *Acidobacteria* related clones

Clones related to uncultured *Chloroflexi* and uncultured *Acidobacteria* were ubiquitous in every clone library, nevertheless they were associated to numerous different T-RFs. *Chloroflexi* clones were found with the T-RFs 160 bp, 451 bp, 510 bp, 515 bp and 530 bp. Similarly, several T-RFs (93 bp, 147 bp, 196 bp, 262 bp, 283 bp and 288 bp) were related to *Acidobacteria*.

3.19.7 *Firmicutes* and *Plantomycetes* related clones

The phyla *Firmicutes* and *Planctomycetes* were recovered at very low abundances (2 %) from glyphosate-treated and untreated samples. The lineage *Firmicutes* was represented by uncultured members of the family *Veillonellaceae* (383 bp), *Paenibacillus* (148 bp) and *Bacillus* related clones (140 bp and 165 bp). On the other hand, *Planctomyces* related clones were found with the T-RF 121 bp and uncultured *Planctomycetes* coincided with the T-RFs 127 bp and 202 bp.

3.19.8 Remaining uncultured bacteria and plastids

In every clone library there were bacterial clones that could not be associated to any specific phylum and that are related to many different T-RFs. The proportion of uncultured bacteria in the clone library retrieved after glyphosate treatement (G416S) was 8 % and in the libraries

originated from control samples there were 4 % (K116S) and 7 % (K316S) of unidentified bacteria.

Additionally, to the bacterial 16S rRNA gene sequences plastid clones were also detected at low abundances (2 %) in every clone library. These sequences were identified as 16S rRNA genes of mitochondria (T-RF 338 bp) and chloroplasts (T-RF 403 bp) from *Glycine max*.

Table 3.2. Clone libraries composition. Phylogenetic affiliation according to ARB with characteristic meassured and predicted T-RF sizes of the 16S rRNA gene clones retrieved from the libraries: K116S, K316S and G416S. The abundance of clones is represented as percent of total sequences from each library.

Phylogenetic lineage		% of clones in indicated library			Characteristic T-RF size (bp)[a]	
		K116S	K316S	G416S	meassured	predicted
Alphaproteobacteria						
Bradyrhizobiaceae	*Bradyrhizobium*		2	6	149	152
Hyphomicrobiaceae	*Rhodoplanes*	2		10	143, 148	149, 153
Hyphomicrobiaceae	*Devosia*		1		435	438
Rhizobiaceace	*Rhizobium*	4	3	2	398	402
Rhizobiales	uncultured	7	1		158, 434	161, 438
Caulobacteraceae	*Brevundimonas*	2			402	404
Caulobacteraceae	*Asticcaulis*	4	3	4	146, 435	148, 437
Erythrobacteraceae	*Erythrobacter*	2			403	405
Alphaproteobacteria	uncultured		2	6	NA	NA
Betaproteobacteria						
Oxalobacteraceae	*Duganella*	7	2	2	487, 490	488, 491
Oxalobacteraceae	uncultured	22		2	429	431
Burkholderiaceae	*Paucimonas*	2			456, 491	459, 493
Burkholderiaceae	*Burkholderia*	2	11	2	139	142
Comamonadaceae	*Variovorax*		1		488	489
Comamonadaceae	*Ramlibacter*	4	1	2	487	489
Burkholderiales incertae sedis	Matsuebacter	2	1		481	483
Burkholderiales incertae sedis	Rubrivivax	2	3		132, 137	135, 140
Burkholderiales incertae sedis	uncultured	2	1		421, 433	431, 442
Hydrogenophilaceae	*Thiobacillus*		1		491	493
Methylophilaceae	*Methylobacillus*		1		491	493
Spirillaceae	*Spirillum*			2	492	494
Betaproteobacteria	uncultured	2	4	4	NA	NA
Gammaproteobacteria						
Legionellaceae	*Legionella*			2	166	169
Legionellaceae	*Ricketsiella*		1		493	494
Xanthomonadaceae	*Lysobacter*	2			231	232
Xanthomonadaceae	*Luteimonas*	2	2		490	491
Xanthomonadaceae	*Frateuria*		3	4	119, 451	123, 453
Chromatiales	uncultured		1		455	457
Deltaproteobacteria						
Myxococcales	uncultured		1	2	181	184
Deltaproteobacteria	uncultured		1		125	129
Actinobacteria						
Actinomycetales	uncultured		1		150	152
Streptomycetaceae	*Streptomyces*		1	2	157	160
Mycobacteriaceae	*Mycobacterium*			2	159	162
Micromonosporaceae	*Micromonospora*		1		274	274
Nocardioidaceae	*Nocardioides*		1	2	156	159
Actinobacteria	uncultured	7	3	6	NA	NA

Continued from previous page

Phylogenetic lineage		% of clones in indicated library			Characteristic T-RF size (bp)[a]	
		K116S	K316S	G416S	meassured	predicted
Bacterioidetes						
Sphingobacteriaceae	uncultured		2		540	540
Sphingobacteriales incertae sedis	uncultured		2	2	89, 91	91, 94
Flexibacteraceae	*Cytophaga*	2			90	92
Bacterioidetes	uncultured		2	2	87	91
Chloroflexi						
Chloroflexi	uncultured		3	4	NA	NA
Acidobacteria						
Acidobacteria	uncultured		8	4	NA	NA
Firmicutes						
Bacillaceae	*Bacillus*		2	2	140, 165	151, 168
Paenibacillaceae	*Paenibacillus*		1		148	150
Veillonellaceae	uncultured		1		383	385
Gemmatimonadetes						
Gemmatimonadetes	uncultured	4	7	8	72	74
Planctomycetes						
Planctomycetaceae	Planctomyces				121	125
Planctomycetes	uncultured	4		2	127, 202	131, 206
Bacteria	uncultured	9	9	6	NA	NA
Plant						
mitochondrion	*Glycine max*		2	2	338	340
chloroplast	*Glycine max*	2	1	2	403	406

[a] Characteristic T-RF lengths (bp) measured from all the clones belonging to the three clone libraries are assigned to a given affiliation. The phylogenetic lineage indicates the closest affiliation for each clone found with ARB. The relative abundance of every clone is indicated as percent for each clone library. The clone library K116S contained clones retrieved from the first harvest of control samples. The K316S library enclosed clones from the third harvest of control samples. The clone library G416S included clones recovered from the fourth harvest of glyphosate-treated plants. NA, not applicable, it was assigned to uncultured clones with more than five possible T-RFs.

3.20 Bacterial community structures as revealed by T-RF fingerprints

A representative T-RFLP fingerprint electropherogram retrieved from the second harvest of glyphosate-treated plants is illustrated in Figure 3.20. The major T-RFs, shown in this fingerprint, could be identified by the clone libraries and were often present in fingerprints retrieved from each harvest.

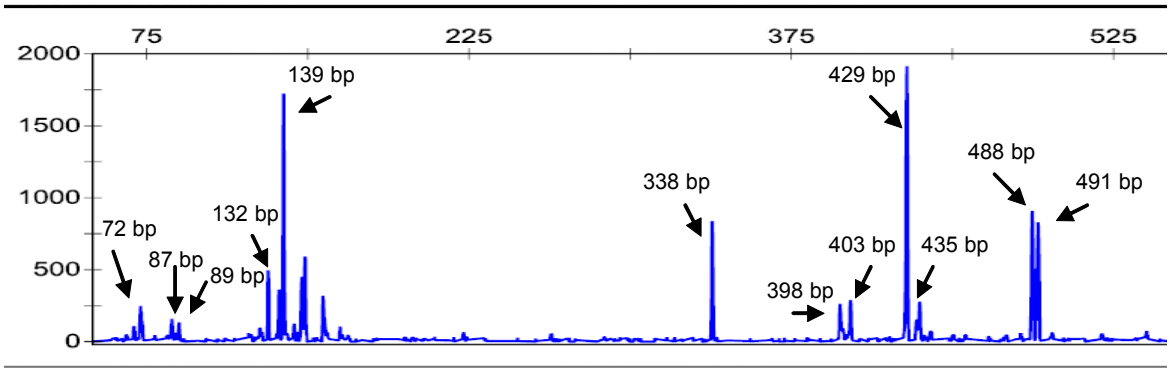

Fig. 3.20. A representative bacterial 16S rRNA T-RFLP fingerprint derived from the second harvest of glyphosate-treated samples. The T-RFs are identified with arrows and the correspondent length is given in bp.

The most abundant T-RF in this fingerprint was the 139 bp, which was related to *Burkholderia* clones. The T-RF 429 bp, dominant in the community as well, corresponds to uncultured *Oxalobacteriaceae* related species. Additionally, uncultured *Gemmatimonadetes* species could be assigned to T-RF 72 bp. The next most abundant peak in this fingerprint was distinguished under the T-RF 89 bp as *Sphingobacteriales incertae sedis*. Furthermore, for the T-RF with a length of 148 bp there were clones associated to different lineages like *Paenibacillus*, *Rhodoplanes* and uncultured bacteria. This phenomenon of multiple organisms being assigned to one specific T-RF was observed several times. Following decreasing abundance, the next T-RF was 132 bp that was related to clones belonging to the genus *Rubrivivax* as well as to uncultured bacteria. The T-RFs 487 bp and 488 bp were both associated to two different members of the family *Comamonadaceae*: *Ramlibacter* and *Variovorax*. Peak 491 bp was associated with different genera of *Betaproteobacteria* in the clone libraries, e.g. *Thiobacillus Methylobacillus* and *Paucimonas* related clones. Clones related to the genus *Streptomyces* were affiliated to T-RF 157 bp. Characteristic for *Rhizobium* species was the T-RF 398 bp. Sequences belonging to *Glycine max* mitochondria were detected in every T-RF fingerprint at low abundances and were associated to the T-RF 338 bp. Chloroplasts of *Glycine max* were identified as T-RF 403 bp which also corresponded to *Erythrobacter* related clones.

3.21 Phylogenetic affiliation of significant clones for this study

Representative sequences from clones associated to the statistically significant T-RFs (72 pb, 139 bp and 429 bp) responsible for the rhizosphere community shifts were compared in a quartet puzzling phylogenetic tree that was created with the ARB software package and the 16S rDNA database. This method applies maximum-likelihood tree reconstruction to all possible quartets that can be formed from selected sequences. In addition to the tree topology the quartet puzzling tree also shows reliability values for each internal branch. The reliability values (number of times the group is reconstructed during the puzzling steps) allow interpretation of the phylogenetic information present in the data.

The reliability percentages of the occurrence of the nodes were estimated after 10.000 puzzling steps. Tree topology was confirmed using maximum-likelihood, maximum-parsimony, and neighbour-joining algorithms as implemented in the ARB software package (Figure. 3.21).

16S rDNA sequences of the archea *Methanobacterium bryantii* and *Methanosarcina barkeri* were used as outgroup references. In the three clone libraries, a total of twelve clone sequences related to *Gemmatimonadetes* (T-RF 72 bp) were found. Besides, eleven sequences affiliated to *Burkholderia* (139 bp) and additional eleven sequences belonging to *Oxalobacteraceae* (149 bp) were retrieved from all libraries. Sequences with the same T-RF were aligned and compare in order to select only sequences showing nucleotide variation. From identical sequences, only one representative sequence was chosen to be included in the phylogenetical tree.

The clone sequences K3-16S-13, K3-16S-61, K3-16S-11 and K3-16S-60 were all selected clones retrieved from the clone library K3-16S, affiliated to *Burkholderia* and characterized by the T-RF 139 bp. Ten *Oxalobacteraceae* related species, characterized by the T-RF 429 bp, were retrieved from the clone library K116S that contained control samples of the first harvest time. These clones were all very closely related to each other, but there was not a clear affiliation to a specific genus. The *Gemmatimonadetes* related clones G416s66b, K316s52, K316s56, G416s62b are all related to *Gemmimonas aurabtiaca*, but as shown in the phylogenetical tree, they are likely to correspond to unknown species.

The frequency of occurrence of significant peaks 72 bp, 139 bp and 429 bp within the established clone libraries proved a high correspondence between the T-RFLP data obtained from fingerprints and the amount of clones retrieved from these libraries. Therefore, the choosen approach to select the best sampling times, using abundance curves for each peak in order to characterize the responsible T-RFs for the rhizosphere community shifts in RR soybean after glyphosate treatment, proved successful.

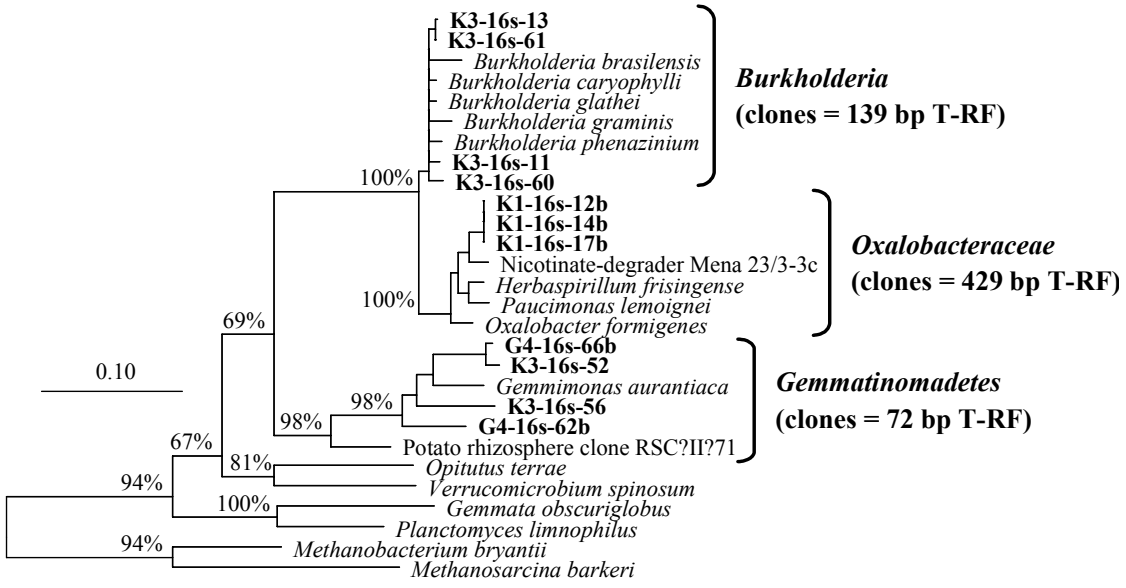

Fig 3.21 Quartet puzzling phylogenetic of the nearly full-length bacterial 16S rRNA sequences retrieved from the clones associated to the three significant T-RFs responsible for the community shifts in this study. Phylogenetic relationship between *Burkholderia* (139 bp), *Oxalobacteriaceae* (429 bp) and *Gemmatimonadetes* (72 bp) related species is shown. The reliability value of each internal branch indicates in percent how often the corresponding cluster was found among the 1.000 intermediate trees. GenBank accession numbers of sequences are indicated in bold letters. 16S rDNA sequences of the archea *Methanobacterium bryantii* and *Methanosarcina barkeri* were used as outgroup references.

4 Discussion

4.1 Assessment of a horizontal gene transfer of the *CP4-EPSPS* gene harboured in RR soybean to *B. japonicum* under laboratory conditions

Bacteria that have developed specific symbiotic or pathogenic relationships with plants expose the best conditions for gene transfer in nature (Kay et al., 2002). *B. japonicum*, as symbiont of soybean and carrier of an EPSPS enzyme sensitive to glyphosate, is therefore an ideal candidate to integrate the *EPSPS* gene from RR soybean DNA under glyphosate selection pressure (Zablotowicz and Reddy, 2004).

The mechanism by which such HGT could occur is likely to be related to natural transformation of bacteria by DNA released from plants (Lorenz and Wackernagel, 1994b). The main two barriers of HGT from plant DNA to microorganisms is recognized to be non-competent status of recipient cells and lack of sequence homology (Nielsen et al., 1998). Correspondently, in this study, attempts to facilitate HGT to *B. japonicum* under controlled laboratory conditions included optimisation of procedures to increase sequence homology between recombinant sequences and evaluation of optimal conditions for natural transformation.

Among the enormous bacterial diversity, more than 40 bacterial species from different environments are known so far to be naturally transformable (de Vries et al., 2001; Lorenz and Wackernagel, 1994b; Nielsen et al., 1998). However, no *Bradyrhizobium* is included within reported naturally transformable species. The closest relative to the genus *Bradyrhizobium* among natural transformable bacteria is *Rhizobium meliloti* (Courtois et al., 1988), although this transformation was achieved only due to a plasmid insertion without subsequent chromosomal recombination (Courtois et al., 1988).

In this study, natural transformation trials were adapted to the cultivation media of *B. japonicum* and its growth rates. Transformation experiments principally consisted of *B. japonicum* exposition to plasmid DNA and initial incubation in non-selective medium. Afterwards, cells were cultivated on selective agar plates (Demaneche et al., 2001; Lorenz and

Wackernagel, 1994b). As nearly every transformable organism has its own specific set of conditions that induces competence (Lorenz and Wackernagel, 1994b), several conditions were required to be tested in order to find the optimal circumstances in which *B. japonicum* undergoes natural transformation. Therefore, *B. japonicum* cultures were exposed to various concentrations of recombinant plasmid DNA in different phases of the exponential growth stage with variable incubation times. Predominantly these three variables, concentration of available DNA for the transformation event, differences in the growth stage and incubation time, have been shown to be differentially required by each transformable bacterium (Demaneche et al., 2001; Lorenz and Wackernagel, 1994b).

The plasmid used in this thesis for *B. japonicum* transformations carried a non-essential DNA region of the *B. japonicum* genome that constitutes the recombination site of the plasmid with the chromosome (Acuña et al., 1987). In the middle of the recombinant region the *CP4-EPSPS* gene of RR soybean was cloned. These additional recombination sequences were used since homologous DNA sequences flanking heterologous DNA have been shown to increase the recombination efficiency (de Vries et al., 2001; de Vries and Wackernagel, 2002). Most well known successful HGT experiments from other authors were performed following this approach with additional homologous sequences between donor and receptor organisms. Those experiments were performed with engineered plasmids in the high natural transformable bacterium *Acinetobacter* sp. BD413, containing a partially deleted antibiotic resistance gene (Chamier et al., 1993; de Vries and Wackernagel, 2002; Gebhard and Smalla, 1998 ; Nielsen et al., 1997). In those studies, after transformation and homologous recombination with the donor plasmid DNA, the *Acinetobacter* antibiotic resistance gene was completed and antibiotic resistance was achieved. This system has been already extended to other soil bacterial species like *Pseudomonas stutzeri* (de Vries et al., 2001) and *Erwinia chrysanthemi* (Schluter et al., 1995). Additionally, *Acinetobacter calcoaceticus* has been transformed with DNA from transgenic plants using the same antibiotic recombination system under controlled laboratory conditions, although transformation frequencies with plant DNA have been shown to be drastically reduced compared to transformation with recombinant plasmid DNA (de Vries and Wackernagel, 2002; Gebhard and Smalla, 1998).

HGT experiments monitoring the transfer of a gene localized on the bacterial chromosome, as is the case for the *EPSPS* gene, require the chromosomal recombination of the inserted gene in

order to achieve high genetic stability without the need for selective pressure (de Vries et al., 2003). The stable maintenance of DNA in bacteria ultimately requires that the DNA in the cytoplasm is linked to an origin of replication, such as via integration into the chromosome or into a plasmid (Nielsen et al., 1997). Since plasmids are often unstable in *B. japonicum* cells (King et al., 2000), it is necessary that the origin of vegetative replication (*OriV*) of the carrier plasmid does not permit autoreplication in *B. japonicum*. This is to guarantee a chromosomal integration, which can be indicated by the respective antibiotic selection (Acuña et al., 1987). That is why *B. japonicum* transformations in our study were performed with an integration vector designed for chromosomal homologous recombination of foreign DNA into the *B. japonicum* genome (Acuña et al., 1987). Nevertheless, under all different conditions tested, no natural transformation event was obtained for *Bradyrhizobium* in this study. Different concentrations of DNA mixed with *Bradyrhizobium* cultures at different growth stages did not yield any transformed *Bradyrhizobium* with recombinant vector DNA. The non natural transformability of *Bradyrhizobium* was expected, since among the vast diversity of bacteria, very few are able to undergo natural transformation (Lorenz and Wackernagel, 1994b).

Natural transformation results of other studies have shown that when a bacterium is able to be transformed with its own homologous DNA, it is expected that the transformability of the same bacterium with foreign DNA will be much reduced (Bertolla et al., 1999; de Vries and Wackernagel, 2002; Gebhard and Smalla, 1998). In the case of *Bradyrhizobium*, there was no development of a natural competent stage under the conditions tested in this study. This indicates that a HGT of a foreign gene like the *CP4-EPSPS* to *Bradyrhizobium* is even less probable than the recombination with the own integration plasmid, as was attempted in this study. There are two natural transformable bacteria able to integrate homologous DNA, yet when they are transformed with plant DNA the transformation efficiency dropped below the detection limit. One of those bacteria is *A. calcoaceticus*, which is a model organism for gene transfer studies (Metzgar et al., 2004), with the highest natural transformation frequency found *in vitro* with homologous chromosomal DNA (10^{-2}) (Nielsen et al., 1997; Palmen and Hellingwerf, 1997). The second most transformable bacterium is *Ralstonia solanacearum*, a bacterium able to develop natural competence *in vitro* (Bertolla et al., 1997), which was transformed with recombinant plasmid DNA but at a much lower transformation rate (10^{-6}) (Bertolla et al., 1999) compared to *A. calcoaceticus*. In both cases, when *Acinetobacter* or

Ralstonia were incubated with non homologous DNA, the transformation rate was much lower than with recombinant plasmid DNA or it was even undetectable (Bertolla et al., 2000; de Vries and Wackernagel, 2002; Gebhard and Smalla, 1998). Foreign DNA integration into the genome of *Acinetobacter* sp. during transformation was at least 10^9-fold lower than that of homologous DNA (Gebhard and Smalla, 1998). In the same way, the natural transformation frequency of *R. solanacearum* (10^{-6}) was reduced to a level, which made the detection of a gene transfer from the transgenic plant to the *R. solanacearum* not possible anymore, when incubated with transgenic plant DNA (Bertolla et al., 2000). Thus, natural transformation with plant DNA at a frequency of 10^{-11} was only found in *A. calcoaceticus* (Gebhard and Smalla, 1998), the bacterium with the highest natural transformation frequency (Nielsen et al., 1997; Palmen and Hellingwerf, 1997). In the case of *B. japonicum*, it can be concluded that if this bacterium was not able to undergo natural transformation with homologous DNA, then the integration of foreign DNA in nature will be even much less probable. Nevertheless it remains possible that *B. japonicum* could eventually be transformable under very specific conditions, which are still unknown.

4.2 The *CP4-EPSPS* gene insertion in *B. japonicum* by biparental mating

Although the natural transformability of *B. japonicum* was not achieved under optimized laboratory conditions, the question about a possible gene transfer under specific conditions in nature remains open. If natural competence of *B. japonicum* could be achieved in nature, a homologous recombination between the *CP4-EPSPS* transgene and the *B. japonicum EPSPS* gene could be facilitated, given that sequence homologies among the bacterial and plant EPSPS synthases are substantial (Gasser et al., 1988; Stallings et al., 1991). Furthermore, there is high similarity between the *CP4-EPSPS* gene inserted into the RR soybean and the *EPSPS* gene of *B. japonicum* (Wagner et al., 2008). Therefore, the introduction of the *CP4-EPSPS* gene into the genome of *B. japonicum*, achieved in this study by mating under optimized laboratory conditions, aims to prove if the *CP4-EPSPS* gene would provide the recipient *B. japonicum* a glyphosate resistance phenotype.

Before biparental mating experiments were achieved, attempts to transform *B. japonicum* by electroporation with recombinant vector DNA were performed following the published

electroporation protocols specific for *B. japonicum* (Guerinot et al., 1990; Hattermann and Stacey, 1990). Yet, no positive *B. japonicum* transformants were achieved by electroporation protocols. Electroporation of *B. japonicum* seems to be very inefficient, since even the authors of the original publications did not use this method for transformation of this bacterium in further work; yet they continued to use the conjugation method for *B. japonicum* transformation (Benson et al., 2005; Franck et al., 2008; Loh and Stacey, 2001). Thus, conjugation still constitutes the standard established method for gene transfer in *B. japonicum* (Miller et al., 2007; Rudolph et al., 2006). This is not surprising since in fact most of DNA sequences in prokaryotic chromosomes which originated from other organisms were acquired by bacterial conjugation (Frost et al., 2005; Jain et al., 1999).

The integration on the *CP4-EPSPS* into the *B. japonicum* genome in this study was achieved by biparental mating followed by homologous recombination. Positive transformants, harbouring the transferred *CP4-EPSPS* were initially selected using kanamycin and tetracycline. Colonies resistant to kanamycin and sensitive to tetracycline contained the *CP4-EPSPS* inserted into the *B. japonicum* genome after homologous recombination into the target region. Sensitivity to tetracycline indicated that the rest of the vector, where the tetracycline marker was contained, was not integrated. The PCR product of the *CP4-EPSPS* in the *B. japonicum* genome confirmed the integration of the gene. Transfer frequencies of about 1.2 x 10^{-6} transconjugants per recipient cell were obtained by biparental mating experiments. Transformation of *B. japonicum* established by biparental mating in other studies reached transformation frequencies of 10^{-7} transconjugants per *B. japonicum* recipient cell (Hattermann and Stacey, 1990).

These conjugation experiments were followed by inhibition tests using different glyphosate concentrations in order to investigate the resistance degree achieved by the transformed *B. japonicum* harbouring the *CP4-EPSPS*. Given that the transfer of the *CP4-EPSPS* was successful, the factor that would maintain the gene within the genome would be a selective advantage due to the expression of the herbicide resistant gene in an environment under glyphosate pressure (Zablotowicz and Reddy, 2004). The studies that reported the transfer of the kanamycin resistance marker from plant to bacteria used a genetic construct which could be expressed in bacteria (Gebhard and Smalla, 1998). In that way the acquisition of the antibiotic resistance sequence was a beneficial adaptation under kanamycin selection pressure

and the antibiotic resistance of the bacteria allowed the monitoring of the transformation event (Gebhard and Smalla, 1998). In the case of *Bradyrhizobium*, the integration of the *CP4-EPSPS* did not provide the recipient *B. japonicum* a glyphosate resistance phenotype. Control and transformed *B. japonicum* were equally sensitive to glyphosate. As the *CP4-EPSPS* is a gene sequence originally contained in *A. tumefaciens* strain CP4 (Padgette et al., 1995), it is possible that this genetic construct could be expressed in other bacteria. In the case of *B. japonicum* the reason for the lack of resistance could be that the engineered transgene in RR soybean is under expression of the promoter CaMV 35S, which might not be active in *B. japonicum*.

The CaMV 35S, inserted in RR soybean, is a strong constitutive promoter and the most widely used for gene regulation in transgenic plants (Schnurr and Guerra, 2000). Promoters of eukaryotic and prokaryotic genes do not exhibit a similar structure with high specific consensus and recognition sequences (Kozak, 1999). Consequently, efficient expression of an eukaryotic gene, transferred to a bacterium genome, requires a prokaryotic promoter (Jacob et al., 2004). However, the CaMV 35S promoter enclose prokaryotic recognition sequences, that allows this promoter to direct gene expression in *Escherichia coli* (Assaad and Signer, 1990) and several other bacteria (Jacob et al., 2004; Lewin et al., 1998). Due to the broad range of activity of the CaMV 35S promoter, it was plausible that it could also induce expression in *B. japonicum*. Yet, under these circumstances, the HGT of the *CP4-EPSPS* from RR soybean to *B. japonicum* would not confer any glyphosate resistance and consequently would not be favoured by natural selection to be kept in the genome.

Gebhard and Smalla (1998), after showing a gene transfer using the rescue system of the kanamycin resistance gene (*nptII*) from a transgenic plant restoring the incomplete *nptII* gene in *A. calcoaceticus*, discussed that their findings should not affect the evaluation of the use of antibiotic resistance genes such as *nptII* as markers in transgenic plants. As promoter sequences used in the commercial transgenic plants, are not active in most bacteria, the transfer of the gene *nptII* from transgenic plants would not provide the recipient bacteria a kanamycin resistance phenotype. Additionally, most of the antibiotic resistance genes used as marker genes are widely disseminated in environmental bacteria (Gebhard and Smalla, 1998). According to this, as the *CP4-EPSPS* from RR soybean is not active in *B. japonicum,* a HGT from plant to this bacterium under glyphosate selection pressure would not maintain the gene

integrated in its genome. However, *EPSPS* and antibiotic resistance genes are widely distributed in different bacteria. Therefore, the acquisition of a glyphosate resistant *EPSPS* gene would be more probable due to bacterial conjugation than by natural transformation with plant DNA. Results of these study confirmed that the only form of successful gene transfer in *Bradyrhizobium* is the conjugative mating. Moreover, the only natural gene transfer process already monitored in soil until now for *Bradyrhizobium* is the plasmid transfer between *Bradyrhizobium* sp. strains (Kinkle et al., 1993).

In order to prove if a bacterial promoter linked to the *CP4-EPSPS* gene would lead to glyphosate resistance in *B. japonicum*, a new vector for homologous recombination containing the *CP4-EPSPS* gene under expression of the *nptII* promoter was performed. The *nptII* promoter was chosen as it was already demonstrated that it is active in *B. japonicum* (Acuña et al., 1987). The new construct *nptII-EPSPS* containing the *CP4-EPSPS* ligated to the *nptII* promoter (*nptII-EPSPS*) was created in *E. coli*. The resulting plasmid with the *nptII-EPSPS* always showed a smaller size than expected when linearized and observed by gel electrophoresis. PCR amplification results proved that the *nptII-EPSPS* construct was present in the integration vector. However, PCR amplification of the integration site showed that the *nptII-EPSPS* construct was not integrated between the two homologous regions for recombination into the *B. japonicum* chromosome (refer to Figure 3.10).

As the kanamycin promoter was also present in the recombinant plasmid for *B. japonicum* a possible homologous recombination between the promoter of the vector and the inserted construct *nptII- EPSPS* could be an explanation for this phenomenon. If a recombination between the homologous sequences of the promoters occurs, part of the plasmid can be restricted and released from the vector construct, explaining the size reduction of the vector. Such an event has been observed in plasmids with repeated sequences introduced into mammalian cells, where intramolecular recombination led to the deletion of the intervening sequences and the loss of one copy of the repeat (Chakrabarti and Seidman, 1986). Additionally, intramolecular recombination within a plasmid has been observed during transformation of *Bacillus subtilis* with a plasmid carrying repeated sequences (Michel et al., 1982). In the case of *B. subtilis*, different recombination events resulted in diverse plasmid reorganizations, including vectors containing the cloned gene in the correct insertion site (Michel et al., 1982). Contrary to the results of recombinant plasmids in *B. subtilis*, all clones

in this study contained the *nptII-EPSPS* construct outside the recombination cloning site. For that reason, homologous recombination experiments with this vector in *B. japonicum* were not continued.

An important observation in all transformation procedures of *B. japonicum* under glyphosate selection was the rather high frequency of spontaneous mutants resistant to glyphosate obtained in control experiments with non transformed bacteria (3.2×10^{-7}). This made the identification of true positive transformants in transformation attempts very difficult. In an ideal transformation experiment the spontaneous mutation rate should be very low in order to easily recognise positive transformants. For example, this was the case for the transformation of *Acinetobacter* sp. strain BD413 with plant DNA at a frequency of 1.5×10^{-10} where the controls had no spontaneous antibiotic resistant mutant (Gebhard and Smalla, 1998). Low spontaneous mutation rates of bacteria have even been defined as a prerequisite for detection of successful transformations. *Erwinia chrysanthemi* was chosen for transformation experiments because the frequency of spontaneous mutations in antibiotic selection medium was below the detection limit of 9×10^{-9} and the frequency of natural transformation was considerably higher at 6.7×10^{-8} (Schluter et al., 1995). The spontaneous mutation frequency observed in *B. japonicum* on glyphosate selection medium (3.2×10^{-7}) is of course much higher when compared to the lack of spontaneous antibiotic resistant mutations in *Acinetobacter* sp. (Gebhard and Smalla, 1998)**.**

Sequence analyses of EPSPS from several bacteria and plants have revealed stretches of conserved amino acid residues (Kishore and Shah, 1988). Random and site-directed mutagenesis investigations of EPSPS isolated from plants and bacteria have identified a region between 90 and 120 amino acid residues to be the site of PEP interaction, where a single amino acid change leads to glyphosate resistance or sensitivity (Comai et al., 1983; Padgette et al., 1991; Selvapandiyan et al., 1995; Stallings et al., 1991). These beneficial mutations for glyphosate resistance can already exist in the bacterial population before the selection pressure is applied, since every prokaryotic population represents a mixture of genetically diverging clonal cell lines on which natural selection acts (Torsvik et al., 2002). The facility of a point mutation conferring glyphosate resistance would explain the observed high spontaneous mutation rate of *B. japonicum* in glyphosate medium. Point mutations contribute to adaptation, which leads to a stepwise improvement of available biological functions on which natural

selection, in this case glyphosate selection, applies evolutionary pressure (Schloter et al., 2000). Nevertheless, in the case of antibiotic resistance, it is common that the phenotype does not always reflect the same genotypes in all selected mutants, because mutations in different genes can produce similar antibiotic resistance phenotypes (Martinez and Baquero, 2000). That would mean that not only mutations in the glyphosate target site in *B. japonicum* could lead to glyphosate resistance. Resistance can be also acquired by active efflux of the toxic substance (Levy, 1992), permeability changes in the outer membrane (Nikaido, 1989) or due to the acquired ability for detoxification or degradation of the inhibitor compound (Martinez and Baquero, 2000). According to these results, in the specific case of *B. japonicum*, the most feasible way to acquired spontaneous glyphosate resistance, at least under the laboratory conditions tested, is due to spontaneous mutations. This observations supports that even in the cases of rigorously controlled laboratory environments, bacteria adapt via point mutations to modify their own abilities within this seemingly constant ecological niche. This supports sustaining the hypothesis that a bacterium will fit into whatever environmental setting is best exploited by its current genetic makeup (Treves et al., 1998).

4.3 Evaluation of the rizospheric community composition of RR soybean roots influenced by glyphosate applications

After glyphosate applications, changes in the bacterial community associated to RR soybean roots were expected, since glyphosate can inhibit growth of bacteria (Coggins et al., 2003) and the presence of this herbicide has been evidenced in root exudates from RR soybean (Grundmann et al., 2008). Assuming the presence of glyphosate-sensitive EPSPS in most bacteria (Coggins et al., 2003; Schulz et al., 1985), herbicide application would restrain the growth of such organisms and would promote the increment of bacteria able to metabolize glyphosate (Forlani et al., 1999).

This study demonstrates bacterial community shifts in the rhizosphere of RR soybean after glyphosate application in an experiment carried out in the greenhouse. Evaluation of T-RFLP analysis based on the amplification of the 16S rRNA gene from total DNA extracted from rhizosphere soil allowed the comparison of the community structures of these treatments. Comparison of T-RFs from the profiles with T-RF sizes calculated from clone library sequences generated from the same samples were performed in order to ensure the correct

phylogenetic identification of each clone. This was necessary since discrepancies between observed and *in silico* predicted fragment sizes are well known (Hiraishi et al., 2000; Lueders and Friedrich, 2000). The deviation between observed and predicted T-RF sizes was in agreement with previous experimental determinations for 16S rRNA genes (Junier et al., 2008; Kaplan and Kitts, 2003; Thies, 2007).

Reported dominant phyla in 16S rDNA soil clone libraries are *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes*, and *Firmicutes* (Janssen, 2006). With the exception of *Verrucomicrobia*, organisms associated to most of these groups were found in the clone libraries obtained from RR soybean rhizosphere.

The aim of this part of the study was to understand the impact and nature of changes on individual bacteria after glyphosate treatment. For that purpose, groups of samples associated to a particular treatment were determined by PCA and T-RFs characteristic for these treatments were identified by indicator species analysis. The T-RFLP method has been shown to be relevant to monitor the diversity, structure, and dynamics of microbial populations in a statistically representative manner (Blackwood et al., 2003). Results of this study confirm that T-RFLP in combination with ordination techniques, indicator species analysis and 16S rRNA gene sequencing is an effective strategy for comparing changes in microbial communities (Battles, 2004; Pett-Ridge and Firestone, 2005; Talbot et al., 2008).

Special attention was given to organisms already reported to be able to metabolize glyphosate, since significant changes in the abundances of these groups were expected. Glyphosate degradation ability is widespread in the family *Rhizobiaceae* (Liu et al., 1991) and furthermore the glyphosate-tolerant soybean was obtained from a modified bacterial *CP4-EPSPS* from the *Agrobacterium* sp. strain CP4 belonging to the *Rhizobiaceae* (Padgette et al., 1995). Several members of this family including *Agrobacterium radiobacter* (McAuliffe et al., 1990), *Rhizobium meliloti*, *Rhizobium leguminosarum*, *Rhizobium galega*, *Rhizobium trifolii*, *Agrobacterium rhizogenes*, and *Agrobacterium tumefaciens* are able to grow on glyphosate as their sole source of phosphorus in the presence of aromatic amino acids (Liu et al., 1991). Other bacteria, reported to be able to degrade glyphosate include *Alcaligenes* sp. (Talbot et al., 1984), *Bacillus subtilis* (Henner et al., 1986), *Pseudomonas* sp. (Jacob et al., 1988), *Arthrobacter atrocyaneus* (Pipke and Amrhein, 1988), *Achromobacter* sp. (McAuliffe et al.,

1990), *Pseudomonas pseudomallei* (Penaloza-Vazquez et al., 1995), *Streptococcus pneumoniae* (Du et al., 2000), *Geobacillus caldoxylosilyticus* (Obojska et al., 2002), *Staphylococcus aureus* (Priestman et al., 2005) and *Spirulina* sp. (Lipok et al., 2007). These bacteria possess a carbon–phosphate lyase that hydrolyzes glyphosate to form sarcosine and inorganic phosphate, allowing them to utilize glyphosate as their sole source of phosphorus (Zablotowicz and Reddy, 2004).

In the three evaluated clone libraries based on 16S rDNA from rhizospheres of treated and untreated RR soybean, clones associated to *Bacillus* and *Rhizobium* were recovered from each library. However, T-RFLP profile analysis did not suggest any significant shift in *Bacillus* or *Rhizobium* populations as response to glyphosate treatment. As the degradation of glyphosate is only reported for *Bacillus subtilis*, it is possible that the *Bacillus* retrieved in this study is not to the same degree able to metabolize glyphosate, as quantitative variation in metabolism within and between closely related *Bacillus* taxa has already been demonstrated (Feldgarden et al., 2003). An enhancement in the *Rhizobium* population abundance in relation to glyphosate application was expected, since the resistance to glyphosate seems to be common for several *Rhizobium* species (Liu et al., 1991), but it has also been suggested that competition with other bacteria limit the growth of small populations of *Rhizobium* in soil (Pena-Cabriales and Alexander, 1983). Additionally, population sizes of *Rhizobium leguminosarum* fluctuated significantly in response to different environmental factors (Lawson et al., 1987). Although glyphosate utilization by single *Rhizobium* species has been demonstrated as an advantage in pure culture studies, competition for carbon sources in the rhizosphere between *Rhizobium* species with overlapping niches can neutralize the population sizes of each species and therefore limit the positive effect of glyphosate resistance. A similar competitive growth restriction was observed for *Pseudomonas* species that catabolize the same carbon source (Wilson and Lindow, 1994).

Comparative sequence analysis and phylogenetic identification of individual clones confirmed that T-RFLP profiles and clone library results were congruent as expected (Macbeth et al., 2004; Stralis-Pavese et al., 2006). This congruency was validated by a comparison of T-RFLP fingerprint data and corresponding clone libraries. From this approach it was expected that the frequency of clones and correspondingly T-RFs should match in a given sample. Three T-RFs (72 bp, 139 bp and 429 bp) were identified by PCA and indicator species analysis as

significantly associated to a particular treatment. Therefore, three samples were chosen for the establishment of clone libraries which were assumed to contain the highest abundance of these phylotypes. The T-RF 72 bp was significantly associated to the glyphosate samples, which means that this T-RF was less abundant in control samples. On the other hand, the T-RF 139 bp was significantly linked to the control samples and the abundance of the T-RF 139 bp was reduced after glyphosate treatment. The abundance of the T-RF 429 was significantly higher at early sampling times and in the later harvests this peak almost disappeared from both, treated and untreated samples.

The clone library K116S, established from DNA of washed roots of the first harvest of control plants, was analysed in detail in order to phylogenetically identify the T-RF 429 bp corresponding to the phylotype that was reduced significantly over time. As expected from the T-RFLP fingerprinting analysis, a very high proportion of clones associated to T-RF 429 bp was retrieved from library K116S. In this library, the *Oxalobacteraceae* group, characterized by the T-RF 429 bp, dominated strongly.

Statistical analysis of the T-RFLP data showed that the abundance of the T-RF 139 bp was notably elevated in the third harvest of control samples. Clones related to the T-RF 139 bp could be identified by the clone library K316S, corresponding to the third harvest of control samples. In the K316S library, *Burkholderia*-related clones sharing the T-RF 139 bp, were the most abundant.

In contrast to the first two libraries, the most abundant phylum in library G416S was *Alphaproteobacteria*. This library was retrieved from the fourth harvest of glyphosate-treated samples, in order to identify T-RF 72 bp. This peak could be assigned to *Gemmatimonadetes* related clones. The abundance of the T-RF 72 bp increased significantly in response to glyphosate treatment, yet *Gemmatimodates* clones, according to the T-RFLP analysis, were not dominant in the bacterial community at any sampling time. Results from the clone libraries were satisfactory as the libraries enabled the identification of the statistically significant T-RFs for the bacterial community shifts. The population shifts of the three statistically significant groups identified in this study, *Burkholderia*, *Oxalobacteriaceae* and *Gemmatimonadetes* related clones are discussed in detail in the next sections.

4.3.1 *Oxalobacteriaceae* (T-RF 429 bp)

Oxalobacteraceae have been frequently identified in root environments (Kamilova et al., 2007; Offre et al., 2007; Rothballer et al., 2006). Clones associated to the T-RF 429 bp were affiliated to *Oxalobacteraceae* members without a clear affiliation to a specific genus. Eleven almost identical sequences of uncultured *Oxalobacteraceae* were retrieved from the clone libraries. Three type sequences were chosen to be included in the phylogenetical tree and they formed a clade with maximum identity. The affiliation to *Oxalobacteraceae* in the phylogenetic tree was supported by a clade shared with *Herbaspirillum*, *Paucimonas*, *Oxalobacter* species (refer to Figure 3.21). Nevertheless, based on 16S rDNA differences, the clones could not be included in any of these genera. This suggests that all organisms found under T-RF 429 bp affiliated to uncultured *Oxalobacteraceae* may belong to the same species, possibly yet unidentified.

Furthermore, all the clones identified by T-RF 429 bp showed the same behaviour in the bacterial community over the time. Uncultured *Oxalobacteraceae* species were significantly related to the early time samplings (p=0,0002). This group was the most abundant at the early plant development stage and almost disappeared over the time. Similar results were obtained by Green and co-workers analysing potting mix samples. At later sampling points the *Oxalobacteraceae* were either absent or faintly detectable (Green et al., 2006).

The reduction of uncultured aerobic *Oxalobacteraeaceae* species over the time, found in this study, which corroborates the findings of Green and co-workers (2006), may point to a group of organisms that could be a biological indicator of a limiting factor whose availability decrease in the rhizosphere over the time (Green et al., 2006). Since oxalotrophic bacteria use oxalate as carbon and energy source (Dimroth and Schink, 1998; Sahin, 2003) it can be expected that this limiting factor might be oxalate.

Oxalic acid and its salts are widespread in nature as they are produced by many plants (Franceschi and Nakata, 2005). Possibly, changes in the oxalate exudates in the plant could induce variations in the *Oxalobacteraceae* populations. Differences in root exudation have been shown to influence the rhizosphere community composition over the time (Baudoin et al., 2002; Bremer et al., 2007). The dominance of uncultured *Oxalobacteraceae* in bacterial communities was found in other studies under variable environmental conditions as well. *Oxalobacteraceae* were also reported to be predominant in aerial parts of hybrid poplars

(Ulrich et al., 2008). It seems probable that aerial parts of plants concentrate high amounts of oxalate that would be available for oxalotrophic bacteria. Results about the accumulation of oxalate in tea plants suggest that oxalate is transported to flushes and leaves and then accumulates in mature leaves as crystal oxalate (Morita and Tuji, 2002). Massey and co-workers (2001) studied the effect of maturity on the oxalate content of spinach and found that the concentration of total and soluble oxalates was higher in the leaves and petioles than in roots on mature plants. They found highest total and soluble oxalates 32 days after planting, with the amounts decreasing as the plants developed vegetatively (Massey et al., 2001). The decrease of oxalate found by Massey and co-workers on mature plants might be correlated to the reduction of *Oxalobacteraceae* at later plant development stages in this study, yet no oxalate measurements were performed.

Lang and co-workers characterized the lichen crust interfaces as a zone with high oxalate content (Lang et al., 2007). Additionally, Nagy and co-workers recovered elevated abundance of uncultured *Oxalobacteraceae* associated to biological crusts in a desert (Nagy et al., 2005). Such crusts are concentrated in the top 1 to 4 mm of soil, at the soil-air interface (Belnap et al., 2004). Therefore these crusts constitute another favourable niche for *Oxalobacteraceae*.

Ilarslan and co-workers found that developing soybean seeds and seedlings accumulate high amounts of soluble oxalate and insoluble crystalline calcium oxalate (Ilarslan et al., 1997). They suggest that the large accumulation of Ca oxalate and oxalate is associated with Ca storage and seed storage protein synthesis. This high availability of oxalate on soybean seedlings could explain the high prevalence of *Oxalobacteraceae* clones retrieved from rhizosphere of younger soybean in this study. Taken together the results of research about oxalate in plants, it seems that oxalate is stored in seeds and highly concentrated in roots at early plant developing stages, but is later accumulated in aerial parts of the plants. Up to date, there is no detailed research reported about the connection between oxalate availability in plants and *Oxalobacteraceae* population changes in the rhizosphere.

Unfortunately, no measurements of oxalate during plant development were performed and it is therefore not possible to conclude that the observed decrease in abundance of uncultured *Oxalobacteraceae* clones over time was connected to a lower availability of oxalate released from plant roots.

4.3.2 *Burkholderia* (T-RF 139 bp)

In this work, the abundance of a *Burkholderia* related T-RF decreased significantly (p=0,0008) after glyphosate application in comparison to a relatively high abundance of *Burkholderia* clones in control samples. This is in contrast to other studies supporting glyphosate resistance of some *Burkholderia* species, as in the case of *B. caryophilli* PG2982, a bacterium well known to be able to metabolize glyphosate (Dotson et al., 1996; Moore et al., 1983). Furthermore, Kuklinsky-Sobral and co-workers recovered *Burkholderia gladioli* and *Pseudomonas oryzihabitans* from culture medium supplemented by glyphosate after pre-planting glyphosate application (Kuklinsky-Sobral et al., 2005). Moreover, it has been demonstrated that sensitivity to inhibition by herbicides varies greatly among strains belonging to the same genus, or even to the same species (Forlani et al., 1995). This may explain that in this study the *Burkholderia* populations present in the rhizosphere behaved differently than reported in the literature for specific species. An example of a *Burkholderia* species including large variety of strains with diverse phenotypic features is *B. cepacia* (Schloter et al., 2000). Different *B. cepacia* strains can initiate plant growth promotion (Sfalanga et al., 1999), plant diseases (Yohalem and Lorbeer, 1994) and they can also act as human pathogens (Frangolias et al., 1999) or opportunistic human pathogens that reside in the rhizospheres (Berg et al., 2005).

Results of the phylogenetic comparison of 16S rRNA gene sequences in this study indicated that the obtained *Burkholderia*-related clones were close relatives to *B. caryophylli*, *B. brasilensis*, *B. glathei*, *B. graminis* and *B. phenazinium*. *B. caryophylli* is known as plant pathogen for *Dianthus caryophyllus* (Ballard et al., 1970) while in contrast, *B. brasilensis* is recognized as endophytic nitrogen-fixing bacterium (Baldani et al., 1997).

The ecological role of the species *B. graminis*, *B. glathei*, and *B. phenazinium* is still unknown (Coenye and Vandamme, 2003). *B. graminis* has been isolated from the rhizosphere of grasses and *B. glathei* strains have been described from fossil lateritic soils (Zolg and Ottow, 1975). *B. graminis* and *B. glathei* have been reported to be very closely related based on comparison of their 16S rDNA sequences (Viallard et al., 1998). The soil bacterium *B. phenazinium* is known for the production of the antibiotic phenazine (Wayne et al., 1987). Moreover Villard and co-workers proved by DNA-DNA hybridization that *B. phenazinium* is closely related to *B. graminis* (Viallard et al., 1998).

Similar contrasting results of bacteria from a same species being resistant to the herbicide linuron *in vitro* yet being susceptible to the same herbicide in soil make the generalization for an ecological role of this species in respect to linuron difficult. Several *Pseudomonas* species were able to grow in a linuron selective culture (Dejonghe et al., 2003), while in an ecological study, two different *Pseudomonas* associated clones were found in the untreated control soil, but were absent in the linuron treated soil (el Fantroussi et al., 1999).

Within the phylum *Bacteria*, strains belonging to the same species defined by their core genome, can differ in the presence and absence of hundreds of accessory genes, and consequently have different ecological capabilities (Prosser et al., 2007). Bacteria with exactly the same 16S rRNA gene sequences can even occupy different ecological niches (Jaspers and Overmann, 2004). Thus, in this study, the 16S rDNA sequencing tool enabled the identification of the T-RF 139 bp down to the genus level *Burkholderia*. Nevertheless, because of the additionally high differentiation of this genus (Coenye and Vandamme, 2003), it was not possible to assign an ecological function to this group of bacteria, whose abundance was significantly reduced by glyphosate treatment.

4.3.3 *Gemmatimonadetes* (T-RF 72 bp)

The abundance of *Gemmatimonadetes* affiliated clones associated to T-RF 72 bp proved to be positively influenced by glyphosate treatment, as demonstrated by PCA and the indicator species analysis.

The *Gemmatimonadetes* constitute a genus of bacteria, given their own class (*Gemmatimonadetes*). The first member of this group was discovered in 2003 in a wastewater treatment operated under enhanced biological phosphorus removal. The bacterium was named *Gemmatimonas aurantiaca* and is a gram-negative aerobe that appears to replicate by budding. *Gemmatimonas aurantiaca* was described as a polyphosphate-accumulating bacterium (Zhang et al., 2003).

Since the *Gemmatimonadetes* population increased after glyphosate treatment, these polyphosphate-accumulating bacteria could take advance of possible phosphate residues from the herbicide. Several polyphosphate-accumulating bacteria have been described in the literature, like *Microlunatus phosphovorus* (Nakamura et al., 1995), *Accumulibacter* sp. (Zeng et al., 2003) and *Rhodocyclus* sp. (Chua et al., 2006). However, initial investigations

attempting to explain the metabolic performance of organisms involved in biological removal of phosphorus have been focused on *Acinetobacter* sp. primarily (Auling et al., 1991; Kortstee et al., 1994), until it was demonstrated that classical culture-dependent methods for bacterial isolation were strongly selective for this genus (Mino, 2000; Onda et al., 2002; Wagner et al., 1994).

In the case of the novel group *Gemmatimonadetes* it is possible that the results of the fingerprinting method underestimate the effect of glyphosate on *Gemmatimonadetes* populations due to primer mismatching in sequences of this not well studied group. The PCA results show a connection between *Gemmatimonadetes* and glyphosate treatment but the significance of this relation obtained by the indicator species analysis (p=0,03) is relatively low. This relative low significance value depends on the relative low abundance of *Gemmatimonadetes* in the community, which was much lower as compared to the typical dominant groups of *Proteobacteria*.

Given that the degeneracy of the primers used can lead to PCR template selection (Lueders and Friedrich, 2003), for further studies on this novel group a re-evaluation of the primer system is highly recommended in order to avoid possible PCR bias. *Gemmatimonadetes* was the only group significantly promoted by glyphosate, unfortunately only one species of this group has been described and very little is known about the ecological importance of the group. The use of group-specific primers has been shown to enable the population dynamics study of less abundant bacterial groups (Hartmann et al., 2006). In the case of *Gemmatimonadetes* the design of specific primers would be very important since it is a mainly unknown group that until now has only been found in very low abundances in microbial community studies (Brons and van Elsas, 2008; Liebner et al., 2008; Polymenakou et al., 2009). Specific primers could enhance the identification of *Gemmatimonadetes* and facilitate the description of more organisms belonging to this group. It would also be very interesting to isolate the *Gemmatimonadetes* group members characterized by the T-RF 72 bp and to test the ability of the group to utilize glyphosate and AMPA as sole source of phosphorus or carbon *in vitro*.

4.3.4 *Bradyrhizobium* (T-RF 149 bp)

Initially, the experimental design of the greenhouse experiments with RR Soybean included six harvests in order to monitor effects of glyphosate in the rhizosphere. Nevertheless, only five samples were taken into account for the statistical analysis, since the T-RFLP fingerprints of the last harvest were completely dominated by a single T-RF 149 bp associated to *Bradyrhizobium* sp. At the time of the sixth harvest, 34 days after germination, well developed nodules were observed at the soybean roots. The resuspension of adhering soil to the roots by agitation in the stomacher treatment has probably disrupted the nodules, resulting in a release of *Bradyrhizobium* sp.. Based on the T-RFLP fingerprint data, *Bradyrhizobium* sp. appeared to represent the complete rhizospheric community, which is not very likely for a real microbial community composition of rhizosphere soil. This result supports the assumption that the T-RFLP method captures only the dominant taxa and loses the rare ones (Bent et al., 2007). For this reason the sixth harvest sample was excluded from the statistical analysis. Other environmental studies using PCR-based rRNA analyses have been limited due to PCR bias by the presence of high DNA abundance of a single dominant group as well. Examples of these include the case of a cyanobacterial population of *Microcoleus chthonoplastes* (Bebout et al., 2002) and the soil flagellate *Heteromita globosa* masking a fungal population (Green et al., 2004). Notable reduction of *Bradyrhizobium* bands in DGGE fingerprinting by the utilisation of Suicide PCR has been reported (Green and Minz, 2005), yet in this study the elimination of *Bradyrhizobium* signal by restriction enzymes, as described by Green and Minz, was not applied in order to avoid extra biases in the method. Five sampling time points were thus considered to be enough to describe bacterial community shifts over time and in response to glyphosate application.

The high abundance of *Bradyrhizobium* recovered from the roots of untreated and glyphosate-treated plants at the sixth harvest contrasts with the inhibition of *B. japonicum* by glyphosate under laboratory conditions. This observation supports the incongruent results between laboratory experiments and field research about *Bradyrhizobium* sensitivity to glyphosate (Zablotowicz and Reddy, 2004). Zablotowicz and Reddy oberserved that *B. japonicum* was completely inhibited at 5 mM glyphosate concentration. Correspondingly, other experiments have shown initial *B. japonicum* inhibition at glyphosate concentrations of 200 μM (Wagner et al., 2008), 1 mM (Moorman et al., 1992) and complete inhibition at 5 mM glyphosate

(Moorman et al., 1992; Zablotowicz and Reddy, 2004). Specific symbiotic indicators suggest the reduction of *B. japonicum* activity in plants after glyphosate application like decline of nodule biomass and leghemoglobin content (Reddy and Zablotowicz, 2003) as well as decrease in nitrogen fixation (King et al., 2001). Measurements of glyphosate after foliar application resulted in concentrations of 400 µM glyphosate in soybean nodules (Grundmann et al., 2008), which constitutes a glyphosate selection pressure for *B. japonicum* as described above. Nevertheless no significant yield reductions due to glyphosate treatment in soybean have occurred under field conditions (Reddy and Zablotowicz, 2003; Zablotowicz and Reddy, 2004; Zablotowicz and Reddy, 2007).

Based on the data of this study, T-RFLP analysis indicate that the glyphosate treatment did not affect the *Bradyrhizobium* populations, as in treated and untreated samples *Bradyrhizobium* related sequences (T-RF 149 bp) dominated the rhizosphere community.

4.4 Time-dependent diversity shifts in the bacterial community associated with RR soybean roots

For diversity estimation based on T-RFLP fingerprints, the Shannon diversity index provided relevant information about the community diversity shifts caused by glyphosate treatments. This index combines information about species richness (T-RFs or OTUs in the community) and takes also the relative abundances of different T-RFs into account (Stirling and Wilsey, 2001). Currently, the Shannon diversity index is the most applied index for interpretation of T-RFLP data (Monard et al., 2008; Sipila et al., 2008; Winderl et al., 2008).

Besides the diversity shifts caused by glyphosate applications, there were additional diversity changes within the community that share a common pattern over time independent of glyphosate treatments. As the experiment was performed with the same RR soybean variety and conducted under identical environmental conditions in the greenhouse, the only factor that might influence community changes over time independent of the glyphosate application is the plant developmental stage. The microbial diversity changes observed in the rhizosphere could be related to rhizodeposition differences at different plant growth stages (Garland, 1996; Lynch and Whipps, 1990), resulting in different rhizosphere effects at the phenological stages (Butler et al., 2003; Cheng et al., 2003). Additionally, the fact that root activity and root

vitality decreases with plant maturity has been supported by the findings of various studies (Hütsch et al., 2002; Kuzyakov and Domanski, 2000).

Interestingly, the bacterial diversity increased to its maximum in both treatments at the second sampling time, corresponding to the flowering stage, independent of herbicide application (refer to Figure 3.12). Smalla and co-workers (2001) also observed higher bacterial diversity in rhizospheres of potato, strawberry and oilseed rape during the plant flowering stage (Smalla et al., 2001). Similarly, in potato rhizospheres, the bacterial diversity reached its maximum level while plants were flowering (Lottmann et al., 2000) and in transgenic potatoes most of the differences in the rhizosphere diversity coincided with the flowering period (Milling et al., 2005). Plant growth stage had the most pronounced impact on active microbial populations in the oilseed rape rhizospheres. In principal, at the early flowering stage bacterial communities were highly different compared to those observed at later plant growth stages (Sessitsch et al., 2005). In this study, Shannon diversity index values of the soybean rhizosphere bacterial community declined slightly after flowering as well. This coincides with the data of Fu and coworkers (2000), who demonstrated that organic substances derived from soybean roots increased significantly from the vegetative stage to flowering stage and declined slightly thereafter (Fu et al., 2002). Correspondingly, exudation rates from 10 different rice cultivars increased with plant development stage from seedling to flowering, but decreased at maturity (Aulakh et al., 2001). In addition, maximum exudation rates of citric and malic acids in rice roots were recorded at flowering stage, which were 13-36 times higher than those at early developing stages (Lu et al., 1999). Since increased microbial biodiversity has been positively correlated to increased and diversified available carbon in the rhizosphere (Grayston et al., 1997; Insam and Domsch, 1988; Zhou et al., 2002), it could be concluded that the increment of microbial diversity in the rhizosphere is a response to the increase of organic root exudates at the plant flowering stage.

4.5 Influence of glyphosate on the diversity shifts of the bacterial rhizosphere associated to RR soybean

The influence of glyphosate on the rhizosphere bacterial community is still not clear. First of all, glyphosate appears to be rapidly degraded by soil microbes regardless of soil type or organic matter content (Araújo et al., 2003; Dick and Quinn, 1995; Forlani et al., 1999). However, such studies cannot be generalized because responses depend not only on herbicide properties, but also on soil properties, plant variety and environmental conditions (Lupwayi et al., 2009). As glyphosate stimulates individual organisms capable of metabolizing the herbicide (Kuklinsky-Sobral et al., 2005) and inhibits bacteria with sensitive EPSPS to glyphosate (Padgette et al., 1995), it is expected, that independently of the soil type or plant variety, glyphosate may modify the bacterial community structure. Since in this study no preemergence herbicide application was performed, the observed effects in the rhizosphere of glyphosate-treated plants are due to the translocation of the herbicide and its degradation products into the roots and thus potentially into the rhizosphere.

The observed diversity shifts in the bacterial community associated to soybean roots were mainly associated to the glyphosate treatments (refer to Figure 3.12). Kuklinsky-Sobral and coworkers (2005) explained the shifts in the endophytic communities of RR soybean after glyphosate application as consequence of some microbial groups being able to use glyphosate as a source of energy and nutrients, whereas this herbicide may be toxic to other groups (Kuklinsky-Sobral et al., 2005). Several studies have shown that glyphosate is available as a substrate to some microorganisms (Araújo et al., 2003; Klimek et al., 2001; Kuklinsky-Sobral et al., 2005; Santos and Flores, 1995). In this study, *Gemmatimonadetes* and *Burkholderia* were found significantly modified by glyphosate application. The statistical significant increment of a single group of *Gemmatimonadetes* organisms, all identified under the same single OTU (T-RF 72 bp), does not explain the increase in diversity of the T-RFs recovered after glyphosate application. The higher bacterial diversity was characterized by the appearance of several minor T-RFs, which represented unidentified bacteria. One explanation for this apparent increase in diversity could result from the significant reduction of the *Burkholderia* population (T-RF 139 bp) in glyphosate-treated samples**.** The absence of high abundance of *Burkholderia* species in glyphosate-treated samples could thus lead to changes in the T-RFLP profile not because the actual prokaryote diversity increased, but because the

method captured only the dominant groups and the rare taxa were not detected (Bent et al., 2007). In the absence of the high abundant group of *Burkholderia* the PCR would amplify templates that were already in the community but were "masked" by the dominant *Burkholderia* group.

The absence of the dominant group and appearance of low abundant diverse organisms could also be explained ecologically, since the niche of *Burkholderia* is then available to be exploited by several other minor species (McNaughton and Wolf, 1970; Tilman, 2004). Yet both these explanations seem to be very unlikely, since when comparing the decrease of *Burkholderia* abundance (Fig. 3.16) with the increase of diversity in the rhizosphere (Fig. 3.12) it is evident that the lowest abundance value of *Burkholderia* coincides with the lowest diversity Shannon index value of the glyphosate-treated plants. This indicates that when *Burkholderia* was less abundant, the community was less diverse as well. Both, the bacterial diversity and the abundance of *Burkholderia* reached its lower points three days after the second glyphosate application.

The main community diversity shifts were caused by glyphosate treatments (refer to Figures 3.12 and 3.16). Three days after both glyphosate applications, the diversity dropped to its lowest Shannon values. Then, 7 and 14 days after glyphosate applications, the bacterial diversity not only recovered after the perturbation, but it increased significantly to double the diversity value compared to untreated samples. Since translocation of glyphosate in plants has been shown to require three days to reach the root and be exudated as glyphosate and AMPA (Devine and Bandeen, 1983; Grundmann et al., 2008; Kremer et al., 2005), it seems that the transient decrease in bacterial diversity is closely connected to the glyphosate application. Other studies reported reduced bacterial diversity levels after application of pollutants and herbicides as well, and the disturbed bacterial communities were able to regain their initial diversity when the perturbation was reduced, returning to the diversity level of the controls (Girvan et al., 2005; Torsvik et al., 1996). Interestingly, in this study, bacterial diversity was not only regained to the level of the controls, but even exceeded those. This is in accordance with van Bruggen and Semenov (2000) who stated that any disturbance of soil will lead to an initial decrease and afterwards increase in biodiversity.

Three days after herbicide application, glyphosate and AMPA are new components released into the plant rhizosphere (Grundmann et al., 2008) and the bacterial community seemed to

adapt to these new quality of carbon compounds, which are capable of inhibiting or favouring different bacterial groups. Changes in the bacterial diversity have often been correlated to changes in the composition of rhizodeposition (Grayston et al., 1998; Paterson et al., 1997). Apparently, after adaptation of the bacterial community to the glyphosate source released by the roots, more diverse groups than in the controls could take advantage of the new carbon sources in the environment. Another interesting application of glyphosate is that low doses of this herbicide are used as ripener in non transgenic sugarcane plantations, whereby a higher sugar and starch yield is obtained (Dissanayake et al., 1998; Dusky et al., 1985; Solomon et al., 2001) and earlier harvests are achieved (Lévesque and Rahe, 1992). A similar ripening phenomenon could have induced the observed earlier flowering time of RR soybeans after glyphosate treatment compared with untreated plants. It is also possible that different sugars and polysaccharides are part of the new heterogeneous compounds accumulated in RR soybeans in response to glyphosate application, since the RR crops still contain their native glyphosate sensitive EPSPS and residues of deregulation of the shikimate acid pathway could be present in the transgenic plants (Dill, 2005). Several authors have concluded that the heterogeneity of carbon sources is the main factor influencing a higher niche variation in soil and consequently a higher bacterial diversity (Benizri et al., 2002; Loranger-Merciris et al., 2006; Torsvik and Øvreås, 2002).

In response to short-term perturbations, biological communities in healthy soils return relatively quickly to the initial state (van Bruggen and Semenov, 2000). After the two short-term glyphosate disturbances that caused transient bacterial diversity diminution, resilience of the community was observed. Correspondently, it has been stated that resilience to stress, high biological diversity and high levels of internal cycling of nutrients are characteristics which describe a healthy soil ecosystem (Lynch and Schepers, 2008).

A natural genetic tool contributing to stress adaptation of microorganisms are point mutations in the genome, which lead to a stepwise improvement of available biological functions on which natural selection applies evolutionary pressure (Arber, 2000). It has been hypothesized that mutation frequency may be increased dramatically under some environmental stress situations, giving rise to episodic rapid evolution (Woese, 1987). Genomic analyses have emphasized the remarkable genetic diversity potentially available in soil microbial communities leading to the development of a diversification of members of these

communities, not only by HGT but also through genomic shuffling mechanisms (deletions, insertions and rearrangements) (Arber, 2000; Arber, 2004). Gene transfer and recombination across species and genus barriers can induce environmental adaptation and the evolution of new traits, like the adaptation or resistance to a herbicide, thereby increasing diversity (Dykhuizen, 1998). Although point mutations are a feasible response to glyphosate selection pressure as adaptative tool to survive in a selective environment, new resistant glyphosate ecotypes will not be detected by the analysis of changes in the 16S rDNA sequence diversity and two ecotypes can be identified as belonging to the same population (Cohan, 2002). This divergence between 16S rRNA gene phylotypes compared to phenotypes and niches occupancy has been reported for different strains of *Brevundimonas alba* (Jaspers and Overmann, 2004).

It is hypothesized that single nucleotide exchanges in the *EPSPS* gene may be the basis of genetic adaptation within a given species. On the other hand, the higher diversity of 16S rDNA sequences retrieved from glyphosate-treated samples could arise from already glyphosate resistant bacteria in the rhizosphere. After a disturbance, diverse bacteria often have different responses to changing environments, so that bacteria with rapid growth rates are able to rapidly fill expanding niches (Bissett et al., 2007). More diverse communities are better able to rapidly recover from disturbances (Girvan et al., 2005) and high functional redundancy of complex bacterial communities contributes to their diversity (Torsvik et al., 1996) and robustness (Bissett et al., 2007). Moreover, a large proportion of bacterial community is inactive at anyone time since several bacterial genera can form resistant spores or switch to slow-growing or dormant vegetative forms that are resistant to environmental stress (Balaban et al., 2004). These dormant bacterial life forms become important when disturbance occurs, providing a rich diversity reserve for resilience (Prosser et al., 2007).

Van Bruggen and Semenov (2000) concluded that disturbance leads to a succession in bacterial communities and the associated food webs. The key to bacterial community resilience is the succession of bacteria utilizing the metabolites released from other organisms able metabolize glyphosate and AMPA, expanding the network and complexity of the bacterial community relations. A recent case study showed an interesting evolution process of microbial interactions under selection pressure, when the persistence of sensitive bacteria becomes dependent on the presence of resistant ones. Benzoate, toxic for a specific

Pseudomonas strain, is rapidly metabolized by *Acinetobacter*. When *Acinetobacter* and *Pseudomonas* were cultivated in a dual culture *Pseudomonas*, which is normally impaired by benzoate, could utilized the benzoate metabolites secreted by the faster growing *Acinetobacter* strain (Hansen et al., 2007). As prerequisite for a functionable interaction, changes of growth patterns and a reduction of the growth rate of *Pseudomonas* were required as well as a heritable change of the phenotype which could allow the rapid evolution of this interaction by possible spontaneous mutations (Hansen et al., 2007). In a similar way, *B. japonicum* ecotypes may get adapted to glyphosate in nature, although glyphosate inhibits its growth when the bacterium is isolated (Zablotowicz and Reddy, 2004). A naturally slow growing bacterium could take advance of metabolized glyphosate products secreted by faster growing bacteria that detoxify the glyphosate in the rhizosphere. Thus, the resilience process after glyphosate selection pressure could include a succession of glyphosate metabolizing bacteria (van Bruggen and Semenov, 2000), accomplished by rapid bacterial adaptation by mutations (Schloter et al., 2000) and bacterial growth changes (Prosser et al., 2007). Therefore, under glyphosate selection pressure in an environment with organisms that are able to metabolize the toxic herbicide fast, the key for enhancing diversity is the succession of metabolites, which can be further utilized by a diverse range of bacteria.

5 Conclusions

B. japonicum is not naturally transformable under any conditions known from the so far reported naturally transformable bacteria. In addition, it was proved in this study that the *CP4-EPSPS* genetic construct of RR soybean is not expressed in *B. japonicum*. It was proposed that expression of the herbicide resistant gene in an environment under glyphosate pressure would be a major factor maintaining the foreign gene in the genome due to a selective advantage (Zablotowicz and Reddy, 2004). Therefore, if there would be a gene transfer of the plant *CP4-EPSPS* to *B. japonicum*, this genetic construct does not confer glyphosate resistance to *B. japonicum* and does not constitute any adaptive advantage to the bacterium under glyphosate selection pressure. As the genetic trait of the glyphosate resistance has been found in several bacteria (Padgette et al., 1995), it would be much more probable that the common mating exchange between bacterial groups could disperse the glyphosate resistance in an environment with glyphosate selection pressure. Thus, the problem of gene transfer of genes originally present in bacteria may be unrelated to transgenic plants (Davison, 2004).

In the specific case of *B. japonicum*, a high mutation rate (3.2×10^{-7}) for glyphosate resistance was observed. Possibly *B. japonicum* can also adapt to the glyphosate selection pressure by mutations under natural conditions. This observation supports the incongruent results between laboratory experiments and field research about *Bradyrhizobium* sensitivity to glyphosate, as nodule formation in the field does not seem to be affected (Zablotowicz and Reddy, 2004). Additionally, it is possible that *B. japonicum* can take advance of less toxic glyphosate metabolites secreted by fast growing bacteria in the rhizosphere.

Comparison of the rhizosphere community of glyphosate-treated and untreated soybean revealed one *Burkholderia* group being significantly inhibited by glyphosate and a group of *Gemmatimonadetes* whose abundance increased significantly after glyphosate application. The significant increment of *Gemmatimonadetes* abundance after glyphosate application could indicate that these organisms are able to metabolize the herbicide. For further characterization of the *Gemmatimonadetes* group it would be interesting to investigate the ability of the group

to utilize glyphosate or AMPA as sole source of phosphorus or carbon (Zhang et al., 2003). However, when discussing the effects of glyphosate on bacterial communities, it is important to consider that the T-RFLP analysis only shows the most dominant species and does not allow insight into the status of the complete genetic diversity, including minor groups present possibly as dormant cells.

After glyphosate application, the bacterial community associated to RR soybean roots not only demonstrated effective resilience after the disturbance, as reported by several authors, but also revealed an increased diversity as compared with the control samples. Complex bacterial community interactions can lead to successful resilience due to succession of metabolization products (Hansen et al., 2007) in an environment with changes of bacterial responses to expanding niches (Bissett et al., 2007). A successful community resilience can also be attributed to reactivation from dormancy stages (Prosser et al., 2007) and changes of growth of pre-existent bacteria, which were present under non disturbed conditions at lower abundances (Bissett et al., 2007). Due to the changed conditions, less competitive bacteria could have obtained improved competitiveness (Bissett et al., 2007).

The rapid restoration of the bacterial community diversity after glyphosate disturbance has been interpreted as an attribute for healthy soils (Lynch and Schepers, 2008; van Bruggen and Semenov, 2000). Yet, it is not possible to conclude that the higher bacterial diversity after the glyphosate disturbance, compared with the control samples, describes a healthier soil, as the mechanisms underlying microbial species richness and the influence of nutrient supply on microbial diversity are still not known. A higher diversity could also lead to still uninvestigated processes like eutrophication in soil (Prosser et al., 2007).

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